Spectroscopic study of stabilities of new synthetic curcumin analogs.

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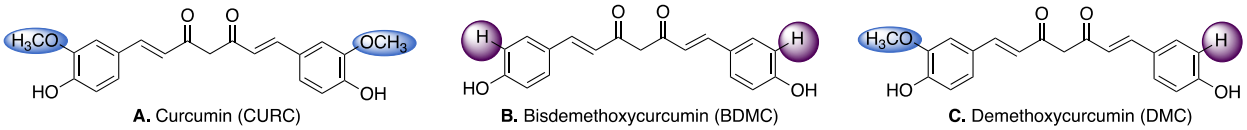
ABSTRACT

Curcumin, bisdemethoxycurcumin, and demethoxycurcumin have demonstrated numerous biological applications. All three compounds are isolated from turmeric with curcumin being the major ingredient. Due to structural similarities, these compounds form a class of curcuminoids and demonstrate similar physical properties. All three curcuminoids are lipophilic and as a result, their pharmaceutical success is limited by poor water solubility. Modification of the organic skeleton allows for the generation of molecules that are not found in nature but can demonstrate biological properties similar to those of natural curcuminoids. However, to be considered valuable candidates for pharmaceutical applications new compounds must demonstrate stability under physiological conditions. This could be monitored using spectroscopic methods, such as nuclear magnetic resonance spectroscopy. We hypothesized that this technique would allow us to track individual hydrogen atoms and identify any changes in their environment when stresses of heat or irradiation are applied to the target compound. Our results demonstrate that synthetic compounds demonstrated remarkable stability under substantial heat and UV radiation exposure in solid and solution form. In summary, synthetic analogs demonstrated superior stability than that of curcumin making them valuable candidates for future biological testing.

INTRODUCTION

Curcumin (Scheme 1a) is the major constituent of the active ingredients in turmeric. The net formula of curcumin is made of twenty-one carbon atoms, twenty hydrogen atoms, and six atoms of oxygen. In its structure, the central carbon-based piece is merged with two carbon-based side arm pieces. Curcumin has several natural analogs known as curcuminoids. They have slight variations in the exact build of the carbon-based side arm pieces. Bisdemethoxycurcumin (Scheme 1b) is symmetric, while demethoxycurcumin (Scheme 1c) is asymmetric. DMC has similarities to both BDMC and CURC, BDMC being the smallest molecule of the three curcuminoids. Curcumin and naturally occurring curcuminoids (Scheme 1) have demonstrated multifaceted utility in medicinal applications [1]. The beneficial health effects of curcuminoids have been widely explored with particular interest shown in their anti-inflammatory and anti-infective effects [2]. These are not the focus of this paper and are discussed in detail elsewhere.

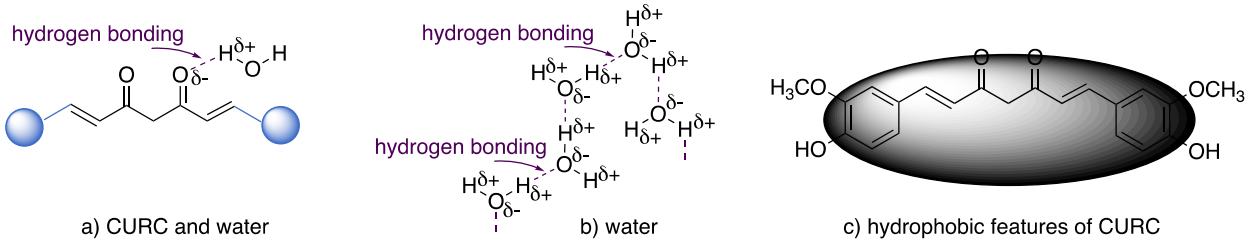


A. Curcumin (CURC) **B.** Bisdemethoxycurcumin (BDMC) **C.** Demethoxycurcumin (DMC)

Scheme 1. Structure of naturally occurring curcuminoid species.

