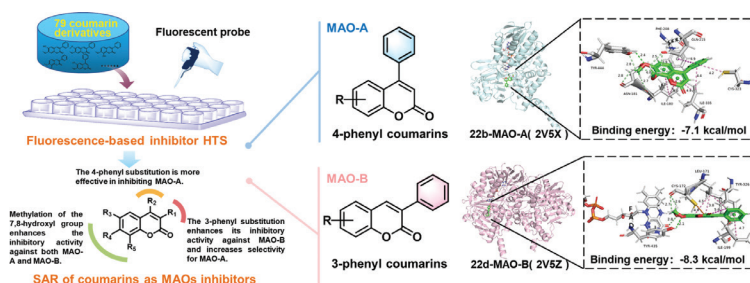


# Coumarin derivatives as inhibitors against monoamine oxidase: structure-activity relationships and inhibitory mechanisms

## Graphical abstract



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## Highlights

- A high throughput screening method for MAO inhibitors based on fluorescence analysis has been established.
- The phenyl group and its substitution positions on coumarin exert a unique effect on the inhibitory activity and selectivity towards MAOs.
- 3-phenyl coumarin **22d** selectively and competitively inhibits MAO-B, with a  $K_i$  value of  $0.19 \pm 0.04 \mu\text{M}$ .
- 4-phenyl coumarin **12b** selectively inhibits MAO-A in a mixed manner, with a  $K_i$  value of  $0.39 \pm 0.04 \mu\text{M}$ .

## In brief

A series of structurally diverse coumarins were synthesized, and their inhibitory activity against MAOs was evaluated using a fluorescence-based screening method. Structure-activity relationship studies indicated that the phenyl group and its substitution positions on coumarin uniquely affected MAO inhibition and selectivity. Specifically, a phenyl group at the C-3 position enhanced MAO-B inhibition, while the one at the C-4 position favored MAO-A.

## Research Article

# Coumarin derivatives as inhibitors against monoamine oxidase: structure-activity relationships and inhibitory mechanisms

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## ABSTRACT

Monoamine oxidase (MAO) is a useful target in the treatment of neurodegenerative diseases and depressive disorders. Understanding the structure-activity relationship (SAR) and mechanisms underlying MAO inhibitors is crucial but challenging. The coumarin scaffold has been shown to be ideal for MAO inhibitors, yet the SAR for MAO inhibition and selectivity based on coumarin remains unclear. In this study a fluorescence-based high throughput screening method was developed using a MAO fluorescent substrate (MR2). Subsequently, three series of coumarin derivatives, including esculetin, daphnetin and 3-substituted coumarin, were synthesized and the inhibitory effects on MAO were assayed. The SAR study revealed that phenyl substitution had a unique effect on MAO inhibitory activity and selectivity. Specifically, 3-phenyl substitution in coumarin derivatives significantly enhanced MAO-B inhibition and increased selectivity for MAO-A, while 4-phenyl substitution is more effective for MAO-A inhibition. Discovery studios were also utilized to investigate the structural requirements for the effective inhibition of MAO by coumarins. Furthermore, the mechanisms underlying inhibition for five phenyl coumarin derivatives were elucidated through enzymatic kinetics analysis and molecular docking simulations. These findings provide new insights into the interactions between coumarins and MAOs and significantly contribute to the development of coumarin-based MAO inhibitors.

**Keywords:** Monoamine oxidase (MAO), Inhibitor, Coumarin, Structure-activity relationship, Inhibition mechanism

## 1. INTRODUCTION

Monoamine oxidases [MAOs] (EC 1.4.3.4.) are members of the flavin adenine dinucleotide (FAD)-dependent enzyme family that are located intracellularly in the mitochondrial outer membranes of neuronal, glial, and other cells and catalyze oxidative deamination of pharmacologically important monoamine neurotransmitters [1]. MAOs exist in two isoforms (MAO-A and MAO-B), share 70% sequence homology, and have highly conserved strand folding, including identical FAD-binding sites [2, 3]. However, MAOs differ in three-dimensional structure, organ and tissue distribution, substrate specificity, and sensitivity to inhibitors. Subtle differences in the size and shape of the hydrophobic cavities of the

two enzyme structures have been reported, as well as the positions relative to the substrate entrance channel. These variations may be the primary reason for different sensitivities to substrates and inhibitors [4, 5]. Both MAO-A and MAO-B oxidatively deaminate dopamine (DA) and tyramine. However, MAO-A preferentially degrades serotonin, epinephrine, and norepinephrine, while MAO-B shows a higher affinity for benzylamine (BA) and phenethylamine (PEA) [6, 7]. Due to differences in the physiologic substrates catalyzed by MAO-A and MAO-B, MAO-A is associated with depression and anxiety, whereas neurodegenerative diseases, whereas Parkinson's and Alzheimer's diseases are associated with MAO-B [8, 9].

The involvement of MAO in the metabolism of key neurotransmitters has made MAO an attractive target for pharmacologic interventions in neurologic disorders [10–12]. MAOs inhibitors (MAOIs) are promising therapeutic agents for preventing and slowing the progression of neurodegenerative diseases. Several selective MAO-A inhibitors, including moclobemide, brofaromine, clorgyline, and toloxatone, that act as antidepressants have been discovered [13–15]. Selective MAO-B inhibitors lazabemide, selegiline, safinamide, and rasagiline, serve as anti-Parkinson agents [16–19]. Additionally, non-selective MAO inhibitors iproniazid, phenelzine and tranylcypromine are also used as clinical [20, 21]. Due to the lack of affinity and selectivity of MAO inhibitors towards specific isoforms, fewer drugs are used in clinical practice and many side effects have been reported [16, 22]. Consequently, the structure-selectivity relationship and mechanisms underlying MAO selective inhibition have been critical and challenging aspects in the development of these inhibitors [23].

Coumarins are natural products with aromatic structures and are widely distributed in the plant kingdom [24]. The bicyclic oxygen bearing heterocyclic scaffold formed by fusion of benzene with the pyrone ring is a unique structure of coumarin [25, 26]. Coumarins have anti-inflammatory [27], anticancer [28], antitumour [29], analgesic [30] and antiviral [31] properties. Among MAO inhibitors, the coumarin scaffold has been shown to be ideal for the development of MAO inhibitors. Several studies have shown that introduction of a substituent at C-3 of the coumarin scaffold inhibits MAOs activity [32–34] and the nature and length of the substituent at position 7 of the coumarin scaffold affects specificity and selectivity [35]. Chimenti et al. [36] synthesized a large series of 3-carboxamido-7-substituted coumarins and tested the coumarins *in vitro* for inhibitory activity against hMAO-A and hMAO-B. Indeed, compound 21 was shown to be the most potent inhibitor ( $IC_{50} = 0.0014 \pm 0.000120 \mu M$ ). Secci et al. showed that the presence of a benzyloxy group at C-7 inhibits MAO-B better than diethylamino, hydroxyl, and methoxy groups, and the best results were obtained by introducing a hydrazinyl molecule at the C-3 position (Compound 53 [ $IC_{50} = 3.22 \pm 0.25 nM$ ]) with respect to inhibitory activity and selectivity of hMAO-B [37]. However, the structure-MAO inhibition activity and selectivity relationship based on the coumarin scaffold is unclear.

To effectively evaluate the inhibitory activity of inhibitors on MAO-A and MAO-B, a fluorescence-based high throughput screening method was developed using an MAO fluorescent substrate (MR2). A series of structurally diverse coumarins was subsequently synthesized to evaluate the impact of various substituents on MAO inhibitory activity. The potential structure-inhibition relationships of these coumarins were discussed and summarized, which showed that phenyl substitution at different positions exhibit a unique effect on MAO inhibitory activity and selectivity between MAO-A and MAO-B. Discovery

studios were also utilized to investigate the structural requirements for the effective inhibition of MAO by coumarins. Furthermore, the mechanisms underlying 5 phenyl coumarin derivative inhibition were further analyzed through enzymatic kinetics analysis and molecular docking simulations. These findings could provide valuable guidance for designing and optimizing potent coumarin-based MAO inhibitors.

