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# Horseradish Peroxidase (HRP): A Tool for Catalyzing the Formation of Novel Bicoumarins

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Horseradish peroxidase (HRP) was used to effectively catalyze the dimerization of coumarins. On the basis of screening and preparative transformation, the catalytic ability of HRP to dimerize various coumarins was investigated. Coumarins that only had one substituted phenolic hydroxyl group could be selectively transformed into novel bicoumarins, while coumarins that had two or more hydroxyls could be transformed into multiple bicoumarins. Eight bicoumarins were obtained and identified by spectroscopic analysis. Among them, bicoumarins **1b**, **2a**, **2b**, **3a** and **3b** are reported here for the first time. The interactions between the different types of coumarins and HRP were also investigated by *in silico* docking analysis. Using the transformed products as standard substances, kinetic analyses of the catalytic reactions were carried out. The optimized reaction conditions for the dimerization of coumarins catalyzed by HRP were: 1 g/L HRP, 0.66 M $H_2O_2$, pH 3, 25 ºC, and 15 min of incubation time. The yields of the bicoumarins ranged from 10 to 40%, especially for bicoumarin **1a**, which was obtained in an approximate 35% yield. In an *in vitro* bioassay, several novel bicoumarins displayed potential $\alpha$-glucosidase inhibitory activities, which can be further used to develop antidiabetic agents.

## Introduction

Industrial bio-catalysis is a key technology of the biotechnology industry. The development of highly efficient catalysts and the control of biochemical reactions at the molecular level are the core of industrial bio-catalysis. Enzymes, which are highly efficient bio-catalysts, can catalyze various reactions at ambient temperature, atmospheric pressure, and neutral pH. This type of catalysis is almost impossible to complete via synthetic chemical methods.[1] Enzymes display high performance and specificity and generate less waste, which comply with the principles of green chemistry.

Peroxidases (PODs) are composed of a group of enzymes that are a series of hemoproteins containing copper or ferric ions at the active sites. They are regarded as highly selective and efficient bio-catalysts. Peroxidases are known to catalyze various oxidative transformations of organic and inorganic compounds using $H_2O_2$ and other related peroxides as oxidizing agents.[2] For example, thyroid peroxidase, lactoperoxidase and myeloperoxidase are obtained from animals, whereas horseradish peroxidase, *momordica charantia* peroxidase and cytochrome C peroxidase are mainly isolated from plants.

Horseradish peroxidase (HRP) is a typical peroxidase enzyme obtained from the roots of *horseradish*. HRP also exists in other *Cruciferae* plants, such as *Arabidopsis thaliana*.[3,4] Therefore, HRP has the advantage of being abundant. There are several peroxidase isoenzymes in the roots of *horseradish*. Isoenzyme C (HRP C) is the most abundant one, and a large number of studies have used the corresponding recombinant enzyme. HRP C is a glycoprotein with a molecular weight of 44 KD and the saccharide moieties consisting of almost 18% of its mass. The carbohydrate is thought to be an important constituent of HRP and imparts stability to the enzyme at high temperatures. The 3D structure of HRP C was first reported by Gajhede *et al*. in 1997.[5] HRP C is known to be composed of 308 amino acid residues, with a maximum UV absorptions at $\lambda_{max}$ 403 nm and 275 nm. The enzyme active site consists of a heme group [iron (III) protoporphyrin IX] and two calcium atoms.

Compared with various peroxidases, HRP is the first choice for the study of peroxidases. Some of the characteristic properties of HRP include its low price, significant resistance to organic solvents, and presence of a large number of substrates.[4] HRP has been widely used in organic synthesis, polymer synthesis, and phenolic waste water treatment. In the presence of hydrogen peroxide, HRP can catalyze the polymerization of phenols, phenylamines, and their corresponding analogues. For example, Wilkens and coworkers obtained two novel trimeric stilbenes by HRP-catalyzed

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Electronic Supplementary Information (ESI) available: Structural identification and $^1$H NMR, $^{13}$C NMR, HSQC, HMBC, HRESIMS spectra of of bicoumarins. See DOI: 10.1039/x0xx00000x

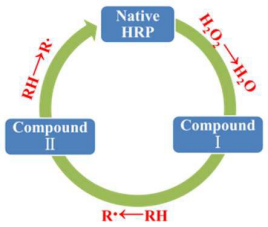



**Fig. 1** Proposed catalytic cycle of horseradish peroxidase (HRP).

biotransformation of trans-resveratrol and (-)-ε-viniferin.[6] Zhou *et al.* performed lignosulfonate polymerization by incubation with HRP/$H_2O_2$.[7] These polymers can be used to prepare organic conductive polymers, organic nonlinear optical materials, and light emitting diodes. The presence of an extensive π conjugated system in the structures of these polymers gives rise to the above-mentioned properties in them. Additionally, HRP is also widely used in immunoassays and in the enzymatic analysis of molecular markers.

The catalytic mechanism of HRP has been well studied.[8-10] HRP can catalyze the oxygen transfer reaction between different organic compounds and $H_2O_2$ or other alkyl peroxides. The reaction is a three-step cyclic reaction by which the enzyme is first oxidized by $H_2O_2$ and then reduced in two sequential one-electron transfer steps from the substrates, which are typically small molecule phenol derivatives. The catalytic cycle of the peroxidases can be summarized in the diagram shown in Fig. 1.

