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Colorimetric Method for Instant Detection of Lysine and Arginine Using Novel Meldrum's Acid-Furfural Conjugate

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In the past few years Meldrum's acid furfural conjugate (MAFC) have been extensively explored as starting material for the synthesis of photo switchable donor acceptor stenhouse adducts (DASA). Hereby, we have explored the interaction of MAFC with various amino acids. To our surprise, nitrogen rich amino acids like lysine and arginine interact spontaneously with MAFC to give colored adduct immediately, whereas other amino acids, including nitrogen rich histidine, didn't show any coloration. Naked eye detection of lysine in benign solvent make this reagent an attractive new entry to the collection of chemosensors for the colorimetric detection of lysine and arginine. Intense coloration corresponds to the absorption at 514 nm under UV-Vis spectrometer. Lowest concentration of 100 μm can be detected with UV-Vis spectrometer. NMR titrations reveals that the appearance of color is due to ring opening of a furfural that leads to the formation of conjugated triene species. Compared to previously reported chemosensors for lysine and arginine, MAFC offers advantages including simple synthesis, easy handling, high speed, low cost, good sensitivity/selectivity.

Introduction

Efficient and selective methods for colorimetric detection of trace elements and substances are the bases of modern assay development.[1] Detection of biochemical species and vital nutrients is not only important in medical science but also in the food industry.[2] Among various important nutrients responsible for proper biochemical functioning, essential amino acids are a vital part of healthy human diet, disbalance in their intake can lead to possible adverse health effects.[3] Therefore, food industry is continuously looking for methods those can quantify the presence of essential amino acids that human body cannot synthesize on its own.

Among various essential amino acids, lysine is of particular interest, as lysine is required for biochemical synthesis of polyamine and is involved in Krebs-Henseleit cycle.[4] During various biochemical processes in liver lysine undergoes catabolism through saccharopine to furnish glutamate and α-amino adipate.[5] Moreover it plays an essential role for the production of carnitine in the body that is required for the conversion of fatty acids into energy.[6] Furthermore, it facilitates calcium absorption[7] and collagen formation[8] for muscle growth and healthy bones. Therefore, often lysine is used as a fortifier to enhance the nutrition value of the products. Often the quantitative analysis of the lysine content is determined by high cost instrumentation like flow injection chemiluminescence,[9,10] amperometric biosensor,[11,12] laser desorption/ionization mass spectrometry,[13] impedimetric,[14] chromatography[15,16] and spectroscopy.[17] A relatively simple colorimetric detection of lysine by gold nanoparticle aggregates was described by Zhou et al., where they used citrate stabilized gold nanoparticles at pH 3 for detection.[18] Inspired from this study various research group have developed the metal assisted colorimetric sensors those can either detect lysine exclusively or in combination with other amino acids.[19,20] However, from practical utility point of view, determination of specific amino acid in aqueous medium through fluorescence or colorimetric means is in great demand.

In addition to metal complexes,[21] various metal free, reaction based chemosensors are explored in the past. Glass et al. reported a fluorescence sensor for lysine, which is based on dimerization of quinolone aldehyde core.[22] Pyrene based fluorescent chemosensor of lysine was reported by Yoon and Lee et al., where they have seen a unique color change from light yellow to pink due to imine formation.[23] Various modification on pyrene sensor were reported,[24,25] however, due to its metabolite binding tendency to DNA[26] alternative sensor probes are continuously searched. Recently, a non-toxic thymol based BODIPY was explored as chemosensor for lysine detection with 0.01 μm colorimetric detection limit.[27] However,

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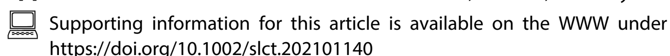
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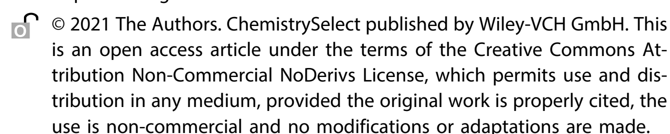
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complex synthesis protocol, relatively fast decomposition and purification hinder their widespread use.