## 2. RESULTS AND DISCUSSION

### 2.1 Method for evaluating MAO inhibitory activity

To effectively evaluate the inhibitory activity of inhibitors on MAO-A and MAO-B, a fluorescence-based inhibitor high throughput screening method was developed using a reported MAO fluorescent probe (MR2) as the substrate [38]. The structural formulae of the substrate and products are shown in Figure 1A. MR2 undergoes oxidative deamination by MAOs to form red resorufin under physiologic conditions (pH 7.4 at 37°C). Due to the crucial importance of kinetic behavior for the quantitative applications of activity-based probes, the enzymatic kinetics of MR2 towards MAO-A and MAO-B were investigated. A representative enzyme kinetics plot was shown in Figure 1B. The kinetics experiments provided the following Michaelis–Menten constant ( $K_m$ ) values for MR2 with MAO-A and MAO-B:  $7.49 \pm 0.21 \mu M$  for MAO-A; and  $1.50 \pm 0.02 \mu M$  for MAO-B. MR2 was then used as a fluorescent substrate for rapid screening of MAO inhibitors using recombinant MAO-A and MAO-B as enzyme sources. Based on the  $K_m$  of MR2, the concentrations were set at  $8.0 \mu M$  for MAO-A and  $2.0 \mu M$  for MAO-B. As shown in Figure 1C, toloxatone and selegiline effectively inhibited MAO-A/B-mediated oxidative deamination of MR2 in a dose-dependent manner with  $IC_{50}$  values of  $6.61 \pm 0.06 \mu M$  for MAO-A and  $20.00 \pm 0.01 nM$  for MAO-B. These values are consistent with the reported inhibitory activities of toloxatone ( $IC_{50} = 6.71 \pm 0.42 \mu M$ ) [39] and selegiline ( $IC_{50} = 60.00 \pm 0.01 nM$ ) [40]. These results demonstrated that the MR2-based assay effectively evaluated the inhibitory activity of inhibitors on MAO-A and MAO-B.

### 2.2 Chemistry

To explore the effects of diverse functional groups and their position on coumarin regarding MAO inhibition activities, a series of 4-substituted esculetin derivatives were first designed and prepared from 1,2,4-phenenyl triacetate via acid catalytic Pechmann condensation (Scheme 1A) [41]. 6 was obtained by treating phenyl triacetate with 1,3-acetonedicarboxylic acid in the presence of perchloric acid. 1,2,4-phenenyl triacetate was reacted with the correctly substituted  $\beta$ -ketoester under perchloric acid catalysis to produce 3a, 3b, 3e, 3f and 3g. 3c and 3d were obtained from chloride 3b by reflux in a mixture of DMF and water (1:3 [v:v]) or treated with sodium azide. Introduction of a

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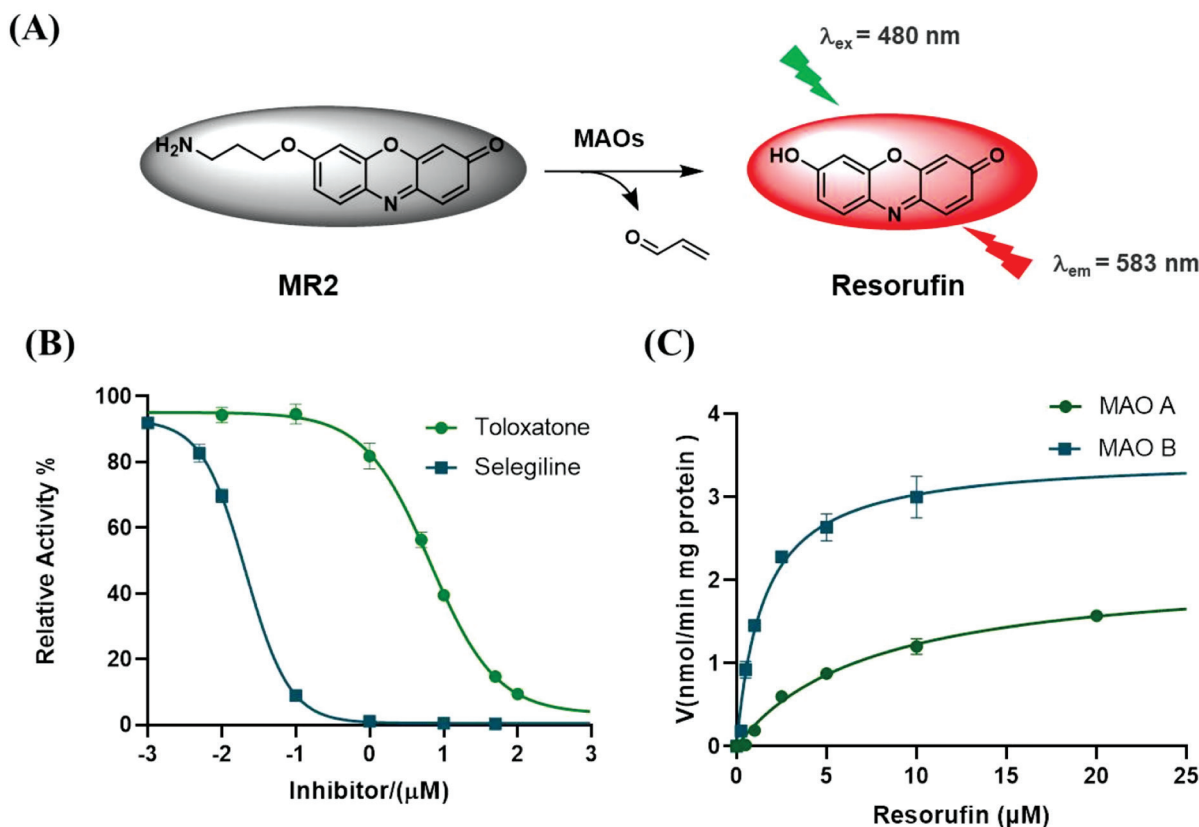


**Figure 1 |** The substrate is oxidized and  $\beta$ -deaminated by the MAOs (A); The enzymatic kinetics of MR2 towards MAO-A and MAO-B (B); The dose-response curves of the positive MAO inhibitor (C).

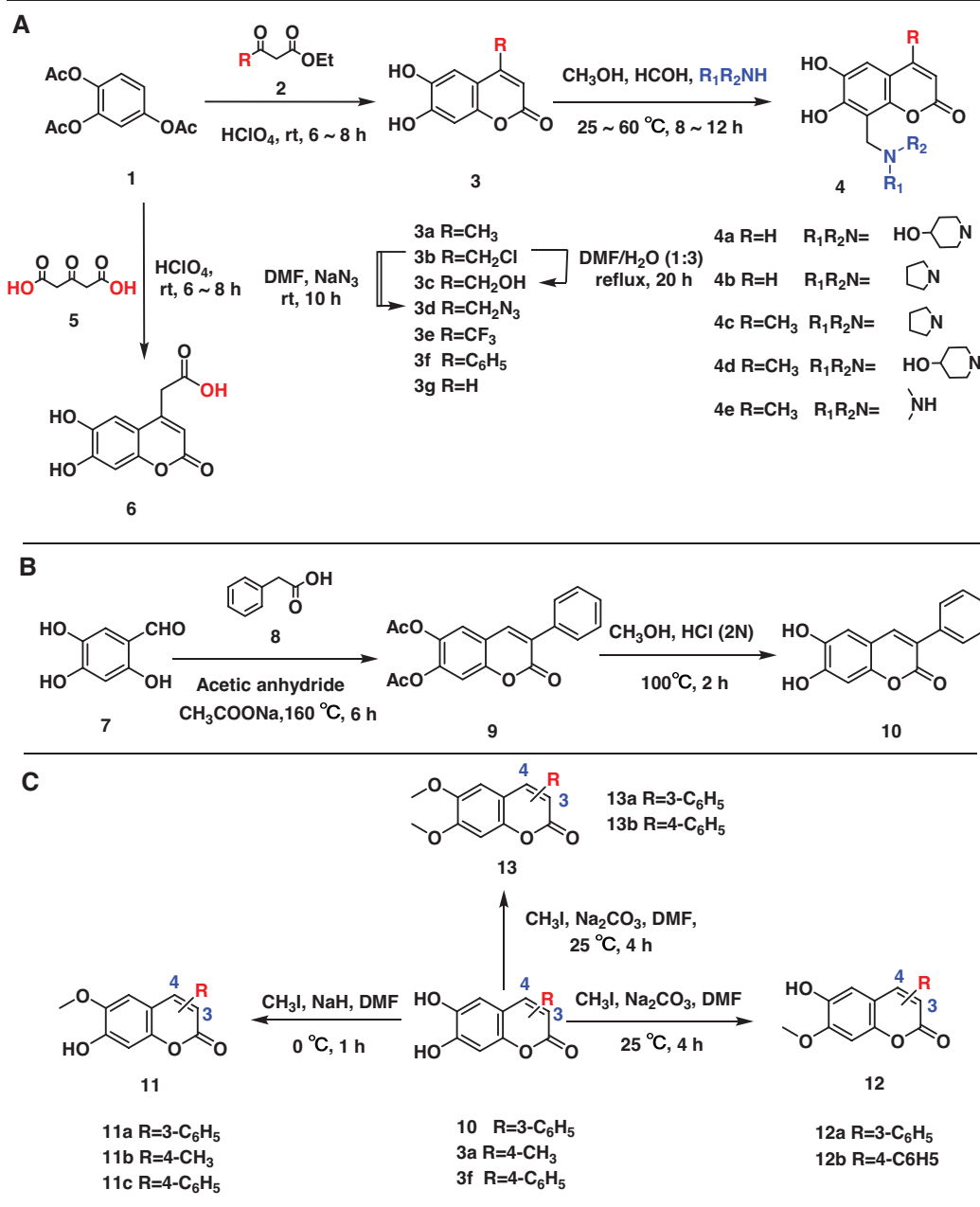
nitrogen-containing group as a hydrogen bond acceptor on the 6,7-dihydroxycoumarins by the Mannich reaction was also carried out. After formaldehyde and the corresponding secondary amine were stirred in ethanol for 0.5 h, the reaction was added to a solution of 6,7-dihydroxycoumarin/4-methyl-6,7-dihydroxycoumarin in ethanol and stirring for 8–12 h at 25–60°C, which obtained **4a–4e**.