Coumarins are well known natural compounds that are widely distributed in many higher plants, such as Rutaceae, Umbelliferae, Leguminosae, and Orchidaceae. Coumarins are well known to have anti-inflammatory, anti-tumor, anti-HIV, anti-biosis, and anti-oxidation properties.[11-14] Coumarins are organic fluorescent chromophores that can be modified by substitutions at different positions of the rings to produce derivatives of various colors and hyperfluorescence. Coumarin derivatives have been used as fluorescent materials in fluorescent brighteners, laser dyes, and fluorescence sensors.[15] Furthermore, it has been reported that 3-phenylcoumarin derivatives have inhibitory effects on the catalytic activity of HRP.[16] Bicoumarin is an important coumarin derivative that has been previously isolated from plants and microorganisms or synthesized by chemical methods.[17-21] Pharmacological investigations have shown that

**Table 1** Screening transformation of various coumarins by HRP

| No. | Name | Structure | Conversion | No. | Name | Structure | Conversion |
|---|---|---|---|---|---|---|---|
| 1 | 6,8-Dimethoxy-7-hydroxycoumarin | | Selective- | 11 | 7,8-Dihydroxy-4-phenyl coumarin | | Multi- |
| 2 | 6-Hydroxy-coumarin | | Selective- | 12 | Esculin | | Multi- |
| 3 | 6-Hydroxy-4-methylcoumarin | | Selective- | 13 | 4-Hydroxycoumarin | | No- |
| 4 | 7-Hydroxy-coumarin | | Selective- | 14 | Ethylcoumarin-3-carboxylate | | No- |
| 5 | 7-Hydroxy-4-methylcoumarin | | Selective- | 15 | Coumarin-3-carboxylic acid | | No- |
| 6 | 6,7-Dihydroxy-coumarin | | Multi- | 16 | Dicoumarol | | No- |
| 7 | 4-Methyl-6,7-dihydroxycoumarin | | Multi- | 17 | 6-Methylcoumarin | | No- |
| 8 | 6,7-Dihydroxy-4-phenylcoumarin | | Multi- | 18 | Coumarin | | No- |
| 9 | 4-Methyl-7,8-dihydroxycoumarin | | Multi- | 19 | 6,8-Dimethoxy-coumarin-7-*β*-D-glucoside | | No- |
| 10 | 7,8-Dihydroxy-6-methoxycoumarin | | Multi- | | | | |

bicoumarins have various useful biological properties. They exhibit anti-coagulating, anti-platelet, anti-inflammatory, and analgesic effects in addition to imparting inhibitory effects on the lyase activity of DNA polymerase *β*, snake venom and human nucleotide pyrophosphatase.[17] In comparison to coumarins, bicoumarins have significantly higher molar absorption coefficients and show red shifts in the absorption and emission wavelengths. Therefore, bicoumarins are also widely used as fluorescent dyes.

Kong *et al*[22] have reported biotransformation of bicoumarins, as catalyzed by *Momordica charantia* peroxidase. Only one coumarin (7-hydroxy-4-methylcoumarin) was transformed by the peroxidase to produce several bicoumarins. The synthesis of bicoumarin requires a multi-step chemical reaction. Considering the biological effects of bicoumarins, HRP has been used in this study to transform coumarins to bicoumarins, and the optimum reaction conditions have been comprehensively investigated. The bioactivities of the synthesized bicoumarins were then evaluated using an *in vitro* bioassay.

## Results and discussion

### Screening experiment

A total of 19 coumarins with different chemical structures were used as substrates in the screening experiment with HRP as the catalyst. Various substituents were attached to the coumarin skeleton, including hydroxyl, methoxy, glucose, phenyl, and carbonyl groups. A coumarin oligomer (coumarin **16**) was also employed. Two milligrams of the substrate and 0.5 mg of HRP were added to 1 ml of buffer for incubation at 25 ºC. The products were extracted with $CH_2Cl_2$ and analyzed by HPLC-DAD. Compared with the starting materials, the chromatograms clearly showed the conversion of different coumarins by HRP (Table 1). Coumarins **1–5** could be catalyzed selectively by HRP to form one or two major products. However, coumarins **6–12** produced multiple products non-selectively, while no product was detected during the oxidation of coumarins **13–19**. Based on the mechanism of HRP catalysis reported earlier in the literature, these transformation reactions can be explained. The oxidative coupling of coumarins catalyzed by HRP in the presence of $H_2O_2$ was a free radical reaction. The phenolic hydroxyl groups on the coumarins played a major role in these reactions. Therefore, the coumarins without a hydroxyl group could not undergo HRP-catalyzed dimerization. Molecular docking was used to calculate the interaction between coumarins and the molecular model of HRP C. These oxidation of coumarins into selective products, multiple non-selective products, and no-products were chosen to dock in the HRP C molecular model.