For biological studies and pharmaceutical applications, aqueous solutions of curcumin and curcuminoids are used. All three curcuminoids suffer from poor solubility in water. The large composition of carbon and hydrogen atoms creates hydrophobic centers which leads to overall lipophilic properties (Scheme 2). In other words, curcuminoids are highly soluble in oil as they resemble it more than water. This matter is further complicated due to a unique property of water – the formation of hydrogen bonding. Water consists of two hydrogen atoms bound to a central oxygen atom. Due to the differences in the size of the atoms they differ in their ability to hold on to electrons. With a mass of 1 atomic unit, hydrogen appears first on the Periodic table and is substantially lighter than oxygen with a mass of nearly 16 atomic units. As a result, hydrogen atoms have a lower ability to attract electrons and adopt a partially positive configuration in water while oxygen atoms have a higher affinity to electrons and become partially

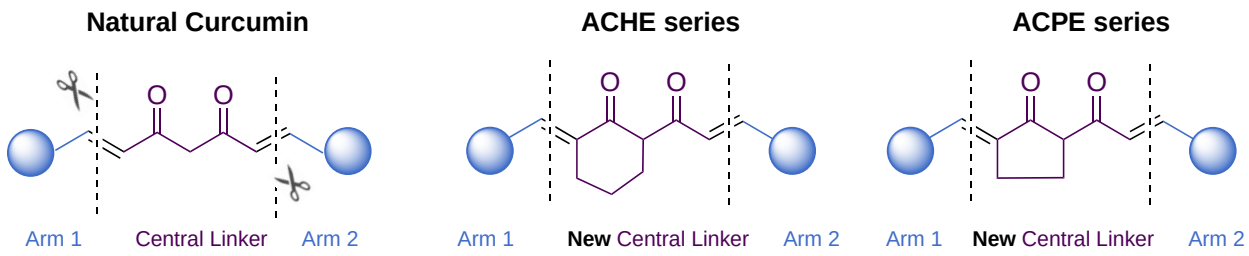
negative (Scheme 2). When CURC or other curcuminoids are introduced to water they not only do not have the same ability to form hydrogen bonding they face a cage-like internal structure of water. To dissolve CURC or other curcuminoids are required to break through the arrangement of hydrogen bonds. While CURC can form hydrogen bonds with water (Scheme 2a) it is less straightforward than the hydrogen bonding between water molecules themselves (Scheme 2b). As a result, the large hydrophobic features of CURC and molecules alike lead to limited hydrogen bonding, which in turn leads to the poorer water solubility of these compounds. In fact, when CURC is added to water it tends to float on top of it just like an oil would on a surface of water.



Scheme 2. Origin of lipophilic properties of curcuminoids: hydrogen bonding and hydrophobic character.

To overcome the solubility limitation of natural curcuminoids several approaches can be taken. A mixture of solvents can be used instead of pure water. In this approach, a sample of CURC is dissolved in an organic solvent followed by the gradual addition of water. These diluted solutions contain only a small fraction of organic solvent and are used as is unless CURC starts to precipitate. Another method utilizes the hydrophobic features of CURC and delivers it as a micelle. However, this type of delivery is only suitable for a limited number of biological applications, and it is often difficult to estimate the exact concentration of active compounds in final samples. The most common approach is to increase solubility by increasing temperature at dissolution. Solubility is a temperature-dependent property and as temperature increases solubility of naturally occurring curcuminoids will increase. Potentially, the highest solubility can be achieved at the boiling point of water (100 ^{o}C), however, it is rarely the case as naturally occurring curcuminoids are temperature sensitive. Finally, a modification of the skeleton to decrease hydrophobic features can result in a line of new compounds that are not available in nature.

We have previously synthesized a series of new curcuminoids containing features to improve their biological properties (Scheme 3). However, since these compounds are unprecedented there are no data available on their properties. In this research project, we investigated the impact of extreme conditions on the stability of novel synthetic curcuminoids in comparison to naturally occurring curcuminoids, such as CURC, BDMC, and DMC. Our hypothesis was that new compounds will have a higher tolerance to extreme conditions due to the modification of a flexible middle chain with a rigid cyclic fragment (Scheme 3). For one series of compounds, a five-membered cyclic structure (ACPE) is introduced, and for the other a six-membered one (ACHE). Our research question was “Do synthetic curcuminoids maintain their structure at extreme temperatures?” To answer this question, we needed to develop a methodology as no data were available for the stability of these compounds in the literature. After analysis of methods and instruments available nuclear magnetic resonance (NMR) spectroscopy was utilized instead of gas chromatography (GC) or high-resolution mass spectrometry (HRMS). While GC and HRMS methods have been demonstrated successful in the determination of decomposition products for naturally occurring curcuminoids they do not offer knowledge of the behavior of these compounds in solution. Due to this limitation, NMR was selected as a preferred method as it allows for a variety of pure solvents, solvent mixtures, and temperatures to be tested. To the best of our knowledge, there have been no previous reports on stability studies of target compounds used in this study.



