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Laccase from Basidiomycetous Fungus Catalyzes the Synthesis of Substituted 5-Deaza-10-oxaflavins *via* a Domino Reaction

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Abstract: The present investigation provides a simple and convenient route for the synthesis of substituted 5-deaza-10-oxaflavins owing to their importance as probable redox coenzymes. The reaction of α,β-unsaturated derivatives of barbituric acid and dimedone with catechol or 1,4-hydroquinones was catalyzed using laccase in aqueous medium. Quinones, generated *in situ* by the oxidation of the corresponding catechol or 1,4-hydroquinones, underwent a domino reaction with chalcones to produce 5-deaza-10-oxaflavins and tetrahydroxanthen-1-ones.

Keywords: aqueous medium; 5-deaza-10-oxaflavins; domino reactions; laccase; tetrahydroxanthen-1-ones

Introduction

Extensive research has been carried out to explore the functions of 5-deazaflavin derivatives **1** in biological system and to develop a biomimetic model possessing the same basic skeleton since the discovery of these derivatives **1** (Figure 1) as one of the naturally occurring essential redox coenzymes.[1] In this context, 5-deaza-10-oxaflavin **2** [2*H*-chromeno[2,3-*d*]pyrimidine-2,4(3*H*)-dione] was considered (Figure 1), in which the nitrogen atom was replaced by an oxygen thus resulting in a substantial oxidation potential due to the stronger electronegativity of oxygen atom.[2] 5-Deaza-10-oxaflavin **2** possesses both isoelectronic and isosteric structures with 5-deazaflavin. It exhibits a strong function of oxidizing alcohols to the corresponding carbonyl compounds. Further derivatisation of the benzopyrano[2,3-*d*]pyrimidines, especially at C-5, led to the formation of several lead compounds with very similar pharmacological profiles as the 5-deazaflavin-based derivatives.[3]

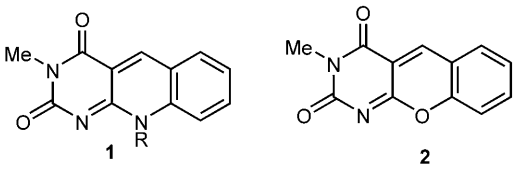


Figure 1. 5-Deazaflavin derivatives **1** and 5-deaza-10-oxaflavin **2**.

Some synthetic work on derivatives in this catagory has been reported, but these precedents did not meet our requirements. In 1980, one of the authors (F. Y.) first reported a method for the synthesis of 5-deaza-10-oxaflavin,[4] which involved three steps including condensation of a phenol to 6-chlorouracil, Vilsmeier–Haack reaction and dehydrative cyclization with polyphosphoric acid (Figure 2). However, isolation of the final product was somewhat troublesome and the overall yield was very low. Blythin and co-workers published a one-step synthesis of a 5-deaza-10-oxaflavin derivative,[5] but this approach was not considered attractive because the starting material used, *N*-cyanoacetylurethane, is not easy to prepare (Figure 2). This necessitated the search for an alternative approach.

The use of biocatalysts is being explored in new millennium industries because they are stable and remain functional in diverse physiological environments including aqueous medium.[6] The unique properties of water in aqueous medium like high dielectric constant and cohensive energy density showed an extraordinary effect on reaction rates. Moreover, its

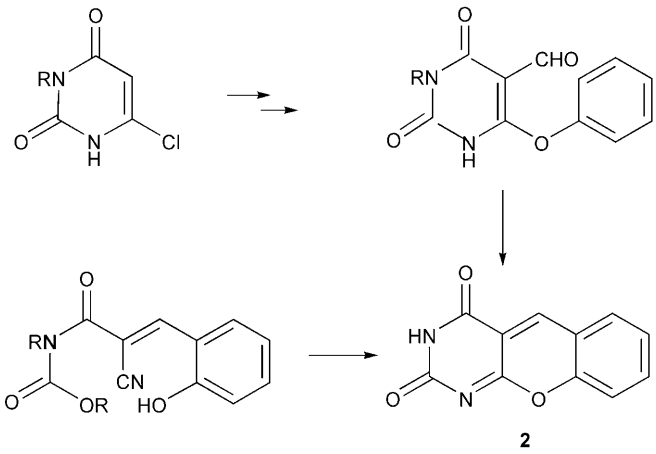


Figure 2. Synthetic approach to 5-deaza-10-oxaflavin **2**.