3-substituted-esculetin **9** was synthesized from phenylacetic acid and 2,4,5-trihydroxybenzaldehyde through Knoevenagel condensation. Compound **9** was then hydrolyzed under acidic conditions to yield **10** (Scheme 1B). Additionally, a series of methylated esculetin derivatives were prepared, as shown in Scheme 1C. Monomethylated products (**11a–11c** and **12a** and **12b**) were obtained from 6,7-dihydroxycoumarin via selective methylation at 6-OH and 7-OH in a good yield with  $\text{CH}_3\text{I}$  in the presence of NaH at 0°C and  $\text{Na}_2\text{CO}_3$  at 25°C, respectively. Dimethylated products (**13a** and **13b**) were also produced under  $\text{Na}_2\text{CO}_3$  at 25°C.

Using a similar approach, a series of daphnetin derivatives were prepared, as shown in Scheme 2. 4-carboxylic daphnetin **15a** and its ester (**15b**) were obtained by treating 1,2,3-phenyl triacetate with 1,3-acetonedicarboxylic acid and dimethyl 1,3-acetonedicarboxylate,

respectively, in the presence of perchloric acid. **16a–16j** were obtained by reacting 1,2,4-phenyl triacetate with the appropriately substituted  $\beta$ -ketoester in the presence of perchloric acid (Scheme 2A). A series of diverse functional groups, including methyl, carboxylic ester, cyano, carboxyl, and phenyl, were introduced to the C-3 site of daphnetin. **18a–18e** were synthesized from phenylacetic acid and 2,4,5-trihydroxybenzaldehyde through the Knoevenagel condensation [42] (Scheme 2B). Moreover, a series of methylated daphnetin derivatives were prepared according to the above-mentioned method for methylation of esculetin (Scheme 2C).

Furthermore, by introducing acidic and phenolic hydroxyl groups in the coumarin scaffold, three subclasses of 3-substituted coumarin derivatives were also synthesized, as described by Wei et al. [43]. As shown in Scheme 3, the 2-oxo-2H-chromene-3-carboxylic acid derivatives (**24a–24g**) were prepared through the Knoevenagel condensation reaction of salicylaldehyde derivatives and Meldrum's acid. The 3-cyano-coumarins (**25a–25i**) were prepared in a one-pot method. The 3-(1H-tetrazol-5-yl)-2H-chromen-2-one derivatives (**26a–26f**) were synthesized from 3-cyano-coumarins via the [3 + 2] cycloaddition of nitriles and sodium azide catalyzed by aluminum chloride at 90°C. The



Scheme 1 | Synthesis of esculentin derivatives.

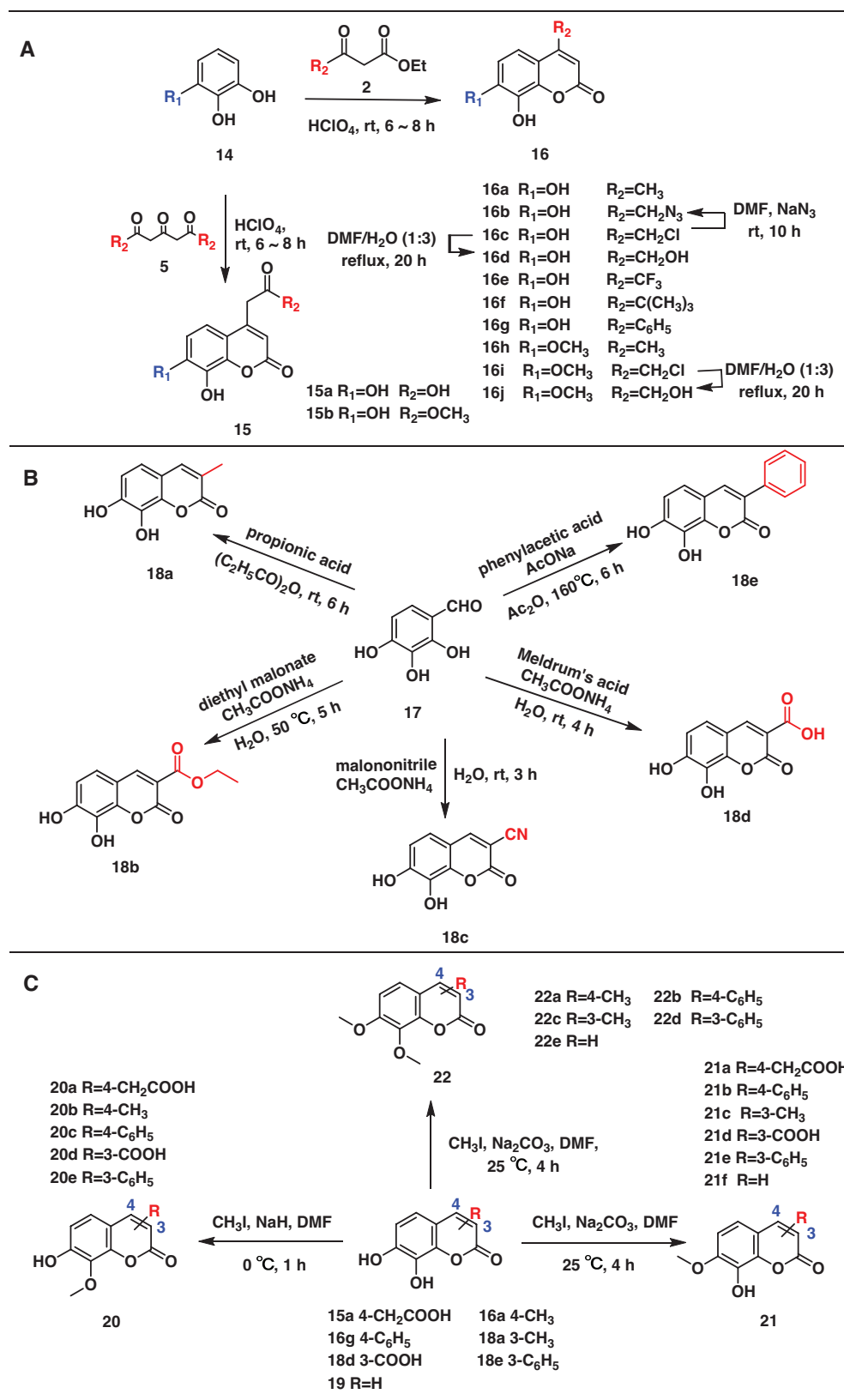
detailed experimental procedures are described in the Supporting Information.