Scheme 3. Series of new synthetic curcuminoids used in this study.

METHODS

Materials. All new curcuminoids were previously synthesized and characterized in Dr. Stepanova's laboratory. The purity of all compounds were tested using elemental analysis provided by external services of Atlantic Microlab. All compounds remained within the accepted standard ±0.4% of each value. Deuterated solvents, such as DMSO-d^6 and $CDCl_3$ were obtained from Sigma-Aldrich and used without purification.

Variable temperature NMR experiments. Measurements were conducted using a 400 MHz Bruker Avance III spectrometer. Measurements of proton signals (^{1}H-NMR) such as integration and chemical shifts were analyzed and compared to before and after treatments. All spectra were calibrated to tetramethyl silane (TMS) as an internal standard. Spectra were analyzed using Topspin or Mnova software.

Conventional heating experiments. Samples were heated using a water bath. A base was built using cardboard to ensure that NMR tubes were sufficiently submerged. Bath temperature was monitored using a calibrated thermometer.

UV-A experiments. Exposure was conducted using UVP Chromato-Vue® cabinet by Analytic Jenna equipped with a 1.08 A UV lamp. All experiments were carried out at a wavelength of 365 nm.

Water addition experiments. DI water and only samples prepared in DMSO-d^6 were used due to the limitation of miscibility. Solutions were prepared at room temperature for all experiments.

EXPERIMENTAL PROCEDURE

Sample preparation. Solution samples were prepared using 10.0 mg of the specified curcuminoid and 0.6 mL DMSO-d^6 or $CDCl_3$ added via syringe. Solution samples were filtered when necessary, using paper filter and concentration was uncorrected.

Variable temperature NMR experiments. After placement of samples in the instrument's cavity, the temperature was modified and monitored using Topspin software. The temperature was gradually increased over several hours and then maintained at 55°C for 45 minutes prior to any measurements taking place. Stability of temperature was always monitored during data collection including overnight experiments.

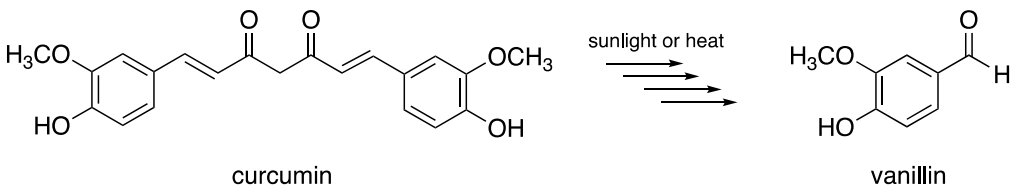
Conventional heating experiments. Samples were uncapped and a cap was replaced with a cotton to allow for an adequate air exchange due to heating and minimize evaporation. A water bath was prepared using boiled water and the temperature was brought down to 55.5°C. Samples were kept in the bath at 58.5°C, after stabilization of the temperature, in between data collection and returned immediately after data collection was completed. Each ^{1}H-NMR spectrum was collected every hour and took approximately 2.5 minutes.

UV-A experiments. Solution samples were placed for 3 hours under a direct source in either a plastic or glass sealed tube. Aliquots of samples were taken after the exposure for NMR experiments.

Water addition experiments. Water was gradually added using a pipettor directly to the sample in the NMR tube. Samples were shaken to ensure mixing of the liquids took place. If precipitation occurred samples were placed in ultrasound chamber to promote redissolving. NMR spectra were collected after each addition took place.