cost-effectiveness, high abundance, non-inflammability and non-toxic nature increased its applicability.[7]

In course of our search for an effective biomimetic model compound, 5-deaza-10-oxaflavin, and to explore chemo-enzymatic reactions in aqueous medium, an efficient laccase-catalyzed synthesis of derivatives of 5-deaza-10-oxaflavin was investigated. This reaction also required H_2O_2, a stoichiometric oxidant.

Result and Discussion

There has been a great deal of interest in the synthesis of 5-deaza-10-oxaflavin {2*H*-chromeno[2,3-*d*]pyrimidine-2,4(3*H*)-dione} owing to its biological importance and function as essential redox coenzymes.[8] A successful synthesis of 5-deaza-10-oxaflavin through fusion of quinones with chalcones was endeavored. Here the action of the laccase, for the *in situ* generation of quinones, is reported.

Laccase, a multicopper oxidase, which reduces oxygen (source of O_2 is from the disproportionation redox reaction of H_2O_2 in acidic medium) to water and simultaneously performs one-electron oxidation of many aromatic substrates such as phenol and aromatic amines. Catechol/hydroquinones have more electron density at the *ortho*-position, so they undergo 1,4-addition reaction with α,β-unsaturated derivatives of barbituric acids leading to non-isolable **8** followed by its oxidation *via* laccase to give **9** which further undergo intramolecular 1,4-addition reaction to form **10**. The intermediate **10** is oxidized by laccase to give **10a** followed by 1,4 addition to give an intermediate that exists in a resonance form consisting of structure **11** in acidic medium.[9] Upon protonation **10a** undergoes transformation to its resonance structure **11.** Due to acidic medium, **11** again gets protonated and leads to the formation of the final 5-deaza-10-oxaflavins **(5a–h/7a–h)** (Figure 3).[10]

The reaction conditions were optimized for the enzymatic synthesis of **5a** (Scheme 1) and **7a** (Scheme 2) from **3a.** An experiment was carried using 1 mmole of **3a** and 1.5 mmole of **5a/7a** in 15 mL of solvent without the addition of laccase or H_2O_2. There was no product formation. The same was also observed when 0.5 mL of 30% of H_2O_2 and heat killed laccase were also added. In another experiment, when 400 U laccase were added without H_2O_2, then only traces of product formation were observed on TLC. The yield of **5a** was significantly improved with the addition of 0.5 mL of 30% H_2O_2 along with 400 U of laccase. Excess of chalcone was required for the reaction; quinones may undergo competing decomposition, dimerization or polymerization due to their intrinsic unstability. Quinones and α,β-unsaturated derivatives of barbituric acid were used in a 1:1.5 ratio to tackle the problem.

The enzymes have been immobilized using several methodologies for their biocatalytic applicability affecting their activity, effectiveness of utilization, deactivation, regeneration kinetics and cost too[11] without considering the toxicity of the immobilization reagents and the waste disposal problem. In this study, the laccase-catalyzed synthesis was carried out in aqueous medium for three consecutive runs and monitored by TLC. Table 1 depicts the yield of the products 5-deaza-10-oxaflavin **5a** and **7a** from the reaction catalyzed by laccase in three consecutive runs. The yield was observed to decrease slightly after each run.