During the last decade, meldrum's acid furfural conjugates (MAFC, **1**) received a significant intention in the field of photo switching chemistry because of their ability to make conjugated triene, so called DASA (donor acceptor stenhouse adducts), upon reaction with nucleophilic secondary amine.[28] We have demonstrated the performance of such photoswitching reaction of such intensely colored DASA even on the polymer surface.[29] Spontaneous transformation from colorless to magenta, either in solution or on surface, due to presence of amines made them ideal candidates for colorimetric amine detection.[30,31] Being motivated by the quick reaction time and intense color reaction with amines, we explored the possibility of using these adducts as colorimetric sensor for detection of amino acids. To our surprise, only lysine and its structural analogue arginine were able to give a quick coloration in the solution, whereas other amino acids remained colorless. Therefore, we feel the need of publishing this new class of lysine and arginine selective chemosensor that was never investigated before. In this short communication, we have investigated the UV-Vis spectra of the adduct to find the detection limit, the influence of the pH on the detection and the probable structure of the adduct through NMR titrations. The factors taken into consideration are sensitivity, linearity, rapidity, repeatability and operational simplicity.

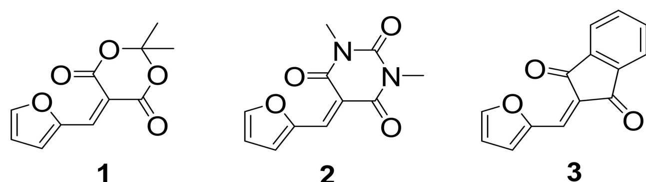

Figure 1. Synthetic activated furans made by condensation of furfural with active methylene compounds.

Result and Discussion

In his original contribution Alaniz et al. has described that activated furans, those are prone to nucleophilic addition to secondary amines, can be synthesized through Knoevenagel condensation of 2-furfural with electron deficient meldrum's acid **1**, barbituric acid **2** and indandione **3** (Figure 1).[28] All these adducts give excellent color reaction with secondary amine. However, MAFC (**1**) has the best solubility of all in ethanol, which can be easily mixed with aqueous samples of amino acids without precipitation. Moreover, facile and high yield synthesis of MAFC 5-furan-(2-ylmethylene)-2,2-dimethyl-1,3-dioxane-4,6-dione by just mixing 2,2-dimethyl-1,3-dioxane-4,6-dione and 2-furfural in water make it of high practical utility. Synthesis is so simple that Helmy et al. proposed to include it in undergraduate organic chemistry practical courses.[32] Bright yellow product was obtained in more than 75 % yield simply by filtration and extraction with organic solvent. Spectroscopic data and NMR measurements (s. supporting info, Figure S1) showed good comparison with literature values.

Initially, we have performed an experiment where ethanolic solution of MAFC was mixed with aqueous solution of random chosen 14 amino acids 4 with different functional groups on chiral carbon. We were expecting a colored reaction with amino acid proline because in our previous study we have shown that DASA formation is more selective to secondary amine as compared to primary amines.[30] However, to our surprise, amino acid lysine and arginine has shown a spontaneous color reaction with MAFC and not any other amino acids including proline (Figure 2A). We have observed this reaction set periodically for more than 24 hours. It was noticed that lysine and arginine gave color reaction immediately after mixing, whereas proline sample has shown a slight coloration after few hours. All other amino acids in the test have shown no color appearance even after 24 hours. Interestingly, the colored products of lysine and arginine were stable until 24 hours and have not shown any precipitation, while other amino acids have shown black gray precipitation that might be resulting from decomposition of MAFC in ethanol.