RESULTS AND DISCUSSION

It was previously demonstrated that curcumin and its naturally occurring analogs (BDMC and DMC) upon exposure to heat or sunlight decompose in its constituents (Scheme 4) which includes an aldehyde. While CURC is considered safe and nontoxic by Food and Drug Administration the aldehyde has very different properties. Even partial decomposition of CURC can have a substantial impact on its biological performance.



Scheme 4. Decomposition product of curcumin.

Considering, the decomposition patterns previously reported for CURC and its natural analogs, two compounds for each central linker were selected. The target compounds contained a furfural-based arm and a cinnamaldehyde-based one, compounds **1** and **2** respectively (Figure 1 A and B). Independently of this study we have previously observed decomposition in solid state for these compounds during shipment for elemental analysis to Georgia during summer months. It was speculated that they will also demonstrate decomposition in solutions during either extreme heating or prolonged irradiation conditions.

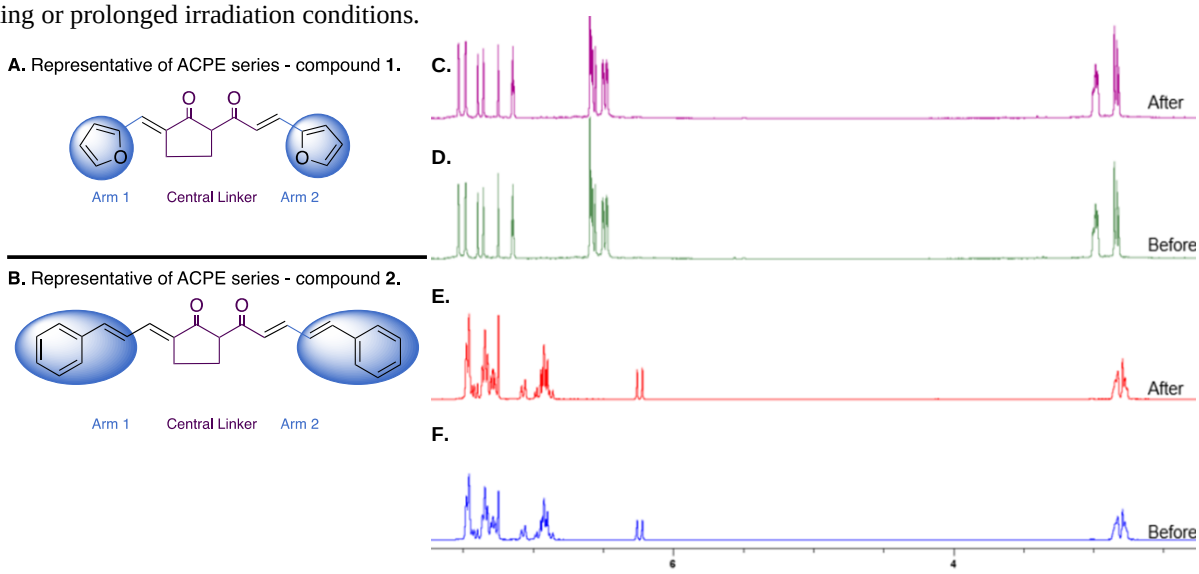


Figure 1. Investigation of stability for compounds of ACPE series using variable NMR technique.
A. chemical structure of compound **1**, B. chemical structure of compound **2**, C–F. NMR spectra obtained.

The spectral information was collected before heating of the samples for each compound and is shown on Figure 1, **D** is for the compound **1** and **F** is for the compound **2**. The spectra obtained after heating are shown in Figure 1 with **C** representing compound **1** and **E** representing compound **2**, respectively. We investigated two case scenarios. The first one was based on an expectation that the signals of H^a–H^b, known as characteristic trans coupling, appearing close to 6.2 ppm on spectrum **D** (Figure 1) for compound **1** and at 6.1 on spectrum **F** (Figure 1) for compound 2 will disappear or their relative intensity to other signals will change. In the second scenario, we expected that the signals corresponding to the constituent aldehyde will appear in the downfield region of 9 to 10 ppm (Figure 2B). The signals of a stand-alone proton H^c (Figure 2A) were not used for the study due to the difficulty of integrating this signal because of the partial signal overlap in aromatic region at 7 to 8 ppm for compound **2**. The collected data demonstrate that the overall appearance of each signal for both compounds remained identical before and after treatment. In addition, even upon expansion of a baseline of the spectrum, no signals of constituent aldehyde were observed as well.