Table 2 shows the effect of different solvent systems on product yield. In DMSO, DMF and MeCN no reaction was observed due to denaturation of laccase. The reaction in aqueous buffer proceeded in higher yield using buffer tablets of NaH_2PO_4, Na_2HPO_4, and potassium phthalate (pH 4). The lower percentage yields in other solvent systems were due to a decrease in laccase activity in non-inter miscible organic and aqueous phases. Moreover, the domino reaction was shown to exhibit higher reactivity and selectivity in aqueous medium rather than in organic solvents.

Furthermore, the effect of reaction temperature was studied by varying it from 5 °C to 60 °C. The optimal reaction temperature was found to be room temperature (25 °C). This could be attributed to the increase in the rate of decomposition and polymerization of the *in situ* generated quinones when a higher temperature was employed while the insolubility of the α,β-unsaturated derivatives of barbituric acids was observed at lower temperature.

The reaction was observed with diverse α,β-unsaturated derivatives of barbituric acids under the optimized conditions (Scheme 3 and Scheme 4). The choice of aldehyde was important based on its biological activity. For example, in chlorobenzaldehyde and 2-chloroquinolin-3-ylaldehyde, the chloro group en-

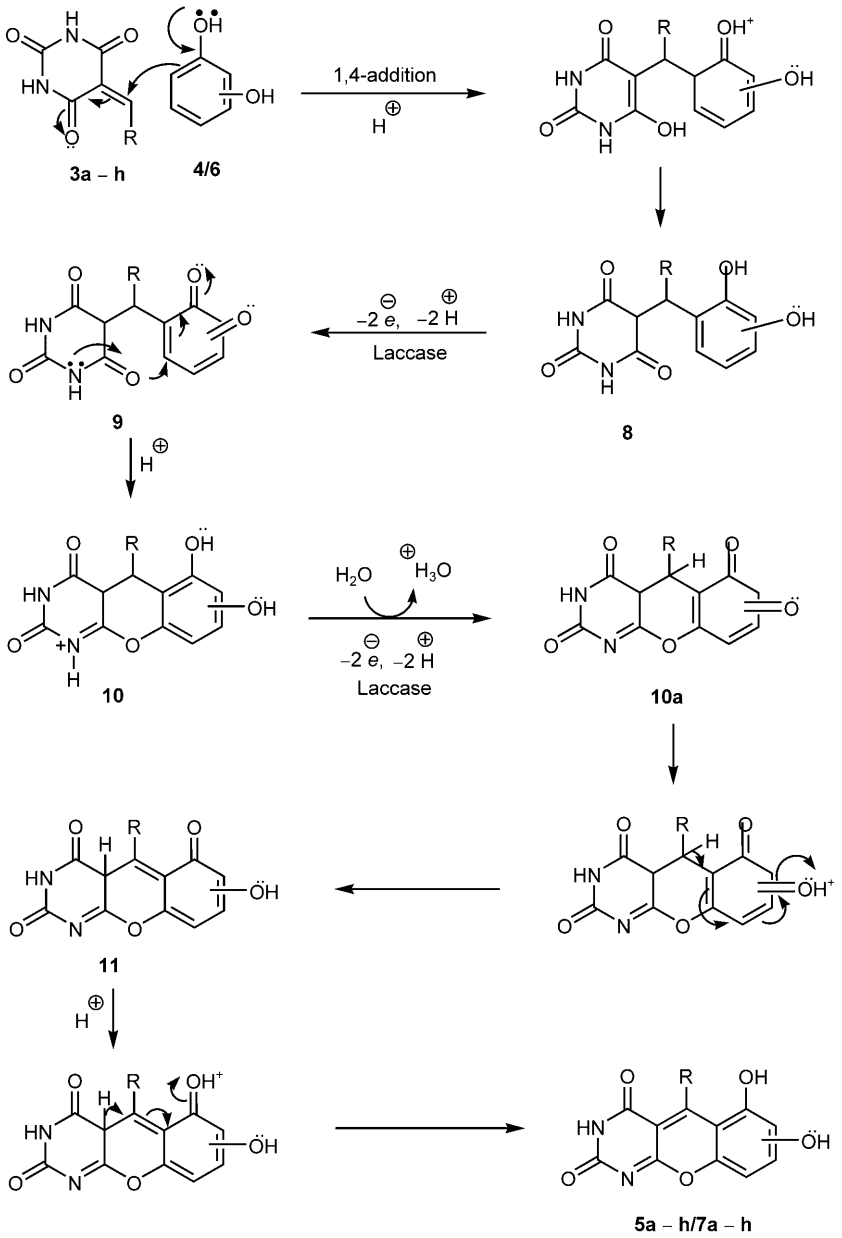
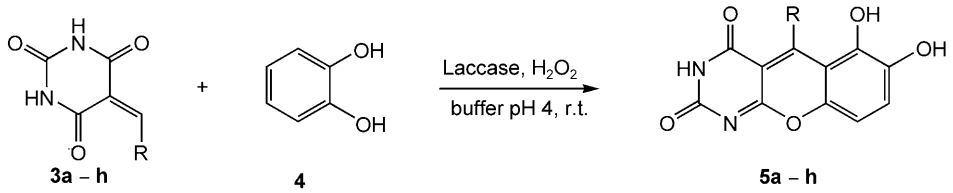