Assuming that these analogs bind with the enzyme using a similar binding mode, we selected a reasonable bioactive mode from the top three modes ranked by the Hammet score. To view the interaction mode more clearly, these coumarins were classified into four groups: (1) substrates with one phenolic hydroxyl group, (2) substrates with two phenolic hydroxyl groups at C-6 and C-7, (3) substrates with two phenolic hydroxyl groups at C-7 and C-8, and (4) substrates with an OH group on the lactone ring (4-Hydroxycoumarin, **13**). The interaction modes are depicted in Fig. 2. As shown in Fig. 2A, 2D, and 2E, 7-OH of coumarins **1**, **4** and **5** interacted with the conserved His42 and Arg38 residues in the catalytic site. Additionally, hydrogen bonds formed among the $H_2O$ molecules of the crystallographic complexes. 6-OH of coumarins **2** and **3** interacted only with catalytic residue His42 (Fig. 2B, 2C). Coumarins **6**, **7** and **8** have two hydroxyl groups at C-6 and C-7. As shown in Fig. 2F, 2G, 2H, these compounds could adopt a conformation such that the two hydroxyl groups formed hydrogen bonds with catalytic residue His42. Compared with compounds **6** and **7**, compound **8** was flipped

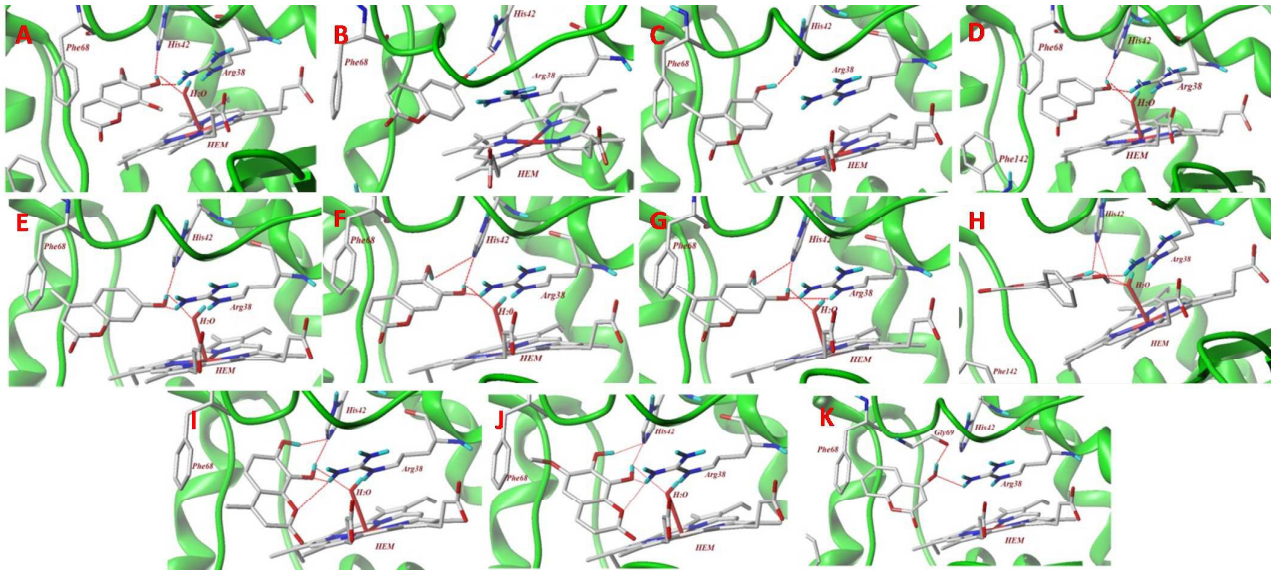


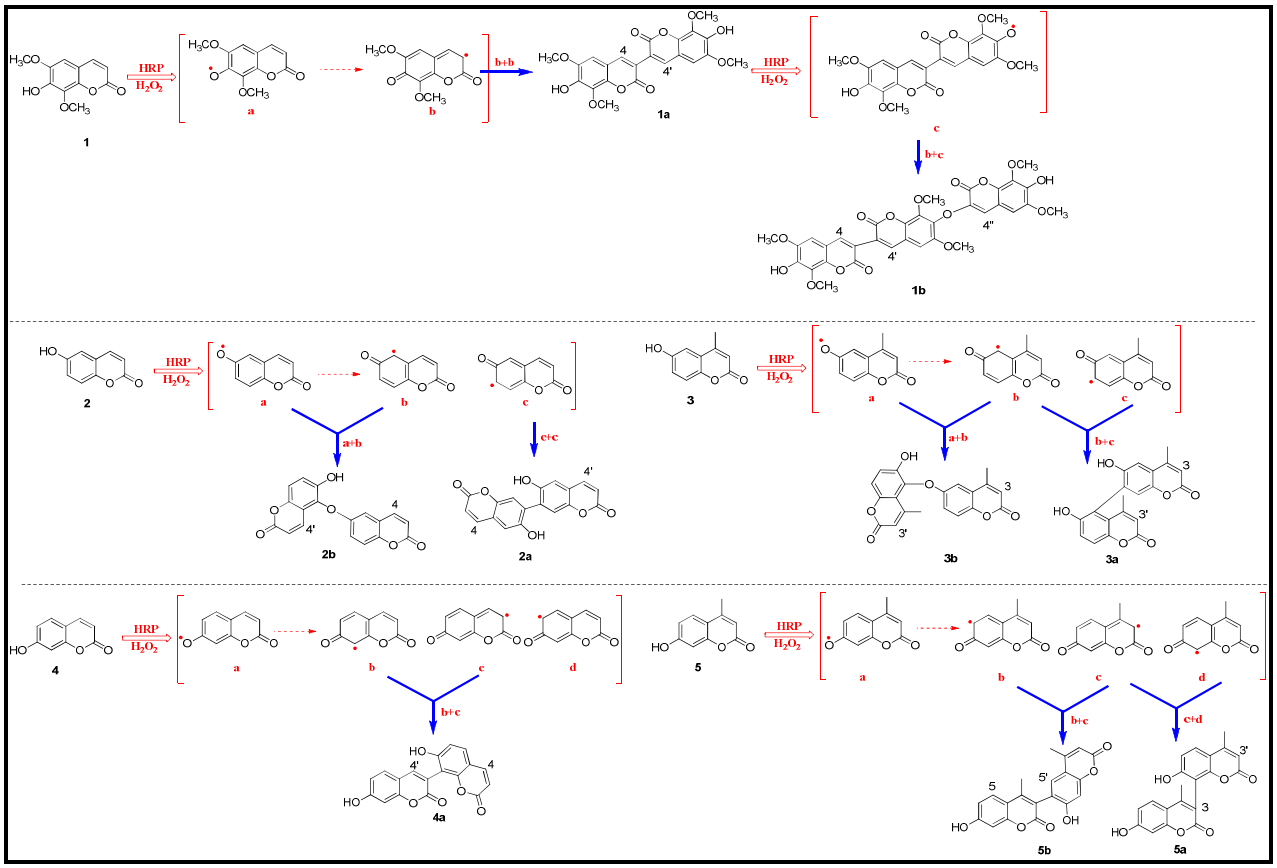

**Fig. 2** The interaction binding modes of coumarins with HRP C. (A) Coumarin **1**. (B) Coumarin **2**. (C) Coumarin **3**. (D) Coumarin **4**. (E) Coumarin **5**. (F) Coumarin **6**. (G) Coumarin **7**. (H) Coumarin **8**. (I) Coumarin **9**. (J) Coumarin **10** and (K) Coumarin **13**.

at 180°. Hydroxyl groups at C-7 and C-8 of compounds **9** and **10** could also simultaneously interact with His42 (Fig. 2I, 2J). As for compound **13**, a hydrogen bond was formed between Arg38 and Gly69. However, no hydrogen bond was observed between the hydroxyl group and His42, as shown in Fig. 2K.

Molecular docking studies revealed that the coumarins adopted similar binding modes in the catalytic site of HRP. In general, these results suggest that the target compounds bind to HRP in a way that their specific hydroxyl groups substituted the positions originally occupied by the oxygen atoms of the benzhydroxamic acid in the crystallographic complexes, close to the heme group.