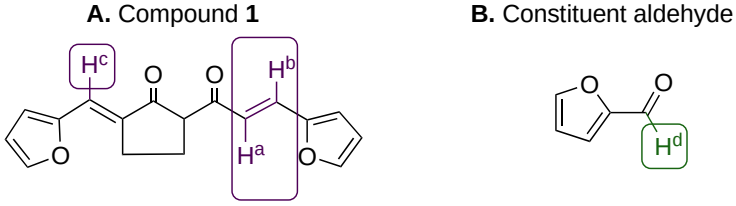


Figure 2. Structural explanation of specific proton signals used in this study.

Inspired by these results we have further adjusted experimental conditions to better represent preparation of samples for biological studies. First, we tested the impact of air and conventional heating (Figure 3A), second, we tested introduction of water into the solution with and without heating, and last we used ultraviolet (UV-A) irradiation (Figure 3B). To test the behavior of solutions using conventional heating and open-air samples prepared for NMR testing were placed in the water bath with caps removed. To ensure that multiple samples could be heated at the same time at the same temperature a contraption was built and used for all samples (Figure 3A). To ensure the high purity of samples, required for NMR investigations, was maintained the caps were replaced with cotton and temperature of the bath was monitored throughout heating instead of the temperature of the samples (Figure 3A).

A. Conventional heating setup.

B. Irradiation setup.

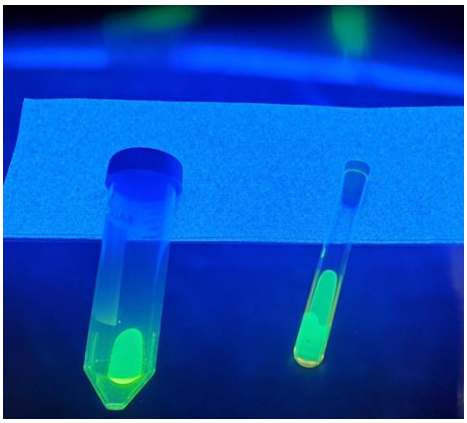


Figure 3. Illustration of setups used for tested curcuminoid:
A. water bath, B. samples under UV-A light in the instrument's chamber.

The collected ^{1}H-NMR data were investigated in a similar manner as described above. Identification of changes in the appearance of trans coupling H^a–H^b signals for both compounds and the detection of signals corresponding to the constituent aldehydes was carried out. Collected data demonstrated that even in the presence of open-air solutions of tested curcuminoids **1** and **2** maintained their composition and only accumulation of water signals over time was observed with no detectable presence of constituent aldehydes.

For the investigation of the water impact, compound **1** was selected due to its higher water solubility. This allowed a gradual increase of water content from 0.1 mL to 0.6 mL prior to the formation of a substantial precipitation. Compound **2** under similar conditions began precipitation upon a second addition of water (total water volume of 0.2 mL). While several spectral datapoints were obtained, the comparison of an initial and the final spectra are shown on Figure 4. The collected data illustrate no changes in appearance of the proton signals for tested compound.