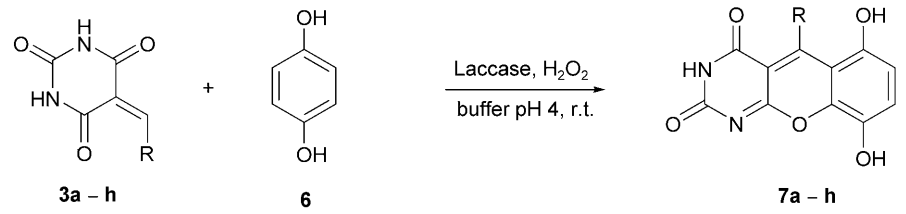
Figure 3. Proposed mechanism pathway.



Scheme 1.

hances the biological activity whereas heteroaromatic aldehydes like thiophenecarboxaldehyde, benzo-[1,3]dioxol-5-aldehyde and 2*H*-indol-3-carboxaldehyde, are employed for superior activity. On other side, nitro and methoxy groups were also found to possess activity. These aldehydes were chosen to carry out the synthesis.[12] The reactivity of the α,β-unsaturated derivatives of barbituric acids with the *in situ* generated quinone is shown in Table 3 and Table 4.

These observations emphasized the need to explore the scope of this process to the chalcone of dimedone, which result in the xanthene moiety, an important



3a – h 6 7a – h

Scheme 2. R = phenyl, 4-methoxyphenyl, benzo[1,3]dioxol-5-yl, 2-thienyl, 3-nitrophenyl, 4-chlorophenyl, 2*H*-indol-3-yl, 2-chloroquinolin-3-yl.

Table 1. 5-Deaza-10-oxaflavin derivatives **5a** and **7a**: synthesis by catalysis of laccase using the optimized conditions.[a]

Runs	**5a** Yield [%][b]	**7a** Yield [%][b]
I (Fresh)	72	76
II	68	69
III	62	61

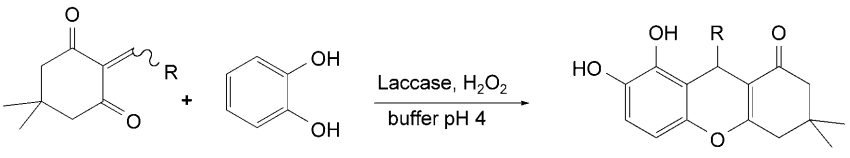
[a] *Reaction conditions:* 1 mmole **4** or **6** and 1.5 mmoles of **3a** were taken in 15 mL of buffer solution and THF was added to dissolve the reactants. 400 U of laccase and 0.5 mL of 30% H_2O_2 were added and the resultant solution was stirred well for 4 h.
[b] Yield refers to the isolated and unoptimized yield.