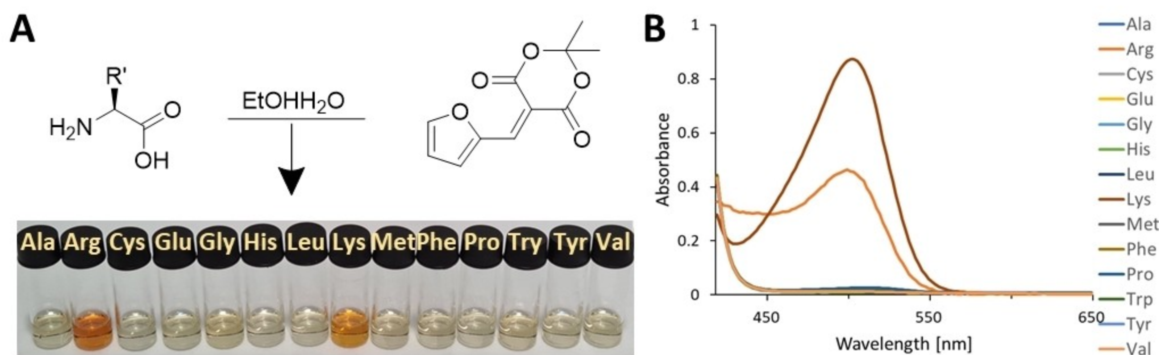

Figure 2. A) naked eye observation of 4 mM mixture of different amino acids with MAFC after 5 minutes B) UV/Vis absorption spectrum of the solutions after 5 minutes.

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These initial experiments have motivated us to investigate this phenomenon with a UV-Vis spectrometer in order to determine the detection limit. To prove the side group-selectivity of the reaction 14 representative amino acids, including hydrophobic and hydrophilic side chain were mixed with MAFC and appearance of any color was analyzed with UV/Vis-spectroscopy. As seen in Figure 2B only MAFC-lysine and MAFC-arginine showed prominent absorption peak and color reaction at 514 nm and 500 nm respectively. Whereas no peak could be observed in other MAFC-amino acid solutions. MAFC-proline shows a minor absorption peak at 512 nm which improved over time, but since this reaction takes place much slower and the peak intensity is not very high, it lacks the typical instant sensor character. It is important to note that other reactive side groups containing amino acids, like histidine, cysteine and glutamic acid were not able to offer any color reaction with MAFC.

After proving the high selectivity of MAFC towards lysine and arginine the influence of other parameters on the reaction such as time and pH were evaluated. The absorbance spectrum shows a big increase in the absorbance peak at 514 nm between 0 min and 30 min. Extending the time beyond 30 minutes did not result in improvement of the peak height, which indicates reaction completion after approx. 30 min. This experiment also depicts that the MAFC-lysine adduct is stable for at least 1 h, which gives the freedom to investigate the chemical bases of colored product. However, appearance of a shoulder peak near 400 nm, at 120 min and 180 min describes the slow decomposition of the product after 60 min (s. supporting info, Figure S2).

Since it is known that pH can influence the sensing capabilities of the probe,[33] we have tested the lysine-MAFC reaction under various pH solutions. Figure 3A displays the absorbance spectra of MAFC-lysine at pH from 1 to 14. It was observed that from pH 4–11, absorbance peak at 514 nm can be seen prominently. Whereas at pH 1–3 and 12–14 no absorption peak at 514 nm was visible, therefore we conclude that our lysine-MAFC system is capable of sensing in wide range of pH from 4 to 11. Figure 3B depicts the same behavior at a single emission wavelength of 514 nm. Similar trend was shown by arginine under different pH conditions (see supporting information Figure S4).

To assess the sensitivity of the potential colorimetric sensor different lysine concentrations were mixed with MAFC and measured via UV-Vis spectrometer. Various concentrations ranging from 0.001–40 mM were tested at neutral pH. From the UV-Vis spectra (Figure 4) we found a detection minimum at a concentration of 100 μM.

Since the absorption peak at 514 nm of MAFC-lysine was slightly higher at pH 6 and 10 compared to pH 7 (Figure 3, B), it was worth testing the peak appearance at these pHs to improve the detection limit below 100 μM. Unfortunately, spectrometric studies have revealed that the detection limit is not improved in slightly acidic or basic solutions (supporting info, Figure S3). Validity of each UV-vis experiment was check in triplicate.

After evaluating the selectivity, pH- and time-dependency and sensitivity of the colorimetric sensor, ^{1}H NMR titrations were required to strengthen our believe for the proposed reaction mechanism between activated furan and selectively reactive amino acid. We have chosen MAFC-lysine system for NMR investigations to avoid associated complex side reaction of the guanidine fragment of arginine amino acid. In the literature appearance of pink/magenta color attributes to the conjugated triene, which is mostly described in the cases where activated furan is reacted with secondary amine.[28,30,31] It is not known if there are any subsidiary reactions happening if a primary amine is implemented instead. Moreover, it was also necessary to understand that among two primary amine groups those are present on the lysine molecule (ε-amino, **5** and α-amino, **6**), which one is causing a colored reaction due to the formation of conjugated triene (Figure 5).