[23] Hydrogen bonds were formed between the εN atom of catalytic His42 and the hydroxyl groups of coumarin molecules.[24] Additional interactions were found between Arg38 and $H_2O$ (Fig. 2A). The extensive hydrogen bonding network with His42 and Arg38 and the distal water molecule above the heme iron makes the extended interaction between coumarin and the heme center of HRP C possible. For coumarins with two phenolic hydroxyls, both hydroxyl groups could form hydrogen bonds with catalytic His42. Hence, different free radicals were formed. The different phenolic hydroxyl radicals linked to form dimers, trimers, and even higher oligomers, which resulted in the formation of more diverse metabolites.[25] For compound **13**, the hydroxyl group was too far away from the N atom of His42 to form a hydrogen bond, whereby a radical was not produced. The molecular modeling studies provide useful information to better understand the interaction modes and features of coumarins with HRP.

**Preparative experiment**

Although bicoumarins were produced by *Momordica charantia* peroxidase catalyzed reactions, few investigations were carried out to understand the selectivity, yields, and reaction conditions.[22] Using a screening assay, preparative biotransformations of coumarins **1–5** were carried out. Briefly, 500 mg substrates (**1–5**) were incubated with HRP and $H_2O_2$, and the products were isolated by preparative chromatography techniques. The transformed products **1a**, **1b**, **2a**, **2b**, **3a**, **3b**, **4a**, **5a**, and **5b** (Scheme 1) were obtained as yellow amorphous powder. Analysis of the spectroscopic data, including HRESIMS, $^1$H NMR (Table S1), $^{13}$C NMR (Table S2), HSQC, and HMBC, determined the structures of these products to be bicoumarins, except for **1b**, which was a trimer. Finally, these bicoumarins were elucidated as 6,8,6',8'-tetramethoxyl-7,7'-dihydroxy-3,3'-bicoumarin (**1a**, isolated yield 30.1%), 6,8,6',8'-tetramethoxyl-7-hydroxy-7'-O-(6,8-dimethoxyl-7-hydroxy-3-coumarinyl)-3,3'-bicoumarin (**1b**, trace product), 6,6'-dihydroxy-7,7'-bicoumarin (**2a**, isolated yield 11.5%), 6-hydroxyl-5-(6-coumarinyloxy)coumarin (**2b**, isolated yield