### 2.3 MAO inhibitory activity assays

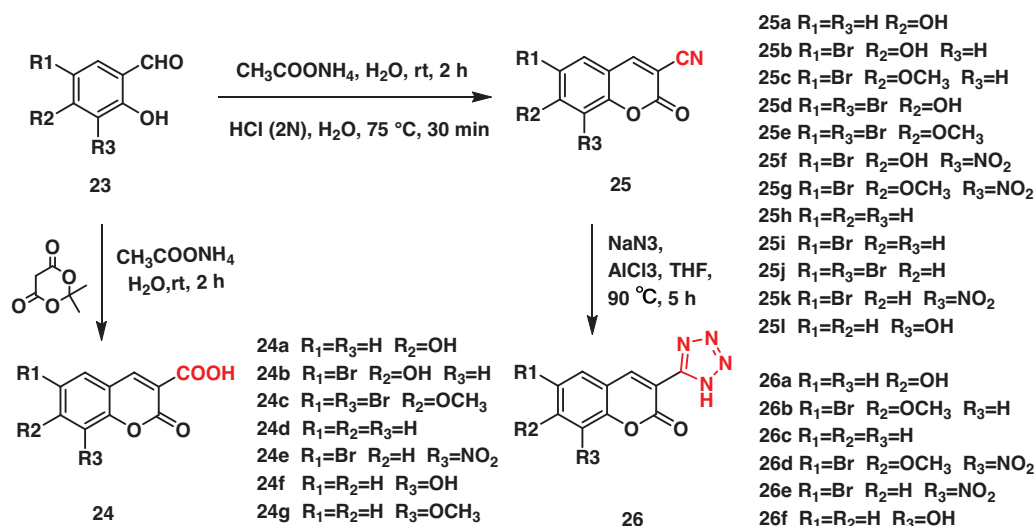
To determine the structure-activity relationships of coumarins in inhibiting MAOs, the inhibitory effects of 79 natural and synthetic coumarins on MAO-A and MAO-B were evaluated. These compounds can be classified into the following three groups: esculentin derivatives

(Scheme 1); daphnetin derivatives (Scheme 2); and 3-substituted coumarin derivatives (Scheme 3). The IC<sub>50</sub> values of coumarin derivatives against MAO-A and MAO-B are shown in Table 1. Compounds 3d and 12b significantly inhibited MAO-A with IC<sub>50</sub> values of  $6.67 \pm 0.03 \mu\text{M}$  and  $5.87 \pm 0.63 \mu\text{M}$ , respectively, which were comparable to the positive inhibitor tolloxatone (IC<sub>50</sub> =  $6.61 \pm 0.06 \mu\text{M}$ ). Dimethylated daphnetin derivatives exhibited moderate MAO-A inhibitory activity with

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Scheme 2 | Synthesis of daphnetin derivatives.



Scheme 3 | Synthesis of 3-substituted coumarin derivatives.

IC<sub>50</sub> values ranging from 12.89–20.74  $\mu$ M, except for 3-phenyl-7,8-dimethoxydaphnetin. In contrast, phenyl-substituted compounds **12a** and **22d** significantly inhibited MAO-B with IC<sub>50</sub> values of 0.76  $\pm$  0.03  $\mu$ M and 0.57  $\pm$  0.03  $\mu$ M, respectively.

It is particularly noteworthy that phenyl substitution at different positions exhibited a very unique effect on MAO inhibitory activity and selectivity between MAO-A and MAO-B. All 3-phenyl-substituted coumarins exhibited significant inhibitory effects on MAO-B (**10**: IC<sub>50</sub> = 5.54  $\pm$  0.09  $\mu$ M, **11a**: IC<sub>50</sub> = 13.56  $\pm$  1.14  $\mu$ M, **12a**: IC<sub>50</sub> = 0.76  $\pm$  0.03  $\mu$ M, **13a**: IC<sub>50</sub> = 3.45  $\pm$  0.20  $\mu$ M, **18e**: IC<sub>50</sub> = 22.28  $\pm$  2.73  $\mu$ M, **20e**: IC<sub>50</sub> = 3.75  $\pm$  0.11  $\mu$ M, **21e**: IC<sub>50</sub> = 7.51  $\pm$  0.04  $\mu$ M, **22d**: IC<sub>50</sub> = 0.57  $\pm$  0.03  $\mu$ M), whereas all of these compounds had negligible inhibitory activity against MAO-A. Simultaneously, all 4-phenyl-substituted coumarins (**3f**, **11c**, **12b**, **13b**, **16g**, **20c**, and **22b**) exhibited minimal inhibitory activity against MAO-B, whereas **3f**, **12b**, **20c**, and **22b** demonstrated marked inhibitory effects on MAO-A with IC<sub>50</sub> values of 16.87  $\pm$  1.34  $\mu$ M, 5.87  $\pm$  0.63  $\mu$ M, 36.70  $\pm$  4.44  $\mu$ M, and 19.17  $\pm$  3.08  $\mu$ M, respectively. These results indicated that 3-phenyl substitution in coumarins significantly enhanced inhibitory activity against MAO-B and increased selectivity for MAO-B, whereas 4-phenyl substitution was more favorable for MAO-A inhibition.

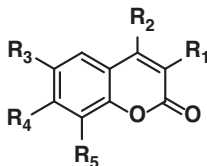
In addition, the hydroxyl group position on the coumarin scaffold and the methylation had an impact on MAO inhibitory activity. Methylation of the 7-OH group appeared to enhance MAO-A inhibitory activity for esculentin derivatives, while methylation of the 6-OH group resulted in a reduction of inhibitory activity, as evidenced by **3f** (IC<sub>50</sub> = 16.87  $\pm$  1.34  $\mu$ M) vs. **12b**

(IC<sub>50</sub> = 5.87  $\pm$  0.63  $\mu$ M) vs. **11c** (IC<sub>50</sub> > 200  $\mu$ M). A similar trend in potency was also observed for MAO-B inhibition, as evidenced by **10** (IC<sub>50</sub> = 5.54  $\pm$  0.09  $\mu$ M) vs. **12a** (IC<sub>50</sub> = 0.76  $\pm$  0.03  $\mu$ M) vs. **11a** (IC<sub>50</sub> = 13.56  $\pm$  1.14  $\mu$ M). Dimethylation of the 7,8-dihydroxy groups significantly enhanced MAO-A inhibitory activity for daphnetin derivatives, as evidenced by **22a** (IC<sub>50</sub> = 15.16  $\pm$  0.26  $\mu$ M) vs. **16a** (IC<sub>50</sub> > 200  $\mu$ M), **22b** (IC<sub>50</sub> = 19.17  $\pm$  3.08  $\mu$ M) vs. **16g** (IC<sub>50</sub> > 200  $\mu$ M), **22c** (IC<sub>50</sub> = 12.89  $\pm$  1.98  $\mu$ M) vs. **18a** (IC<sub>50</sub> > 200  $\mu$ M), and **22e** (IC<sub>50</sub> = 20.74  $\pm$  0.11  $\mu$ M) vs. **19** (IC<sub>50</sub> > 200  $\mu$ M). Similarly, methylation of 3-phenyl daphnetin can progressively enhance inhibitory activity against MAO-B, as shown by **18e** (IC<sub>50</sub> = 22.28  $\pm$  2.73  $\mu$ M) vs. **20e** (IC<sub>50</sub> = 3.75  $\pm$  0.11  $\mu$ M) and **21e** (IC<sub>50</sub> = 7.51  $\pm$  0.04  $\mu$ M) vs. **22d** (IC<sub>50</sub> = 0.57  $\pm$  0.03  $\mu$ M). In addition, for 3-substituted coumarin derivatives, no compounds exhibited significant inhibition of MAO-A, while some 3-cyanosubstituted coumarins showed moderate inhibitory activity against MAO-B. This finding suggests that the 3-cyanide group may be a preferred moiety for MAO-B.