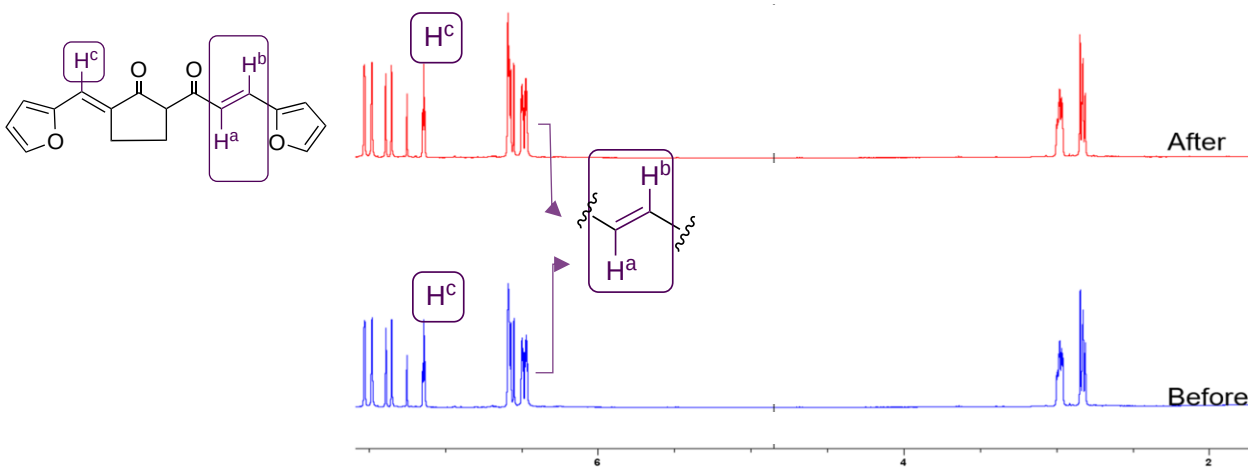


Figure 4. NMR spectra collected for water experiments, before – initial spectrum, after –a final data point.

Exposure to UV-A was carried out using a plastic (Figure 3B left) and a glass container (Figure 3B right). The UV-A source was chosen due to potential of tested compounds to be applied for food safety purposes. The details of this method are not the focus of this study and will be published separately. We tested a naturally occurring analog of curcumin and its synthetic analog (Figure 5). The samples were irradiated in the solution and a characteristic glow was observed (Figure 3B). Due to the size of the chamber no mixing of the samples occurred as it was not possible to place a stirring plate inside. After irradiation was complete, NMR spectra were taken, and a summary is shown in Figure 5. The comparison of spectra was done similarly as described above.