**Scheme 1** Proposed mechanism for the transformation of coumarins **1–5**.

15%), 6,6'-dihydroxy-4,4'-dimethyl-7,5'-bicoumarin (**3a**, isolated yield 16%), 6-hydroxy-4-methyl-5-(4-methyl-6-coumarinyloxy) coumarin (**3b**, isolated yield 23%), 7,7'-dihydroxy-3,8'-bicoumarin (**4a**, isolated yield 21%), 7,7'-dihydroxy-4,4'-dimethyl-3,8'-bicoumarin (**5a**, isolated yield 22%), and 7,7'-dihydroxy-4,4'-dimethyl-3,6'-bicoumarin (**5b**, isolated yield 17%). The structures of **1a** and **4a** were further confirmed by comparison with the natural products obtained from *Chimonanthus praecox*[26] and *Gnidia socotrana*.[27] The elucidation of structures, $^{1}H$ and $^{13}C$ NMR data and all of the spectra for the bicoumarins can be observed in the supporting information. When the oxidative coupling reaction of 6,8-dimethoxy-7-hydroxycoumarin (**1**) was catalyzed by HRP in the presence of $H_2O_2$, one dimer (**1a**) and one trimer (**1b**) were obtained, all of which were connected by C-C bonds. **1a** was the major product (isolated yield 30.1%), while **1b** was a trace product. The major oxidation products of coumarins (**2**–**5**) were dimers, formed with C-C or C-O bonds. 7-Hydroxy-coumarin (**4**) produced a major bicoumarin with an isolated yield of 21%. Analysis of the reaction extract by HPLC-DAD suggested that no residual substrate was detected, and few by-products were observed. One of the factors that affect the yield of the dimerization reaction could be that the coumarin radicals were partially and non-specifically absorbed by HRP. If it is possible to avoid irreversible adsorption, biotransformation with HRP would lead to higher yields. Considering the formation of radicals, a mechanism of dimerization has been proposed (Scheme 1). HRP transferred electrons, and $H_2O_2$ was the electron acceptor. Coumarins (**1**–**5**) were dehydrogenated to produce various radicals, which underwent self-coupling reactions to produce dimers via C-C or C-O bonds. Huang previously reported a non-catalyzed chemical method to produce bi(dihydroxy)coumarins under $N_2$, with heating at 130 ºC for 48 h.[28] Pasciak and coworkers obtained hydrodimers by electrochemical reduction of coumarins.[29] Compared to these chemical methods, the HRP catalyzed method succeeded in dimerizing various hydroxycoumarins into bicoumarins using more mild reaction conditions.

**Kinetic analysis of the formation of bicoumarins in HRP catalyzed reactions**

To investigate the transformation characteristics of HRP towards the different coumarin substrates (**1**-**5**), kinetic analyses were carried out. Most substrates (**2a**, **2b**, **3a**, **3b**, **4a**, **5a**, **5b**), which have similar chemical structures, followed Michaelis–Menten kinetics within the range of substrate concentrations tested in the kinetic analyses (Fig. 3B, 3C, 3D, 3E) on the basis of Eadie–Hofstee plots (Fig. S1). Furthermore, kinetic parameters were also analyzed systematically (Table 2). Interestingly, the dimerization of coumarin **1**, which has 6,8-dimethoxy groups, followed the Hill kinetic model (Fig. 3A). Unlike the classical Michaelis–Menten kinetic model, the Hill equation supported a sigmoidal kinetic profile for the formation of **1a**, which suggested a substrate activation step in the oxidation of coumarin during the HRP-catalyzed reaction process. A sharp increase in velocity was observed in the

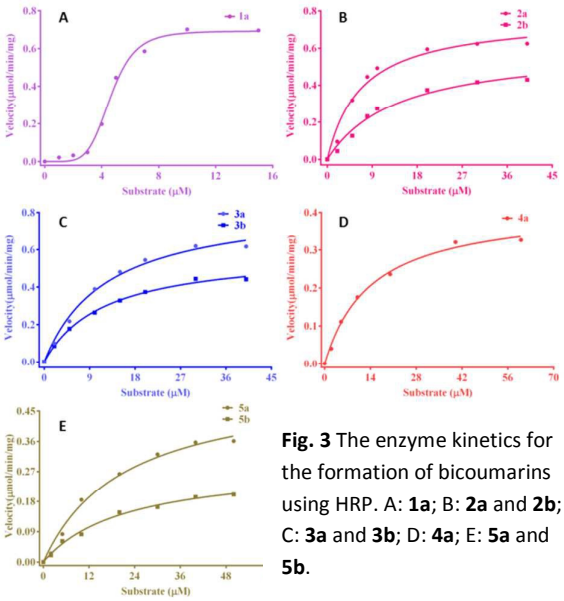



**Fig. 3** The enzyme kinetics for the formation of bicoumarins using HRP. A: **1a**; B: **2a** and **2b**; C: **3a** and **3b**; D: **4a**; E: **5a** and **5b**.

**Table 2** Kinetic parameters of bicoumarins determined using HRP

| Bicoumarins | $V_{max}$ (μmol/min/mg) | $K_m$ (μM) | n |
|---|---|---|---|
| **1a** | 0.69 ± 0.02 | 4.64 ± 0.12 | 5.42 ± 0.71 |
| **2a** | 0.77 ± 0.05 | 6.90 ± 1.46 | — |
| **2b** | 0.61 ± 0.05 | 14.25 ± 2.82 | — |
| **3a** | 0.85 ± 0.05 | 12.34 ± 1.04 | — |
| **3b** | 0.59 ± 0.02 | 11.86 ± 1.09 | — |
| **4a** | 0.42 ± 0.02 | 14.18 ± 1.60 | — |
| **5a** | 0.54 ± 004 | 21.46 ± 3.72 | — |
| **5b** | 0.29 ± 0.02 | 21.80 ± 3.27 | — |

formation of **1a** at a concentration of 5 μM (corresponding to n) along with a significant color burm of the reaction solution, which indicated the increased degree of cooperativity.