## 2.4 Establishment of the 3D-QSAR model

To elucidate the structure-MAO inhibitory activity relationship of coumarins, quantitative structure-activity relationship (QSAR) studies were conducted using Drug Discovery Studio, which has been extensively applied to explore the correlations between biological activities and molecular descriptors of compounds. The coumarins aligned with the isosurfaces of the 3D QSAR model coefficients on van der Waals and electrostatic potential grids are shown in Figure 2A and B. Regions in which high electron density (negative charge) favored MAO inhibition are shown as red contours in the electrostatic

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**Table 1** | The IC<sub>50</sub> values of coumarin derivatives against MAOs.

| Compound   | R <sub>1</sub>                | R <sub>2</sub>                     | R <sub>3</sub>    | R <sub>4</sub>    | R <sub>5</sub>                    | MAO-A                 | MAO-B                 |
|------------|-------------------------------|------------------------------------|-------------------|-------------------|-----------------------------------|-----------------------|-----------------------|
|            |                               |                                    |                   |                   |                                   | IC <sub>50</sub> (μM) | IC <sub>50</sub> (μM) |
| <b>3a</b>  | H                             | CH <sub>3</sub>                    | OH                | OH                | H                                 | 110.20 ± 4.75         | >200                  |
| <b>3b</b>  | H                             | CH <sub>2</sub> Cl                 | OH                | OH                | H                                 | >200                  | >200                  |
| <b>3c</b>  | H                             | CH <sub>2</sub> OH                 | OH                | OH                | H                                 | >200                  | >200                  |
| <b>3d</b>  | H                             | CH <sub>2</sub> N <sub>3</sub>     | OH                | OH                | H                                 | 6.67 ± 0.04           | >200                  |
| <b>3e</b>  | H                             | CF <sub>3</sub>                    | OH                | OH                | H                                 | >200                  | >200                  |
| <b>3f</b>  | H                             | C <sub>6</sub> H <sub>5</sub>      | OH                | OH                | H                                 | 16.87 ± 1.34          | >200                  |
| <b>3g</b>  | H                             | H                                  | OH                | OH                | H                                 | >200                  | >200                  |
| <b>4a</b>  | H                             | H                                  | OH                | OH                | C <sub>6</sub> H <sub>12</sub> NO | >200                  | >200                  |
| <b>4b</b>  | H                             | H                                  | OH                | OH                | C <sub>5</sub> H <sub>10</sub> N  | >200                  | >200                  |
| <b>4c</b>  | H                             | CH <sub>3</sub>                    | OH                | OH                | C <sub>5</sub> H <sub>10</sub> N  | >200                  | >200                  |
| <b>4d</b>  | H                             | CH <sub>3</sub>                    | OH                | OH                | C <sub>6</sub> H <sub>12</sub> O  | >200                  | >200                  |
| <b>4e</b>  | H                             | CH <sub>3</sub>                    | OH                | OH                | C <sub>3</sub> H <sub>8</sub> N   | >200                  | >200                  |
| <b>6</b>   | H                             | CH <sub>2</sub> COOH               | OH                | OH                | H                                 | >200                  | >200                  |
| <b>10</b>  | C <sub>6</sub> H <sub>5</sub> | H                                  | OH                | OH                | H                                 | >200                  | 5.54 ± 0.09           |
| <b>11a</b> | C <sub>6</sub> H <sub>5</sub> | H                                  | CH <sub>3</sub> O | OH                | H                                 | >200                  | 13.56 ± 1.14          |
| <b>11b</b> | H                             | CH <sub>3</sub>                    | CH <sub>3</sub> O | OH                | H                                 | >200                  | >200                  |
| <b>11c</b> | H                             | C <sub>6</sub> H <sub>5</sub>      | CH <sub>3</sub> O | OH                | H                                 | >200                  | >200                  |
| <b>12a</b> | C <sub>6</sub> H <sub>5</sub> | H                                  | OH                | CH <sub>3</sub> O | H                                 | >200                  | 0.76 ± 0.03           |
| <b>12b</b> | H                             | C <sub>6</sub> H <sub>5</sub>      | OH                | CH <sub>3</sub> O | H                                 | 5.87 ± 0.63           | >200                  |
| <b>13a</b> | C <sub>6</sub> H <sub>5</sub> | H                                  | CH <sub>3</sub> O | CH <sub>3</sub> O | H                                 | >200                  | 3.45 ± 0.20           |
| <b>13b</b> | H                             | C <sub>6</sub> H <sub>5</sub>      | CH <sub>3</sub> O | CH <sub>3</sub> O | H                                 | >200                  | >200                  |
| <b>15a</b> | H                             | CH <sub>2</sub> COOH               | H                 | OH                | OH                                | >200                  | >200                  |
| <b>15b</b> | H                             | CH <sub>2</sub> COOCH <sub>3</sub> | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16a</b> | H                             | CH <sub>3</sub>                    | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16b</b> | H                             | CH <sub>2</sub> N <sub>3</sub>     | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16c</b> | H                             | CH <sub>2</sub> Cl                 | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16d</b> | H                             | CH <sub>2</sub> OH                 | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16e</b> | H                             | CF <sub>3</sub>                    | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16f</b> | H                             | C(CH <sub>3</sub> ) <sub>3</sub>   | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16g</b> | H                             | C <sub>6</sub> H <sub>5</sub>      | H                 | OH                | OH                                | >200                  | >200                  |
| <b>16h</b> | H                             | CH <sub>3</sub>                    | H                 | CH <sub>3</sub> O | OH                                | >200                  | >200                  |
| <b>16i</b> | H                             | CH <sub>2</sub> OH                 | H                 | CH <sub>3</sub> O | OH                                | >200                  | >200                  |

Table 1 | Continued

| Compound | R <sub>1</sub>                     | R <sub>2</sub>                               | R <sub>3</sub>    | R <sub>4</sub>    | R <sub>5</sub>    | MAO-A                 | MAO-B                 |
|----------|------------------------------------|----------------------------------------------|-------------------|-------------------|-------------------|-----------------------|-----------------------|
|          |                                    |                                              |                   |                   |                   | IC <sub>50</sub> (μM) | IC <sub>50</sub> (μM) |
| 16j      | H                                  | CH <sub>2</sub> Cl                           | H                 | CH <sub>3</sub> O | OH                | 30.31 ± 2.54          | >200                  |
| 16k      | H                                  | H                                            | CH <sub>3</sub> O | OH                | OH                | >200                  | >200                  |
| 18a      | CH <sub>3</sub>                    | H                                            | H                 | OH                | OH                | >200                  | >200                  |
| 18b      | CH <sub>2</sub> COOCH <sub>3</sub> | H                                            | H                 | OH                | OH                | >200                  | >200                  |
| 18c      | CN                                 | H                                            | H                 | OH                | OH                | >200                  | >200                  |
| 18d      | COOH                               | H                                            | H                 | OH                | OH                | >200                  | >200                  |
| 18e      | C <sub>6</sub> H <sub>5</sub>      | H                                            | H                 | OH                | OH                | >200                  | 22.28 ± 2.73          |
| 19       | H                                  | H                                            | H                 | OH                | OH                | >200                  | >200                  |
| 20a      | H                                  | C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> | H                 | OH                | CH <sub>3</sub> O | >200                  | >200                  |
| 20b      | H                                  | CH <sub>3</sub>                              | H                 | OH                | CH <sub>3</sub> O | >200                  | >200                  |
| 20c      | H                                  | C <sub>6</sub> H <sub>5</sub>                | H                 | OH                | CH <sub>3</sub> O | 36.70 ± 4.44          | >200                  |
| 20d      | COOH                               | H                                            | H                 | OH                | CH <sub>3</sub> O | >200                  | >200                  |
| 20e      | C <sub>6</sub> H <sub>5</sub>      | H                                            | H                 | OH                | CH <sub>3</sub> O | >200                  | 3.75 ± 0.11           |
| 21a      | H                                  | C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> | H                 | CH <sub>3</sub> O | OH                | >200                  | >200                  |
| 21b      | H                                  | C <sub>6</sub> H <sub>5</sub>                | H                 | CH <sub>3</sub> O | OH                | >200                  | >200                  |
| 21c      | CH <sub>3</sub>                    | H                                            | H                 | CH <sub>3</sub> O | OH                | >200                  | >200                  |
| 21d      | COOH                               | H                                            | H                 | CH <sub>3</sub> O | OH                | >200                  | >200                  |
| 21e      | C <sub>6</sub> H <sub>5</sub>      | H                                            | H                 | CH <sub>3</sub> O | OH                | >200                  | 7.51 ± 0.04           |
| 21f      | H                                  | H                                            | H                 | CH <sub>3</sub> O | OH                | >200                  | >200                  |
| 22a      | H                                  | CH <sub>3</sub>                              | H                 | CH <sub>3</sub> O | CH <sub>3</sub> O | 15.16 ± 0.26          | >200                  |
| 22b      | H                                  | C <sub>6</sub> H <sub>5</sub>                | H                 | CH <sub>3</sub> O | CH <sub>3</sub> O | 19.17 ± 3.08          | >200                  |
| 22c      | CH <sub>3</sub>                    | H                                            | H                 | CH <sub>3</sub> O | CH <sub>3</sub> O | 12.89 ± 1.98          | >200                  |
| 22d      | C <sub>6</sub> H <sub>5</sub>      | H                                            | H                 | CH <sub>3</sub> O | CH <sub>3</sub> O | >200                  | 0.57±0.03             |
| 22e      | H                                  | H                                            | H                 | CH <sub>3</sub> O | CH <sub>3</sub> O | 20.74 ± 0.11          | >200                  |
| 24a      | COOH                               | H                                            | H                 | OH                | H                 | >200                  | >200                  |
| 24b      | COOH                               | H                                            | Br                | OH                | H                 | >200                  | >200                  |
| 24c      | COOH                               | H                                            | Br                | CH <sub>3</sub> O | Br                | >200                  | >200                  |
| 24d      | COOH                               | H                                            | H                 | H                 | H                 | >200                  | >200                  |
| 24e      | COOH                               | H                                            | Br                | H                 | NO <sub>2</sub>   | >200                  | >200                  |
| 24f      | COOH                               | H                                            | H                 | H                 | OH                | >200                  | >200                  |
| 24g      | COOH                               | H                                            | H                 | H                 | CH <sub>3</sub> O | >200                  | >200                  |
| 25a      | CN                                 | H                                            | H                 | OH                | H                 | >200                  | >200                  |
| 25b      | CN                                 | H                                            | Br                | OH                | H                 | >200                  | >200                  |
| 25c      | CN                                 | H                                            | Br                | CH <sub>3</sub> O | H                 | >200                  | 64.20 ± 2.36          |
| 25d      | CN                                 | H                                            | Br                | OH                | Br                | >200                  | >200                  |
| 25e      | CN                                 | H                                            | Br                | CH <sub>3</sub> O | Br                | >200                  | 10.05 ± 0.36          |