Mixing lysine with MAFC and measuring it with NMR, instant appearance of color and representative peaks for conjugated triene at 5.35 ppm (s, 1H), 6.45–6.49 ppm (m, 2H) and 7.55 ppm (s, 1H) in the NMR-spectra indicates an immediate reaction between MAFC and lysine (Figure 6 B). Moreover disappearance of the signals of furan ring of MAFC (Figure 6 A at 6.87 ppm (m, 1H), 8.09 ppm (m, 1H) and 8.43 (d, 1H), within first few minutes of the reaction (including the measurement time), depicts the sensing capabilities of this reaction system. Periodic recording of the NMR have shown that the resulting colored product is stable in the reaction mixture at room temperature for at least 1 hour, without

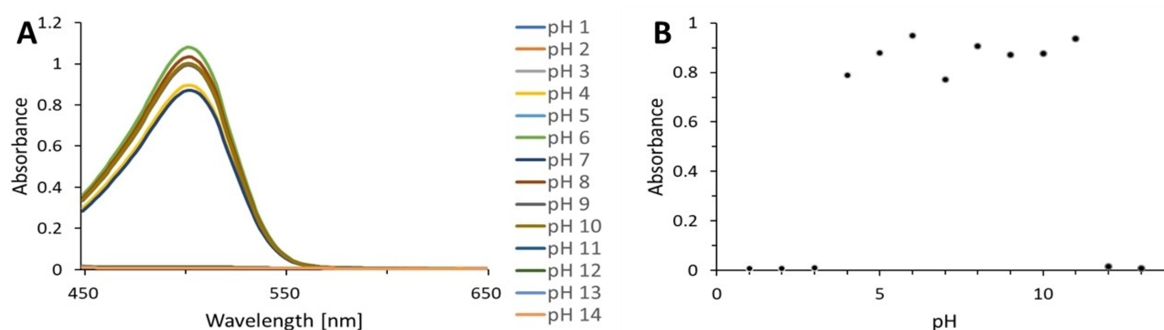

Figure 3. A) UV-Vis absorption spectrum of MAFC-lysine solutions at pH 1–14 B) absorption behavior of MAFC-lysine solution with pH 1–14 at 514 nm.

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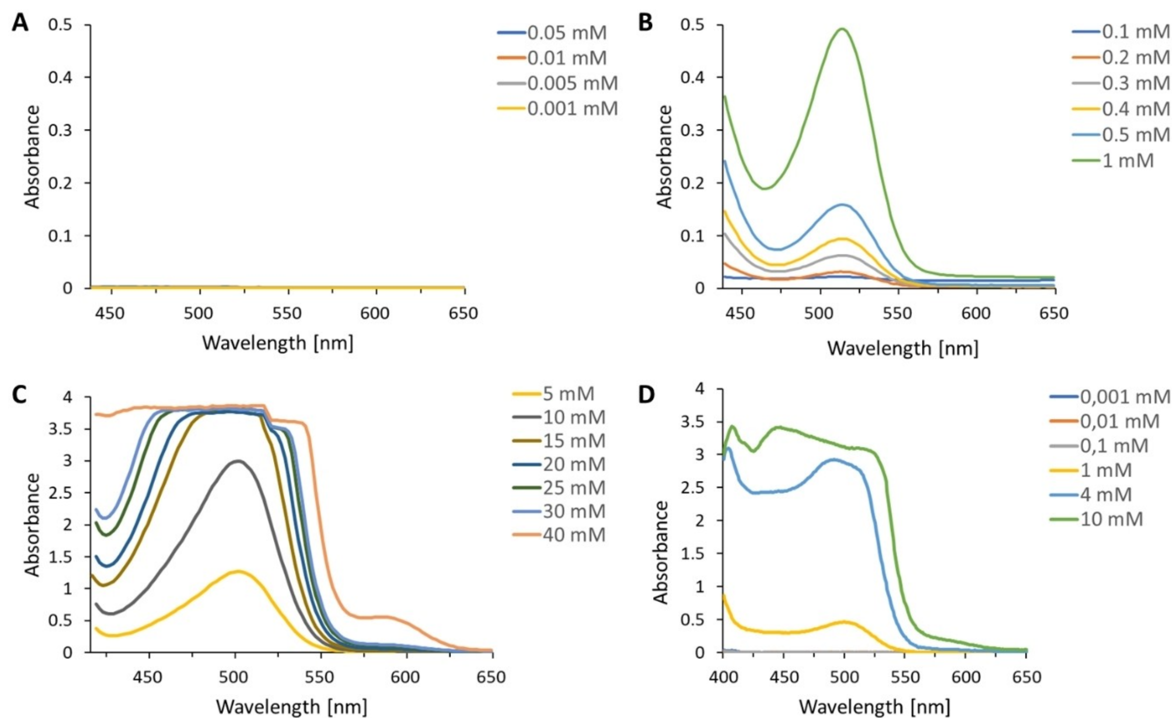