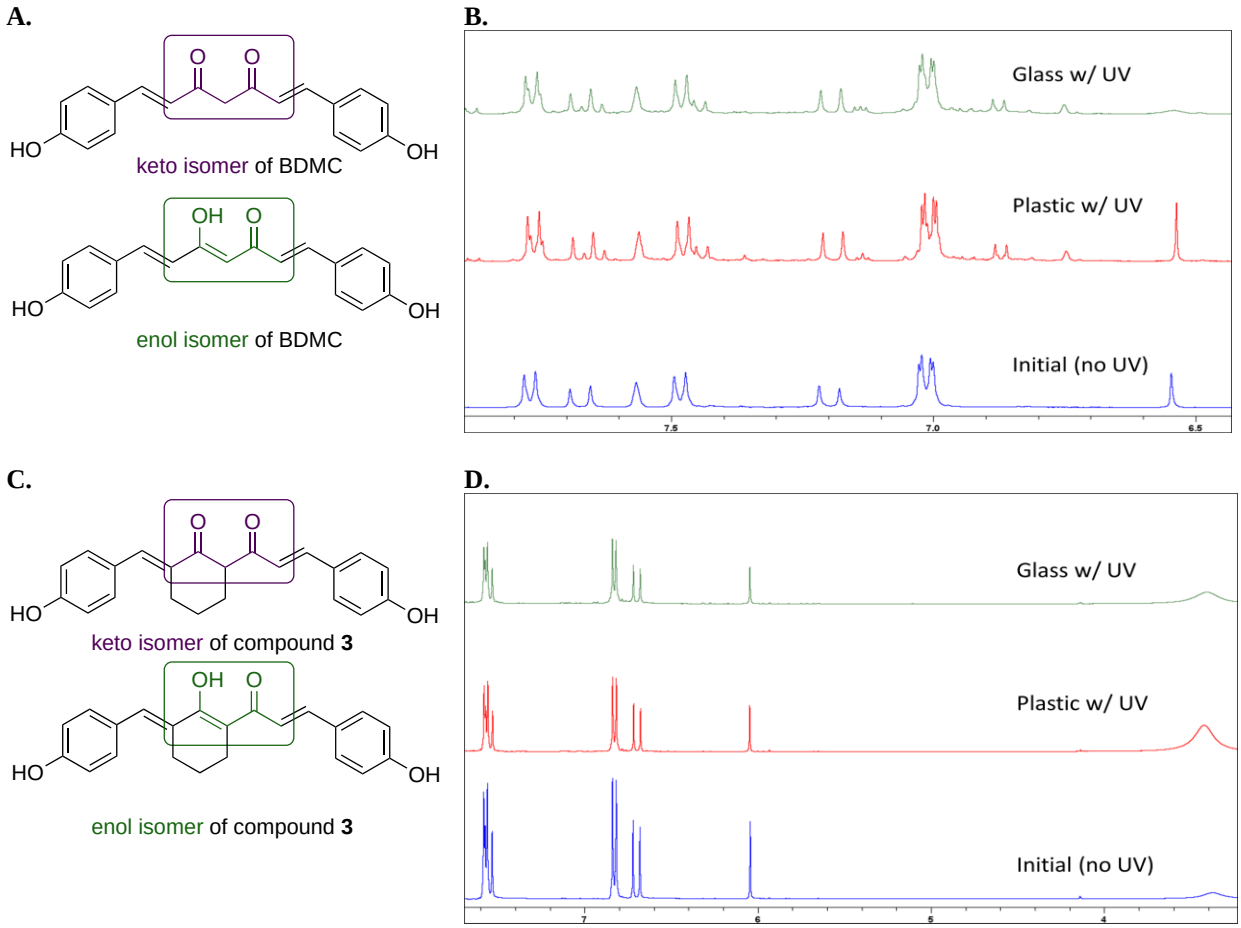


Figure 5. Analysis of stability upon exposure to UV-A:
A. chemical structure of isomers of BDMC, B. ^{1}H NMR spectra for irradiated samples of BDMC,
D. chemical structure of isomers of compound **3**, E. ^{1}H NMR spectra for irradiated samples of compound **3**.

Exposure of BDMC samples to UV-A resulted in appearance of additional signals for both plastic and glass vessels (Figure 5B). Relatively small signals were observed in all regions of the NMR spectrum indicating that not a decomposition but rather isomerization was taken place. One possibility is formation of a keto isomer (Figure 5A) of BDMC. Further investigation will be required to confirm that hypothesis as information on keto isomer of BDMC is limited as it was never isolated or characterized as an individual component. Compound **3** resembles similarity to BDMC but is different in the linker used as a flexible carbon bridge is replaced with a rigid cyclic structure. While it is possible for compound **3** to exist as a keto and an enol isomer (only one out of two possible one is shown) the spectra indicate no substantial amount of keto isomer prior or after irradiation took place (Figure 5D). The only changes observed was increasing amount of water which corresponded to a raising broad peak near 3 ppm.

To conclude our study, we investigated the impact of an exposure to direct sunlight on the composition of solution samples of naturally occurring curcumin and its four synthetic analogs. The summary of full spectra along with structure of tested compounds is summarized on Figure 6. For each compound a before and after spectrum is

provided on the left side and the corresponding structure is shown on the left. All synthetic compounds had a central flexible bridge replaced. Two of the synthetic compounds contained a five-membered rigid linker (compounds **1** and **2**) and one of them had a six-membered one (compound **4**). For the cross-comparison of the impact of the side arms compounds **1** and **2** were compared. For the impact of a central linker compounds **1** and **4** were analyzed. The data were analyzed for the appearance of constituent aldehydes which would point to the decomposition of compounds. In addition, regions of 6 to 8 ppm were analyzed as well for detection of isomers of tested compounds. Our data indicate that prolonged exposure to direct sunlight results in a decomposition of all samples. As it is illustrated on Figure 6 with an expansion box all compounds demonstrated a characteristic aldehyde signal at 9 to 10 ppm range. These data indicate that while synthetic compounds do have higher stability towards UV-A they still remain somewhat light sensitive as the naturally occurring curcuminoids.