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Table 1 | Continued

| Compound   | R <sub>1</sub>    | R <sub>2</sub> | R <sub>3</sub> | R <sub>4</sub>    | R <sub>5</sub>  | MAO-A                 | MAO-B                 |
|------------|-------------------|----------------|----------------|-------------------|-----------------|-----------------------|-----------------------|
|            |                   |                |                |                   |                 | IC <sub>50</sub> (μM) | IC <sub>50</sub> (μM) |
| <b>25f</b> | CN                | H              | Br             | OH                | NO <sub>2</sub> | 58.57 ± 5.66          | 10.79 ± 0.01          |
| <b>25g</b> | CN                | H              | Br             | CH <sub>3</sub> O | NO <sub>2</sub> | >200                  | 28.27 ± 1.24          |
| <b>25h</b> | CN                | H              | H              | H                 | H               | >200                  | >200                  |
| <b>25i</b> | CN                | H              | Br             | H                 | H               | >200                  | 3.45 ± 0.90           |
| <b>25j</b> | CN                | H              | Br             | H                 | Br              | >200                  | 23.15 ± 0.85          |
| <b>26a</b> | CN <sub>4</sub> H | H              | H              | OH                | H               | >200                  | >200                  |
| <b>26b</b> | CN <sub>4</sub> H | H              | Br             | CH <sub>3</sub> O | H               | >200                  | >200                  |
| <b>26c</b> | CN <sub>4</sub> H | H              | H              | H                 | H               | >200                  | >200                  |
| <b>26d</b> | CN <sub>4</sub> H | H              | Br             | CH <sub>3</sub> O | NO <sub>2</sub> | >200                  | >200                  |
| <b>26e</b> | CN <sub>4</sub> H | H              | Br             | H                 | NO <sub>2</sub> | >200                  | >200                  |
| <b>26f</b> | CN <sub>4</sub> H | H              | H              | H                 | OH              | >200                  | >200                  |
| Toloxatone |                   |                |                |                   |                 | 6.71 ± 0.42           |                       |
| Selegiline |                   |                |                |                   |                 |                       | 0.06 ± 0.01           |

field plot (2A), while regions in which low electron density (negative charge) were unfavorable for MAO inhibition are depicted as blue contours. Positively charged groups at positions C-3 and C-6, and negatively charged groups at position C-8 of coumarin were introduced to enhance inhibitory activity against MAO-A. This result aligns with the finding that the introduction of a methyl group at position C-3 and a methoxy group at position C-8 successfully inhibited MAO-A. Introduction of positively charged groups at the C-4 and C-6 positions, along with negatively charged groups at the C-3, C-7, and C-8 positions of the coumarins, were favorable for inhibiting MAO-B activity. This was consistent with experimental results and explains why coumarins with phenyl substitution at the C-3 position and methoxy substitution at the C-7 and C-8 positions exhibit stronger inhibition of MAO-B. Similarly, the green-yellow steric contours depicted in **Figure 2B** illustrated areas where steric bulk was predicted to increase (green) or decrease (yellow) activity. The introduction of a large group at the C-4 position and a small group at the C-6 and C-7 positions of coumarins favors MAO-A inhibition, as evidenced by compound **12b**. Similarly, substitution at the C-3, C-6, and C-7 positions of coumarins with a large group and at the C-4 and C-8 positions with a small group favored inhibition of MAO-B, which aligned with the experimental results.

After combining all the results, the structure-MAO inhibition relationship of the coumarins is summarized in **Figure 2C**. Notably, phenyl substitution had a unique effect on MAO inhibitory activity and selectivity of

coumarins. The introduction of a phenyl group at the C-3 position of coumarins significantly enhanced MAO-B inhibition and increased selectivity for MAO-A, whereas a phenyl group at the C-4 position was more favorable for MAO-A inhibition. Moreover, the simultaneous introduction of a hydroxyl group at the C-6 position and a methoxy group at the C-7 position favored the inhibition of both MAO-A and MAO-B, while the introduction of methyl groups at the C-7 and C-8 positions further enhanced the inhibition of both enzymes. Additionally, the introduction of a cyano group at the C-3 position favored the inhibition of MAO-B, while the introduction of methyl groups at the C-3 and C-4 positions favored the inhibition of MAO-A. These findings offer new insights into how the steric-electrostatic properties and various substituents of coumarins affect MAO inhibitory activity, which could aid in the rational design of novel coumarins as potent and selective MAO inhibitors.

## 2.5 Enzyme kinetic analysis

The unique inhibitory effects of phenyl-substituted coumarins promoted us to further explore the mechanism underlying inhibition. The inhibitory mechanisms of **12a**, **13a**, **21e**, and **22d** against MAO-B and **12b** against MAO-A were investigated using the probe substrate, **MR2**, which serves as a typical tool molecule occupying the substrate pocket and undergoing oxidative deamination catalyzed by MAOs. The MAO for each compound had a linear relationship and the Lineweaver-Burk plot (B) showed a quadratic relationship with inhibitor