Table 2. Solvent optimization for the synthesis of 5-deaza-10-oxaflavin.[a]

Entry	Solvent	**5a** Yield [%][b]	**7a** Yield [%][b]
1	1 M Acetate buffer pH 4	25	29
2	Water	–	–
3	THF	53	57
4	Ethanol	40	46
5	1 M Phosphate/Citrate buffer pH 5.5	–	–
6	1 M Buffer tablet of pH 4 + THF	72	76
7	Methanol	23	27
8	DMSO	–	–
9	MTBE	15	23
10	MeCN	–	–
11	1,4-Dioxane	37	35
12	DMF	–	–
13	*t*-BuOH	39	42

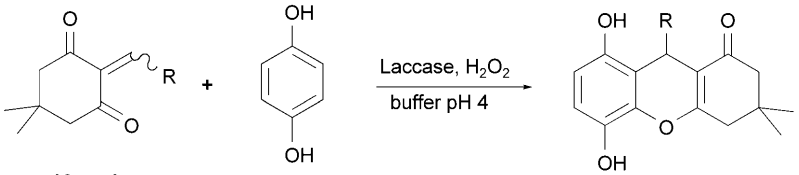
[a] *Reaction conditions:* 1 mmole **4** or **6** and 1.5 mmoles of **3a** were taken in 15 mL of solvents. 400 U of laccase and 0.5 mL of 30% H_2O_2 were added and the resultant solution was stirred well for 4 h.
[b] Yield refers to the isolated and unoptimized yield.

synthon for the synthesis of various heterocyclic compounds. Xanthene-based fluorescent dyes are widely used in chemical biology to detect biological substances, such as proteins, DNA and sugars with high sensitivity and selectivity.[13] The classical synthetic approaches are numerous with the use of harsh organic solvents such as *p*-TSA, polyaniline-*p*-toluenesulfonate salt, sulfamic acid, glacial acetic acid,[14] AcOH-H_2SO_4 and Amberlyst-15 etc. under acidic conditions. Here, the desired derivatives of 9-aryl-7,8-dihydroxy-3,3-dimethyl-2,3,4,9-tetrahydro-xanthen-1-one were obtained in a reduced reaction time period and with improved yield.



12a – d 4 13a – d

Scheme 3.



12a – d 6 14a – d

Scheme 4. R = phenyl, 4-methoxyphenyl, benzo[1,3]dioxol-5-yl, thiophen-2-yl.

Table 3. Laccase-catalyzed synthesis of 5-deaza-10-oxaflavin derivatives **5a–h** and **7a–h**.[a]

S. No.	Entry No.	R	**5a–h**		**7a–h**	
			Yield [%][b]	Time [h]	Yield [%][b]	Time [h]
1	**a**	Phenyl	72	4	76	4.5
2	**b**	4-Methoxyphenyl	63	4.5	26	6.5
3	**c**	Benzo[1,3]dioxol-5-yl	65	5.5	57	5
4	**d**	Thiophen-2-yl	55	3.5	24	4.5
5	**e**	3-Nitrophenyl	25	7.5	48	8.5
6	**f**	4-Chlorophenyl	43	6	9.5	6
7	**g**	2*H*-Indol-3-yl	52	4	65	5
8	**h**	2-Chloroquinolin-3-yl	64	4.5	58	4

[a] *Reaction conditions:* 1 mmole **4** or **6** and 1.5 mmoles of **3a–d** were taken in 15 mL of solvents. 400 U of laccase and 0.5 mL of 30% H_2O_2 were added and the resultant solution was stirred well for 4 h.
[b] Yield refers to the isolated and unoptimized yield.