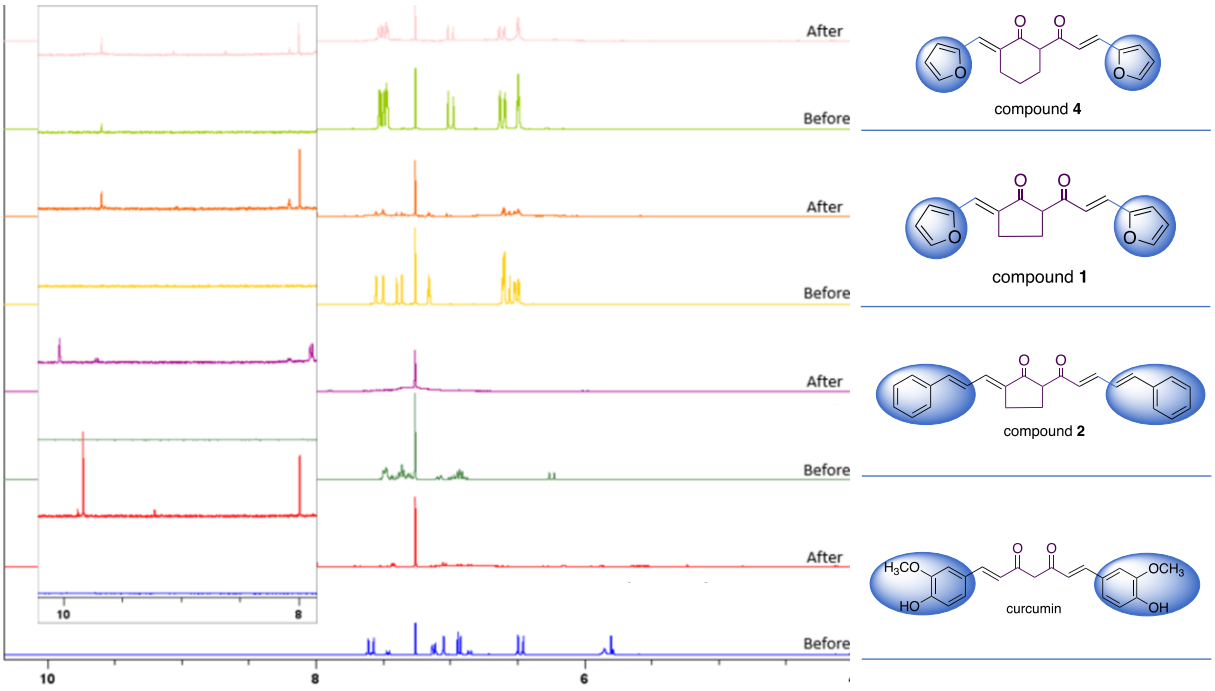


Figure 6. Exposure to sunlight of synthetic curcuminoids (compounds 1 through 4) and natural curcumin.

LIMITATIONS

Due to the constraints of time on usage of the NMR spectrometer and other factors out of our control the sample size of tested compound was limited to four. Future investigations will need to expand the library of tested substances to ensure that the whole series based on a five-membered rigid linker (ACPE) and a six-membered rigid linker (ACHE) are included. This will provide a more holistic view of stabilities of these compounds as a new class of curcumin analogs and facilitate their future biological testing.

CONCLUSION

New synthetic curcuminoids containing a modified central linker and arms have been subjected to extreme conditions to investigate their stability in solution. The collected data demonstrate that compounds **1** through **4** have a superior stability when compared to curcumin or its natural analogs (BDMC and DMC). The collected ^{1}H-NMR spectra demonstrated absence of change in overall appearance. The corresponding trans coupling H^{a}–H^{b} signals also remained unchanged and absence of any signals of constituent aldehydes was observed. Data were collected for pure organic solvents that are commonly used in organic laboratories, such as deuterated chloroform ($CDCl_3$), and biological studies, such as deuterated dimethyl sulfoxide (DMSO-d^6).

Based on the data collected for the solvent mixtures using DMSO-d^6 and deionized water or deuterated water (D_2O) our study also demonstrated that new synthetic compounds **1** through **4** have superior stability under conditions resembling biological studies. The stability of synthetic compounds towards sunlight is similar to that of naturally occurring curcuminoids. However, at this point collected data are not sufficient to draw a conclusion on the rate of decomposition or the extent of it. To the best of our knowledge, this is the first study that investigated decomposition process of unnatural (synthetic) curcumin analogs, and it provides useful insights for future biological studies of these novel compounds.

ACKNOWLEDGEMENT

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