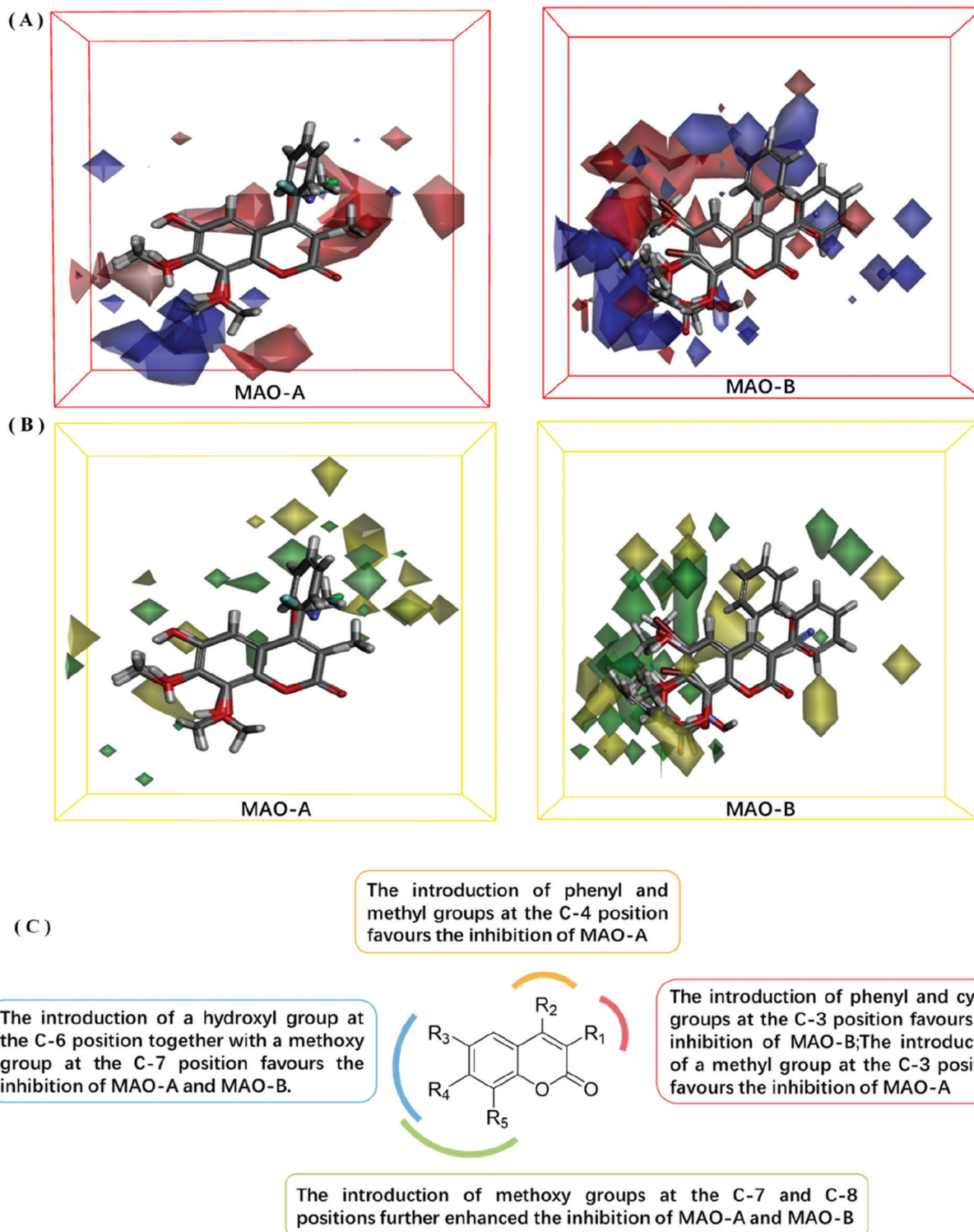


Figure 2 | The coumarin derivatives aligned with the isosurfaces of the 3D QSAR model coefficients on van der Waals (A) and electrostatic potential grids (B), and a summary of the SARs of coumarins as MAOs inhibitors (C).

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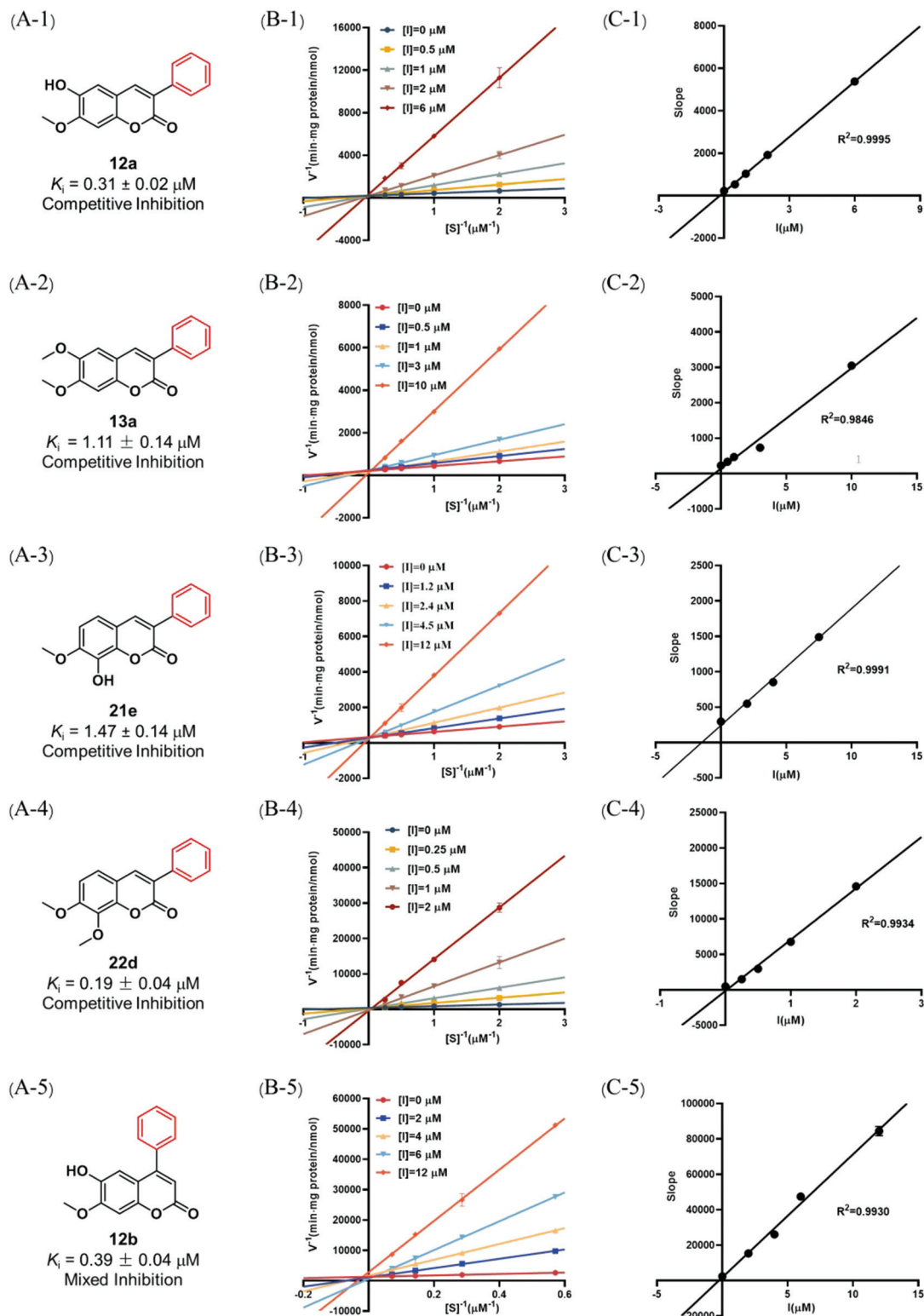


**Figure 3 | Mode of inhibition of the selected inhibitor against MAOs. Compound structural formulae and  $K_i$  values and types of inhibition (A), compound Lineweaver-Burk plots (B), and quadratic slope plots of inhibitor concentrations (C).**

concentration (C), as shown in Figure 3. Compounds **12a**, **13a**, **21e**, and **22d** displayed competitive inhibition against MAO-B, with  $K_i$  values of  $0.31 \pm 0.02 \mu\text{M}$ ,  $1.11 \pm 0.13 \mu\text{M}$ ,  $1.47 \pm 0.13 \mu\text{M}$ , and  $0.19 \pm 0.04 \mu\text{M}$ , respectively. The Lineweaver-Burk plot lines intersected in quadrant II for compound **12b**, suggesting that compound **12b** inhibits MAO-A-catalyzed MR2 oxidative deamination in a mixed manner (competitive plus non-competitive) with a  $K_i$  value of  $0.39 \pm 0.04 \mu\text{M}$ . These findings provide valuable information for analyzing enzyme-small molecule interactions.

## 2.6 Molecular docking analysis

To further elucidate the interaction patterns of phenyl-substituted coumarins with MAOs and the effects on selectivity at the molecular level, molecular docking simulations were performed using the crystal structures of human MAO-A (PDB: 2Z5X) and human MAO-B (PDB: 2V5Z). Compounds **12a**, **18e**, **20e**, and **22d** were selected as model compounds with 3-phenyl substitutions and compounds **12b**, **16g**, **20c**, and **22b** were selected with

4-phenyl substitutions to perform molecular docking simulations with MAO-A and MAO-B, respectively, to determine the mechanisms by which the phenyl substitution position influences enzyme selectivity. The conformations of phenyl-substituted coumarins varied based on whether the phenyl group was substituted at the C-3 or C-4 position with 3-phenyl coumarins exhibiting a narrower conformation (Figure 4A and C), while 4-phenyl coumarins displayed a more compact conformation (Figure 4B and D). Docking simulations conducted in the hMAO-A crystal structure revealed that the 4-phenyl coumarins consistently occupied the same binding position within the enzyme (Figure 4B), whereas the 3-phenyl coumarins exhibited a significant conformational shift during docking, indicating reduced affinity for MAO-A (Figure 4A). Amino acids (Phe-208, Cys-323, and Ile-335) in hMAO-A interact strongly with the 4-position phenyl group of coumarin. Furthermore, docking simulations performed in the hMAO-B crystal structure more clearly indicated that the 3-phenyl coumarins are oriented towards the FAD cofactor with