Figure 4. UV/Vis absorption spectra of A)-C) MAFC-lysine solutions with concentrations of 0.001–40 mᴍ at pH 7 and D) MAFC-Arginine solutions with concentrations of 0.001–10 mᴍ.

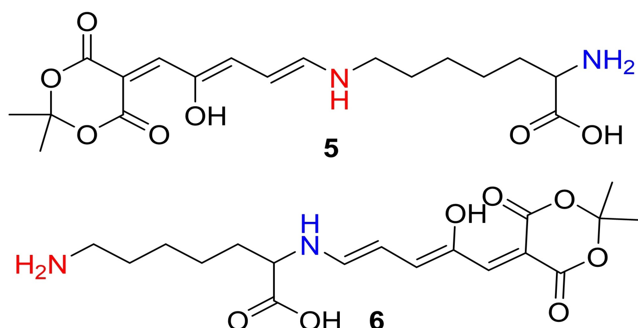

Figure 5. Possible conjugated triene adducts could be resulted from the reactions between ε-amino and α-amino groups of ʟ-lysine with MAFC.

appearance of any additional peaks (supporting info, Figure S5). These results are in conformance with the outcome of the UV/Vis experiments. A fast reaction time, high sensitivity and colorimetric indicator that is stable over time are favorable conditions for exploring the system as chemical sensors. Because it allows a high detection speed whilst giving enough time for a thorough quantitative analysis. Assignment of the peaks for conjugated triene for MAFC-lysine conjugate are compared with ring opening reaction of activated furan of the MAFC with standard diethylamine system (DEA, **7**) to gain the confidence on elucidated structure (Figure 6 C). Appearance of conjugated triene signals at 4.64 ppm (1H), 6.35 ppm (2H) and 7.61 ppm (1H) are in accordance with the NMR-spectrum of MAFC-lysine system (Figure 6 B and C). Slight up field shift in the peak at 4.64 ppm could be resulting from the two electron donating ethyl groups on the amine of DEA (**7**). In most of the studies on reactions of MAFC, secondary amines like DEA were used as nucleophile, most likely due to single substitution possibility compared to the primary amines. During the reaction of lysine with MAFC no such double adduct peaks were observed on ^{1}H NMR spectra.

In an independent ^{1}H NMR experiment we aim to distinguish the reactivity of two primary amino groups present on the lysine (Figure 5, compound **5** and **6**). From the current literature it appears that ε-amine has higher reactivity as compared to α-amino group due to higher pKa value, which becomes an obvious reason for selectivity of this reaction for lysine only because other amino acids are lacking such functional group. However, in order to confirm it, we have recorded the ^{1}H NMR spectra of the reaction of MAFC with chemical fragments of lysine, where ε-amine and α-amine can interact with MAFC separately. We have considered pentylamine **8** and glycine **9** as chemical fragments of lysine and they were reacted in equimolar quantities with MAFC **1** (Figure 6, D and E, supporting info, Figure S6). These spectra were compared with the spectrum of MAFC-lysine adduct. As expected, glycine showed no reaction with MAFC neither in terms of color adduct formation nor in the NMR-spectrum, the signals of furan ring at 6.87, 8.09 and 8.43 ppm remain intact (Figure 6, E). On the other hand, the reaction of MAFC with pentylamine **8** showed a prominent color reaction similar to MAFC-lysine adduct and at the same time typical triene peaks were observed those correspond well to the triene of MAFC-lysine (Figure 6, D).