Analyses of $K_m$ provided the affinity of HRP for the different substrates in the order of 1>2>3≈4>5. Compared to other coumarins, **1** has the largest affinity, where the two methoxy groups could enhance the interaction between **1** and HRP. Moreover, the $K_m$ values of different substrates also suggested that same radicals were prone to couple to form bicoumarins (**1a**, **2a**). This evidence suggested that the radicals have better stability, a longer lifetime, and higher affinity for each other. And the $V_{max}$ also exerted the consistent with Km in the content, stability, and lifetime As shown in scheme 1 and table 2, only two bicoumarins (**2b** and **3b**) were produced by the coupling of oxyradicals, while the other bicoumarins were produced by the coupling of carbon radicals. This suggested a greater stability of the carbon radicals. Therefore, it was proposed that the oxyradicals were absorbed by the enzyme, which decreased their concentrations in solution.

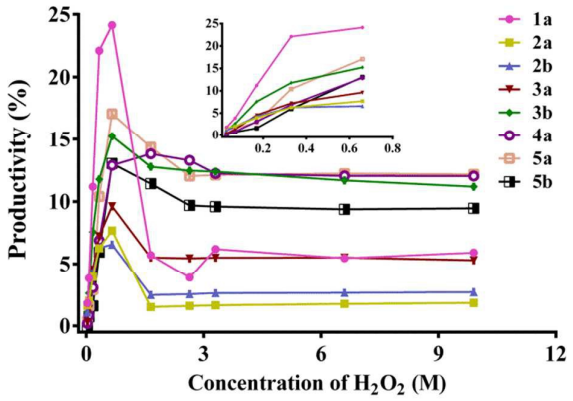



**Fig. 4** Optimization of the reaction condition: concentration of $H_2O_2$.

**Investigation of the multiple conditions of biotransformation**

Coumarins containing phenolic hydroxyl groups could be converted into bicoumarins in HRP-catalyzed reactions with the presence of $H_2O_2$. Considering the numerous applications of bicoumarins, it was very necessary to optimize these dimerization reactions. Therefore, five factors that may influence the biotransformation, such as the $H_2O_2$ concentration, pH, incubation time, incubation temperature, and HRP concentration, were investigated separately. The transformed bicoumarins (**1a**, **2a**, **2b**, **3a**, **3b**, **4a**, **5a**, **5b**) obtained from the preparative experiments were used as the standard compounds. The optimization of these five factors was carried out by monitoring the formation of the bicoumarins by HPLC-DAD.

The effect of the $H_2O_2$ concentration was studied from the conversion of coumarins **1–5** under the following conditions: $H_2O_2$ concentration ranging from 0.03 M to 9.9 M, HRP at 5 g/L, 25 ºC, 45 min, and pH 6.0. As shown in Fig. 4, the production of the bicoumarins reached a maximum when approximately 0.66 M $H_2O_2$ was used.

To obtain the optimum pH value for the formation of bicoumarins, the conversions were carried out over a pH range of 1 to 8.5, with 0.66 M $H_2O_2$ and 5 g/L HRP, at 25 ºC and 45 min. The pH values were regulated by changing the proportion of the $NaH_2PO_3$ aqueous solution (0.2 mol/L) and citric acid aqueous solution (0.1 mol/L). The analytical results suggested that acidity and alkalinity influenced the formation of bicoumarins significantly (Fig. 5A). When the pH values were less than 1 or greater than 8, HRP was deactivated. Therefore, the pH value for the dimerization of coumarins was optimized at pH 3.

The incubation time for the transformation was studied with 0.66 M $H_2O_2$ and 5 g/L HRP at 25 ºC and pH 3. The mixture was extracted by $CH_2Cl_2$ at different time points of incubation. The results indicated that bicoumarins were formed when the coumarins came into contact with HRP and $H_2O_2$ (Fig. 5B). The