Table 4. Laccase-catalyzed synthesis of 5-deaza-10-oxaflavin derivatives **13a–d** and **14a–d**.[a]

S. No.	Entry No.	R	**13a–d**		**14a–d**	
			Yield [%][b]	Time [h]	Yield [%][b]	Time [h]
1	**a**	Phenyl	68	4	56	4.5
2	**b**	4-Methoxyphenyl	55	4.5	47	6.5
3	**c**	Benzo[1,3]dioxol-5-yl	57	5.5	43	5
4	**d**	Thiophen-2-yl	42	3.5	37	4.5

[a] *Reaction conditions:* 1 mmole **4** or **6** and 1.5 mmoles of **11a-d** were taken in 15 mL of solvents. 400 U of laccase and 0.5 mL of 30% H_2O_2 were added and the resultant solution was stirred well for 4 h.
[b] Yield refers to the isolated and unoptimized yield.

Conclusions

In the present work, the simple reaction conditions, easy work-up procedure and product yield in consecutive runs made our methodology a valid contribution to the existing organic synthesis approach of 5-deaza-10-oxaflavin.

Experimental Section

The chalcone was prepared according to the literature procedure.[15] Guaiacol was purchased from Hi Media Laboratories Pvt. Ltd. (Mumbai, India). All media components and chemicals used were of analytical grade.

Microorganism

The basidiomycetous fungus, RCK-1, isolated from deteriorated sugarcane bagasse, collected from Sugar Mill Industry, Sonipat, Haryana, India, was grown and maintained on malt extract agar (MEA) containing ($g\,L^{-1}$): malt extract 20.0, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, $Ca(NO_3)_2{\cdot}4H_2O$ 0.5, agar 20.0 (pH 7.0) at 30 °C.[16] Pure cultures were stored at 4 °C and sub-cultured every fortnight.

Laccase Production

Static cultivation was carried out at 30 °C in 250-mL Erlenmeyer flasks with 50 mL malt extract broth (MEB) containing ($g\,L^{-1}$): malt extract 20.0, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, $Ca(NO_3)_2{\cdot}4H_2O$ 0.5 (pH 7.0). The flasks were inoculated with two fungal discs (8 mm diameter each) from the periphery of 4-day-old fungal culture and incubated at 30 °C. After 96 h of growth, 50 μL copper sulphate (1 M) were added to the MEB. The cultures were harvested by filtering through Whatman No. 1 filter paper after 264 h of growth. The culture filtrate was centrifuged at $12{,}000\times g$ for 20 min at 4 °C and the supernatant obtained was assayed for laccase activity.

Purification of Laccase

The culture filtrate (900 mL) containing laccase enzyme was precipitated by addition of ammonium sulphate (80% cut-off) and centrifuged at $10{,}000\times g$ for 20 min 4 °C. The precipitate was resuspended in 50 mM citrate phosphate buffer (pH 5.5) and dialyzed at 4 °C against the same buffer. This partially purified enzyme (78 mL) was concentrated to 20 mL using 10 kDa filter membrane (Vivaspin, Vivascience, Sartorius Group, Stone house, UK) at 4 °C and thereafter, the concentrated enzyme sample was loaded to an anion-exchange DEAE-sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) column ($25\times 2\,cm^2$), equilibrated with 10 mM Tris HCl buffer (pH 7.5). The proteins were eluted

by NaCl gradient (0–1 M dissolved in equilibrating buffer) at a flow rate of 0.5 mL min^{-1} with each 1 mL fraction. Fractions with laccase activity were pooled, desalted and assayed for laccase activity.

Enzyme Assay

Guaiacol was used as a substrate for assaying laccase activity.[16] One unit (U) of laccase was defined as the change in absorbance of 0.01 mL^{-1} min^{-1} at 470 nm.