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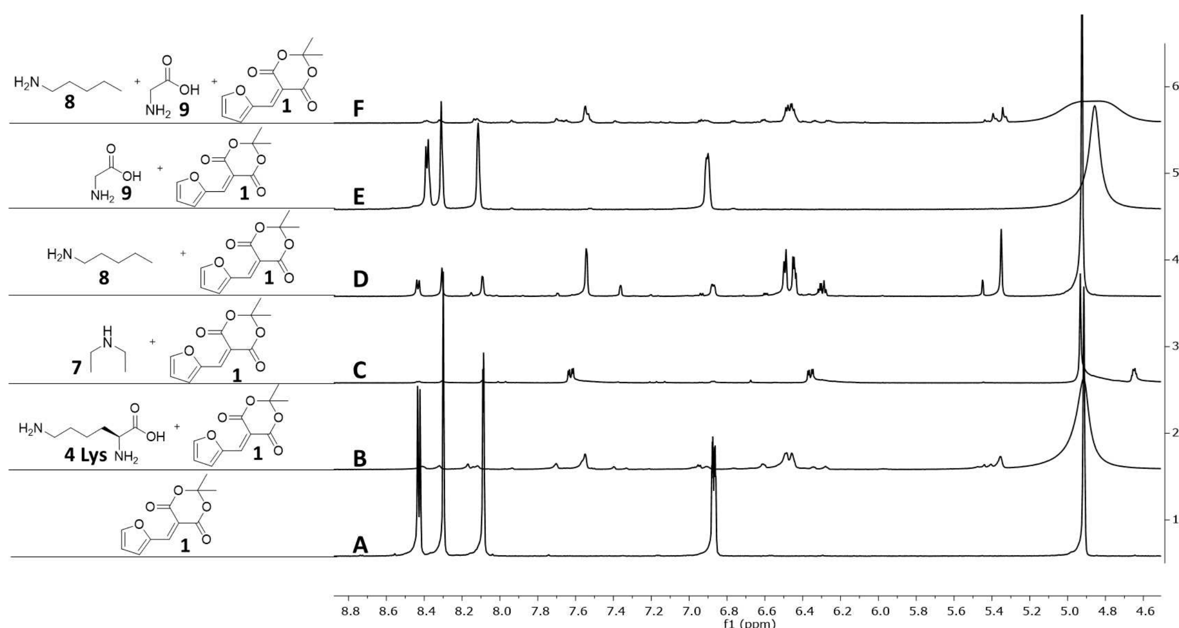

Figure 6. ^{1}H NMR of MAFC-lysine and MAFC-DEA.

Further, to exclude any possible effect of presence of α-amino group on the reaction capability of ε-amine, a mixture of glycine **9** and pentylamine **8** was added to MAFC. The reaction mixture has not shown any additional peak other than the typical peaks at 5.35 ppm, 6.45–6.49 ppm and 7.55 ppm with pentylamine only (Figure 6, F). It concludes that α-amino group from most amino acids would not be able to perform ring opening reaction and has no effect on the reaction sequence. The reason for this is probably the comparative electron deficiency of the α-amine due to the adjacent carboxyl group of amino acid. The positive inductive effect of alkyl chain length to ε-amine should have an impact on the reaction performance.

Further, we have investigated the effect of decreased lysine concentration with respect to the MAFC in order to observe if there is any secondary reaction happening on the MAFC-lysine adduct when excess amount of MAFC is available in the reaction mixture. (supporting info, Figure S7). The spectra clearly showed an incomplete reaction when 0.25 and 0.5 eq. of lysine was implemented, even elongating the reaction time to 1 hour does not offer any side reaction. Using equimolar quantities of MAFC and lysine drive the reaction to maximum extent. Increasing lysine content any further results in no improvement in signals of conjugated triene. From these experiments, we speculate that the MAFC-lysine product is a 1:1 adduct **5** of lysine ε-amine on activated furan ring of MAFC (Figure 7). Any further reaction on the adduct due to the presence of additional MAFC is ruled out.