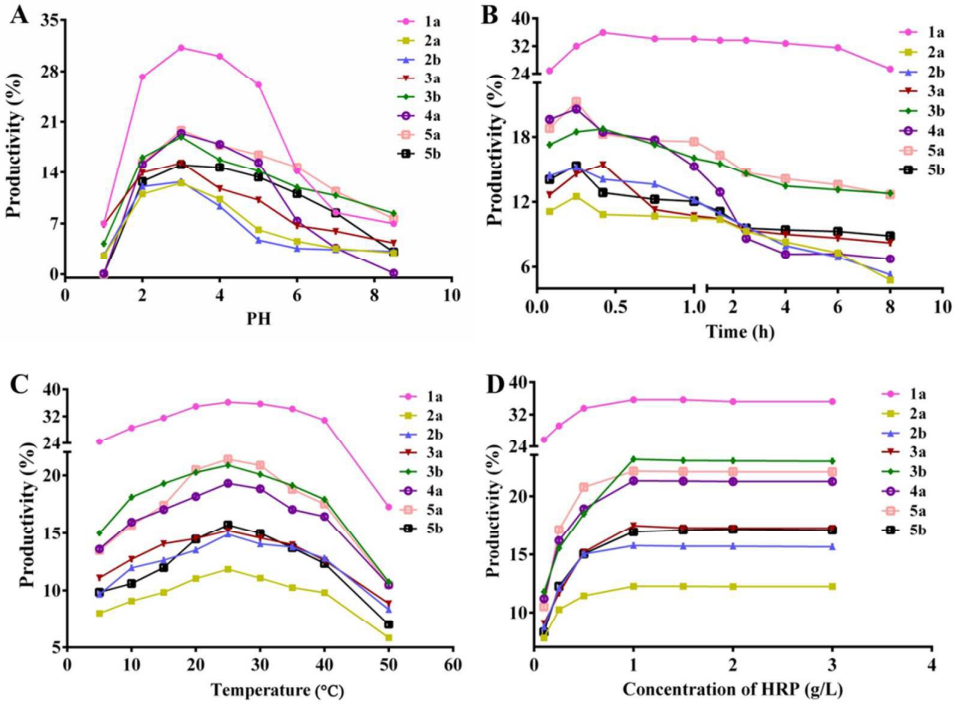



**Fig. 5** Optimization of the reaction conditions: A. pH; B. Time; C. Temperature; D. Concentration of HRP.

dimerization was almost completed in the first 0.5 h. Moreover, the data suggested that the production of bicoumarins decreased when the incubation time was too long. There is a possibility that the products could be absorbed by HRP. As shown in Fig. 5B, bicoumarins **2a**, **2b**, **4a**, **5a**, and **5b** were produced within an optimum time of 15 min, while **1a**, **3a** and **3b** reached maximum production at 25 min of their incubation times.

The incubation temperature was also investigated with 0.66 M $H_2O_2$, HRP 5 g/L, pH 3, and 15 min of incubation time. All of the substrates were converted by HRP at different incubation temperatures, from 5-50ºC (Fig. 5C). A comparison of the conversion indicated an optimal temperature of 25 ºC (Fig. 5C). Additionally, the poor yield of bicoumarins at higher temperatures suggested that HRP was deactivated at 50 ºC.

Finally, the conversion of the coumarins at different HRP concentrations was carried out with 0.66 M $H_2O_2$, at pH 3, 25 ºC, and 15 min of incubation time. As shown in Fig. 5D, the production of bicoumarins increased with increasing HRP concentration until it reached 1 g/L. No further increase was observed when the HRP concentration was increased from 1 g/L to 3 g/L. This suggested that the optimal HRP concentration was 1 g/L for the dimerization of coumarins.

In summary, five factors, including the $H_2O_2$ concentration, pH, incubation time, incubation temperature, and HRP concentration, were investigated for the oxidation of coumarins. Compound **1a** was produced with the maximum yield, which was approximately 35% yield, using the optimized reaction conditions. On the basis of the above optimization conditions, bicoumarins could be prepared by HRP catalyzed reactions with the yields in the range of 10 to 40%. To our knowledge, the yield of this reaction can be improved if the adsorption of coumarins by HRP could be effectively avoided.

Some problems still exist, although HRP had the potential to catalyze the dimerization of coumarins. A mutant pH-dependent horseradish peroxidase isoenzyme C in which Arg38 has been replaced with lysine had been obtained by Sanders.[30] Therefore, it was necessary to prepare mutant horseradish peroxidase with good stability and a high capability for the further conversion.

### *α*-glucosidase inhibitory effects of the transformed products

Diabetes with high concentrations of blood sugar can cause serious complications in the kidneys, eyes, and cardiovascular system. Therefore, the treatment of diabetes primarily focuses on reducing fluctuations in blood sugar. α-Glucosidase is the most important element for the absorption of glucose in the small intestine. Therefore, inhibition of α-glucosidase can significantly decrease postprandial hyperglycemia after a mixed carbohydrate diet and can be a key strategy in the control of diabetes mellitus. Acarbose, one of the α-glucosidase inhibitors, is widely used for the treatment of diabetes. The inhibitory effects of coumarins **1**–**5** and the dimerized products against α-Glucosidase were tested using *p*-NPG as substrate and acarbose as a positive control. In this *in vitro* bioassay, coumarins **4**–**5** and bicoumarins **3b**, **4a**, **5a** and **5b** showed potential inhibitory effects against $\alpha$-Glucosidase and were more effective than the positive control acarbose (Table S3). In particular, product **4a** had the strongest α-glucosidase inhibition, and its $IC_{50}$ value (36.99 μM) was approximately eighteen times lower than that of acarbose ($IC_{50}$ 685.5 μM). Furthermore, bicoumarins displayed stronger inhibitory effects than their corresponding starting materials. Structure analysis suggested that substituents on C-3, C-4 and C-7 may improve the inhibitory effect of coumarins. This understanding will be helpful in designing new and improved types of α-glucosidase inhibitors from coumarins.

## Experimental

### Apparatus

NMR spectra were acquired on Bruker 501 spectrometer. HRESIMS spectra were collected on an Agilent 1100 series LC/MSD ion trap mass spectrometer. Preparative HPLC was conducted using an Agel instrument with a UV detector and a YMC-Pack ODS-A column (250 × 20 mm, 5 $\mu$m). Analytical liquid chromatography was performed on a Ultimate 3000 HPLC instrument equipped with diode array detector (DAD). Samples were separated on a DIONEX Acclaim 120 column (Ø250 mm × 4.6 mm). Constant temperature oscillator (ZHWY-2102C) was obtained from Shanghai ZHICHENG Analysis Instrument Manufacturing Co., Ltd (China). The constant temperature mixing apparatus (MSC-100) was obtained from Hangzhou Allsheng Instruments Co., Ltd (China).