General Procedure for the Synthesis of 2*H*-Chromeno[2,3-*d*]pyrimidine-2,4(3*H*)-dione and Tetrahydroxanthen-1-one

Catechol or hydroquinone (1 mmole) and chalcone (1.5 mmoles) were taken in 15 mL of buffer solution where THF was added to dissolve the reactants. Then, purified laccase (400U) was added to the resultant solution and stirred well. 0.5 mL of 30% H_2O_2 was added to oxygenate the reaction mixture. The progress of the reaction was checked by TLC examination at an interval of every 30 min. Upon completion of reaction, the reaction mixture was extracted with ethyl acetate (3×15). The organic layer was dried over Na_2SO_4. The product was purified by column chromatography using a mobile phase of hexane:ethyl acetate (80:20) and recrystallized with ethanol. The aqueous phase was further used for another run without any pre-treatment.

5,6-Dihydroxy-10-phenyl-9-oxa-1,3-diaza-anthracene-2,4-dione (5a): HR-MS: m/z = 324.2322 (M^+); anal. calcd for $C_{17}H_{10}N_2O_5$: C 62.96, N 8.64, H 3.73%; found: C 62.85, N 8.59, H 3.86%; IR (KBr pellet): ν = 3322.44 (OH), 3205.54 (NH), 1733.56 (C=O), 1672.34 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 6.38–7.54 (m, 7H, Ar), 8.81 (s, 2H. OH), 9.85 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 113.41,114.42, 115.68, 119.39, 128.39, 129.59, 131.47, 133.48, 135.82, 137.06, 143.25, 144.77, 164.18, 165.51, 166.99, 168.77, 191.20.

5,8-Dihydroxy-5-phenyl-9-oxa-1,3-diaza-anthracene-2,4-dione (7a): HR-MS: m/z = 324.1452 (M^+); anal. calcd. for $C_{17}H_{10}N_2O_5$: C 62.96, N 8.64, H 3.73%; found: C 62.75, N 8.69, H 3.76%; IR (KBr pellet): ν = 3312.24 (OH), 3203.45 (NH), 1730.65 (C=O), 1673.42 cm^{-1} (C=O); ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 6.38–8.20 (m, 7H, Ar), 8.90 (s, 2H. OH), 10.25 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 113.41, 114.42, 115.68, 119.39, 128.39, 129.59, 131.47, 133.48, 135.82, 137.06, 143.25, 144.77, 164.18, 165.51, 166.99, 168.77, 191.20.

7,8-Dihydroxy-3,3-dimethyl-9-phenyl-2,3,4,9-tetrahydro-xanthen-1-one (13a): HR-MS: m/z = 336.1211 (M^+); anal. calcd. for $C_{21}H_{20}O_4$: C 74.98, H 5.99%; found: C 75.05, H 5.89%; IR (KBr pellet): ν = 3310.94 (OH), 1738.14 (C=O), 1674.65 cm^{-1} (C=C); ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.93 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 5.08 (1H, s, C_9-H), 6.66–9.20 (m, 11H, Ar), 10.23 (s, 2H. OH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 25.01, 29.18, 50.21, 111.84, 117.31, 122.56, 123.24, 123.84, 125.68, 126.89, 127.51, 127.62, 127.84, 128.36, 129.46, 130.12, 131.25, 148.51, 154.67, 164.56, 195.71.

5,8-Dihydroxy-3,3-dimethyl-9-phenyl-2,3,4,9-tetrahydro-xanthen-1-one (14a): HR-MS: m/z = 336.1211 (M^+); anal. calcd. for $C_{21}H_{20}O_4$: C 74.98, H 5.99%; found: C 75.05, H 5.89%; IR (KBr pellet): ν = 3310.94 (OH), 1738.14 (C=O), 1673.83 cm^{-1} (C=C); ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.96 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 4.86 (1H, s, C_9-H), 6.66–9.20 (m, 17H, Ar), 9.97 (s, 2H. OH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 25.14, 29.13, 50.14, 111.64, 117.25, 122.43, 123.12, 123.96, 125.35, 126.39, 127.46, 127.53, 127.93, 128.78, 129.35, 130.38, 131.46, 148.35, 154.43, 164.56, 195.71.

Supporting Information

Additional experimental procedures and spectral data are available as Supporting Information.

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