The isolation of the MAFC-lysine adduct via crystallization or chromatography was not possible without decomposition. Therefore, all structure elucidation experiments were performed on crude reaction. Further, a LCMS chromatogram of the reaction was recorded to finalize our structure hypothesis

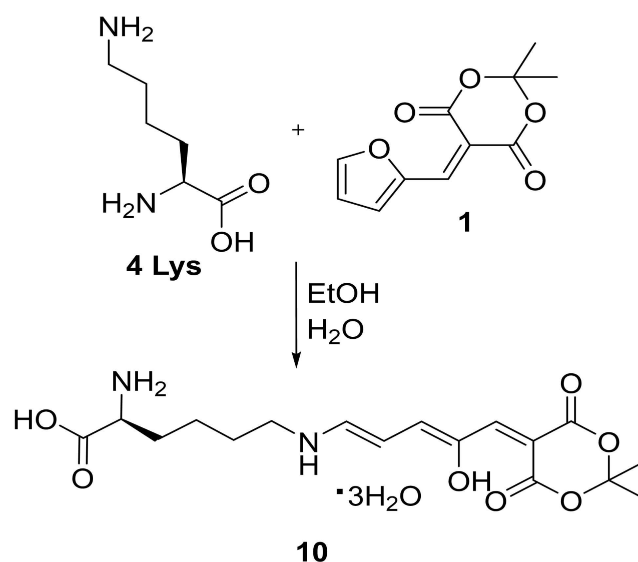

Figure 7. Proposed structure for the colored product formation by the reaction between lysine and MAFC at neutral pH.

(supporting info, Figure S8). It was noticed that the reaction went to completion after 15 minutes only and a product peak at 423$^+$ (MH$^+$) was apparent to the structure MAFC-lysine trihydrate **10**. Therefore, we finally concluded the following reaction that results in the formation of colored product selectively with amino acid lysine.

Further we have tested the validity of our reagent with example proteins mixtures, namely collagen, and pea protein. Rice and pea protein are in generally used in completion to each other as protein reach nutritional supplements because rice protein is rich in sulfur containing methionine but with low

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Figure 8. MAFC-Collagen (left) and MAFC-Pea protein solutions (right).

amount of lysine while pea protein has a high amount of lysine (7360 mg/100 g protein) and lacking the sulfur containing amino acid. On the other hand collagen which has abundance of glycine, proline, and hydroxyproline and poor in lysine content compared to the rice and pea protein can be our test model. In our experiment, it was observed that pea protein gives most colored product whereas no color reaction was visible in collagen-MAFC mixture (Figure 8), which is in good agreement with the content of lysine in these protein sample. The experiment showed that hereby reported reagent MAFC can be used to detect the presence of lysine not only in its free form but also in large sequences of peptides or proteins.

Conclusion

Discovering colorimetric chemosensors for biologically and nutritionally important molecule is an ongoing research in the interest of biotechnology and food industry. Further, in the present academic and pharmaceutical research low molecular weight species are abundantly used either for bioactivity or for chemosensing. In many instances during the total multicomponent peptide and solid phase synthesis, the amino acids play a crucial role in making desired linkages. Therefore, new non-chromatographic methods for detection of trace amounts of amino acids are most sought by the chemist and forensic experts. We have explored an unprecedented MAFC as a colorimetric sensor for selective detection of amino acid lysine and arginine with rapid detection time, high sensitivity, repeatability and good efficacy. As compared to previously known organic chemosensors, minimal synthetic requirement, and low cost and synthetic simplicity for production of MAFC makes this reagent highly attractive compared to the previously known chemosensors for amino acids detection. Further, operational simplicity in benign solvents like water and ethanol makes this method user friendly. The color formation can be easily seen by the naked eye in high concentrations; however, a detection limit of 100 μM was achieved by using standard UV/Vis spectrometer. The structure of the adduct has been elucidated using ^{1}H NMR spectroscopy and LCMS analysis. With this study, we have offered a new chemosensor for selective detection of lysine and arginine, which could be improved to enhance the detection limit.

Supporting Information Summary

In the supporting information experimental details of all experiments described in this publication as well as aforementioned but not extensively discussed results are listed. This includes synthesis and ^{1}H-NMR analysis of MAFC as well as time- and pH dependent UV-Vis measurements of MAFC-lysine and MAFC-arginine. Additionally ^{1}H-NMR measurements of MAFC-lysine at different molar ratios and MAFC-glycine and –pentylamine are depicted. LCMS analysis of the proposed MAFC-lysine trihydrate adduct can be found at the end of the supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Amino Acids · Chemoselectivity · DASA · Lysine · MAFC

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