### Chemicals

All solvents, including dichloromethane, trichloromethane, ethyl acetate, petroleum ether (60-90 ºC), acetone, methanol, and chemical reagents, such as aquaehydrogenii dioxide ($H_2O_2$), citric acid, disodium hydrogen phosphate ($Na_2HPO_3$), and dimethyl sulfoxide (DMSO), were A.R. grade and obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. Dimethyl Sulfoxide-$d_6$ (DMSO-$d_6$) was produced by Cambridge Isotope Laboratories, Inc. Methanol and acetonitrile for HPLC analysis were of chromatographic grade (SIGMA, USA).

### Enzyme

Horseradish peroxidase (HRP, >250 u/mg) was purchased from J&K Scientific.

### Biotransformation of coumarins by HRP

Screen scale biotransformation was conducted in a 1-ml reaction system. Two milliliters of buffer consisted of 1.263 ml of $Na_2HPO_3$ (0.2 mol/L) and 0.737 ml of a citric acid aqueous solution (0.1 mol/L); 0.5 mg of HRP was dissolved in 94 μl of buffer. Two milligrams of substrate (coumarin) was dissolved in 376 μl of water, 376 μl of acetone, and 150 $\mu$L of buffer. The HRP solution and substrate solution was mixed to give the 1-ml reaction system, which was stirred as 120 rpm at 25 ºC. Five minutes later, 4 μl of a 10% $H_2O_2$ aqueous solution was added to the above mixture. The solution was extracted with $CH_2Cl_2$ after stirring for 45 minutes. After the evaporation of $CH_2Cl_2$ *in vacuo*, the extract was analyzed by HPLC-DAD.

Preparative scale biotransformation was conducted with the above mentioned conditions, but 500 mg of the substrates

were used. The dimerized products were purified by the pre-HPLC instrument with a RP-18 column (detected at 210 nm, 8 ml/min). Compounds **1a** (isolated yield 30.1%) and **1b** were obtained at 15 min and 23 min, respectively with MeOH−water (55%:45%, 0.05% trifluoroacetic acid v/v). Compounds **2a** (isolated yield 11.5%) and **2b** (isolated yield 15%) were isolated with $CH_3CN$-water (30%:70%, 0.05% trifluoroacetic acid v/v) at 18 min and 45 min, respectively. Compounds **3a** (isolated yield 16%) and **3b** (isolated yield 23%) were purified with $CH_3CN$-water (30%:70%, 0.05% trifluoroacetic acid v/v) at 15 min and 38 min, respectively. Compound **4a** (isolated yield 21%) was obtained at 8 min with MeOH−water (55%:45%, 0.05% trifluoroacetic acid v/v). Compounds **5a** (isolated yield 22%) and **5b** (isolated yield 17%) were isolated at 36 min and 45 min with $CH_3CN$-water (30%:70%, 0.05% trifluoroacetic acid v/v).

**Yeast α-Glucosidase Inhibitory Activity.**

Coumarins and bicoumarins were tested for their ability to inhibit α-glucosidase. This assay was carried out as previously described.[22]

**Molecular Modeling**

The molecular docking studies were performed using the Surflex-Dock of the SYBYL procedure to explore the potential binding mode between coumarin derivatives and HRP C protein complex. Surflex-Dock uses an empirical scoring function and a patented search engine to dock ligands into a protein's binding site. The structure of HRP C in a complex with benzhydroxamic acid, solved at 2.0 Å resolution (PDB code 2ATJ), was used as receptor.[31] The active pocket for substrate binding was generated around the crystallographic ligand in an automatic mode with the setup of float radius as zero. Coumarin derivatives were docked into the active site of HRP C to give a sight into the interaction mode and each ligand was generated with 20 binding modes.

## Conclusion

In conclusion, HRP was used to catalyze the dimerization of coumarins. And some novel bicoumarins were obtained as the major products. Screening scale experiments revealed that the coumarins that possessed phenolic hydroxyl groups could be converted into bicoumarins by HRP. Incidentally, coumarins with one phenolic hydroxyl group produced specific bicoumarins, while coumarins with two or more hydroxyl groups produced multiple bicoumarins non-specifically. The dimerization mechanism involved phenolic radicals that participated in the reactions catalyzed by HRP in the presence of $H_2O_2$, which has been previously reported. The interactions between different coumarins and HRP C enzyme were investigated by the *in silico* docking analysis, which could be helpful in explaining the reaction mechanism. For selective dimerization, the experiment was carried out at a preparative scale to obtain the products. Five coumarins were used as substrates in the HRP catalyzed reaction, and totally nine products were obtained. The structures of the products were elucidated by HRESIMS, 1D-NMR, 2D-NMR, and literature analyses. All of the products obtained were bicoumarins, except **1b**, which was identified as a trimer. Additionally, products **1b**, **2a**, **2b**, **3a** and **3b** are new compounds. Using the dimerized products as standards, kinetic analyses of the catalytic reactions were conducted, which can be helpful to discuss the radical reaction. Additionally, the reaction conditions were investigated. An optimized reaction condition was obtained for the dimerization of coumarins with 1 g/L HRP, 0.66 M $H_2O_2$, pH 3, 25 ºC, and 15 min of incubation time. The yield of bicoumarins ranged from 10 to 40%, with bicoumarin **1a** produced at an approximately 35% yield. Finally, in an *in vitro* bioassay, several new bicoumarins displayed potential α-glucosidase inhibitory effects, which can be further developed into antidiabetic agents.

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## Notes and references

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Horseradish peroxidase was used to catalyze the formation of bicoumarins. The kinetic analysis and optimization of the transformation conditions were carried out in the present work.