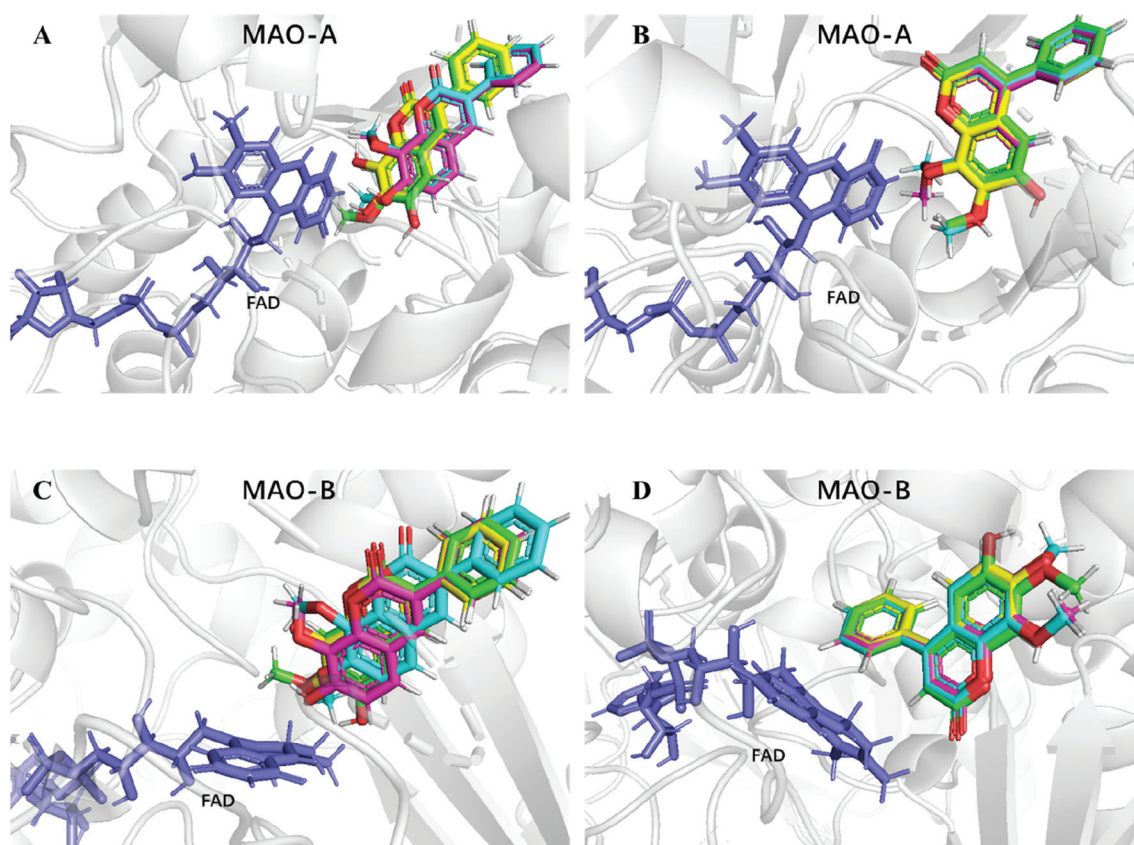


Figure 4 | Docking simulation of MAOs with phenyl-substituted coumarins. Docking diagrams of 3-phenyl substituted coumarins with MAO-A and MAO-B (A and C), Docking diagrams of 4-phenyl substituted coumarins with MAO-A and MAO-B (B and D).

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the 3-phenyl ring facing the hydrophobic entry cavity (Figure 4C), in agreement with previous results [33]. In contrast, the opposite orientation was observed for the 4-phenyl coumarins (Figure 4D). These docking simulation results aligned well with the SAR analysis, emphasizing that the position of the phenyl group is critical for the selectivity of coumarins towards MAOs. Specifically, a phenyl group at the C-3 position enhanced binding to MAO-B, while a phenyl group at the C-4 position was more favorable for MAO-A.

The detailed interaction modes of the 4-phenyl daphnetin, **16g**, and the methylated derivatives (**20c** and **22b**) with MAO-A were further investigated to elucidate the effect of methyl substitution on MAO-A inhibition. As

shown in Figure 5, the coumarin core and the 4-phenyl substituent of the three compounds mainly interacted with the MAO-A amino acid residues (Ile-180, Phe-208, Cys-323, and Ile-335). Notably, compared to the 4-phenyl daphnetin, **16g** (Figure 5A), the 8-methylated derivative, **20c** (Figure 5B), and the 7,8-dimethylated derivative, **22b** (Figure 5C), exhibited increased hydrogen bonding interactions with the amino acid residues (Gln-215 and Tyr-444) of MAO-A. Additionally, the binding energy progressively decreased with increasing degrees of methylation (**16g** = −6.3 kcal/mol, **20c** = −6.9 kcal/mol, and **22b** = −7.7 kcal/mol). These results indicated that 4-phenyl daphnetin methylation enhanced interactions with MAO-A while reducing binding energy, which

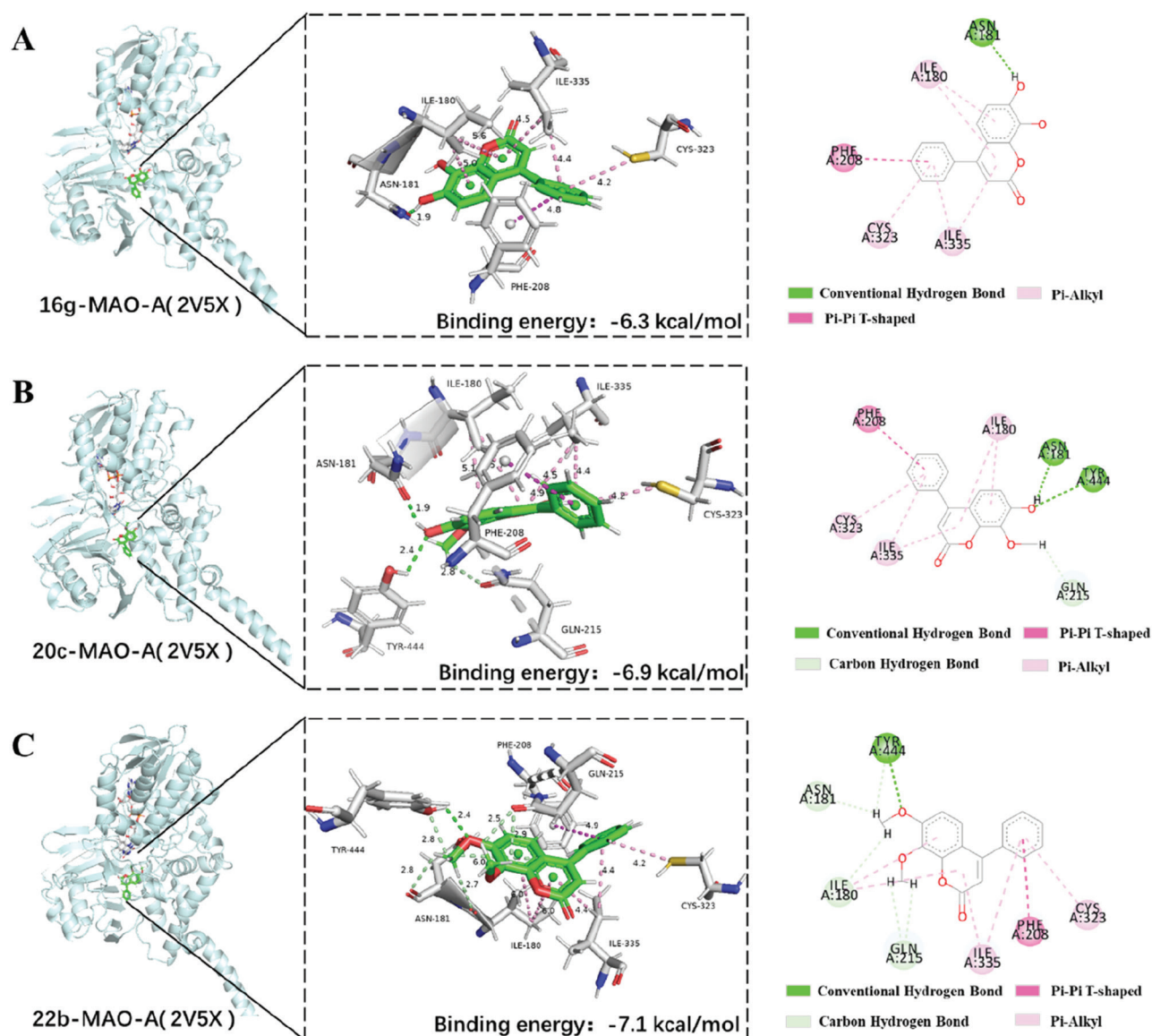


Figure 5 | Detailed view of the binding of **16g** (A), **20c** (B), and **22b** (C) to the catalytic cavity of MAO-A and the interaction.

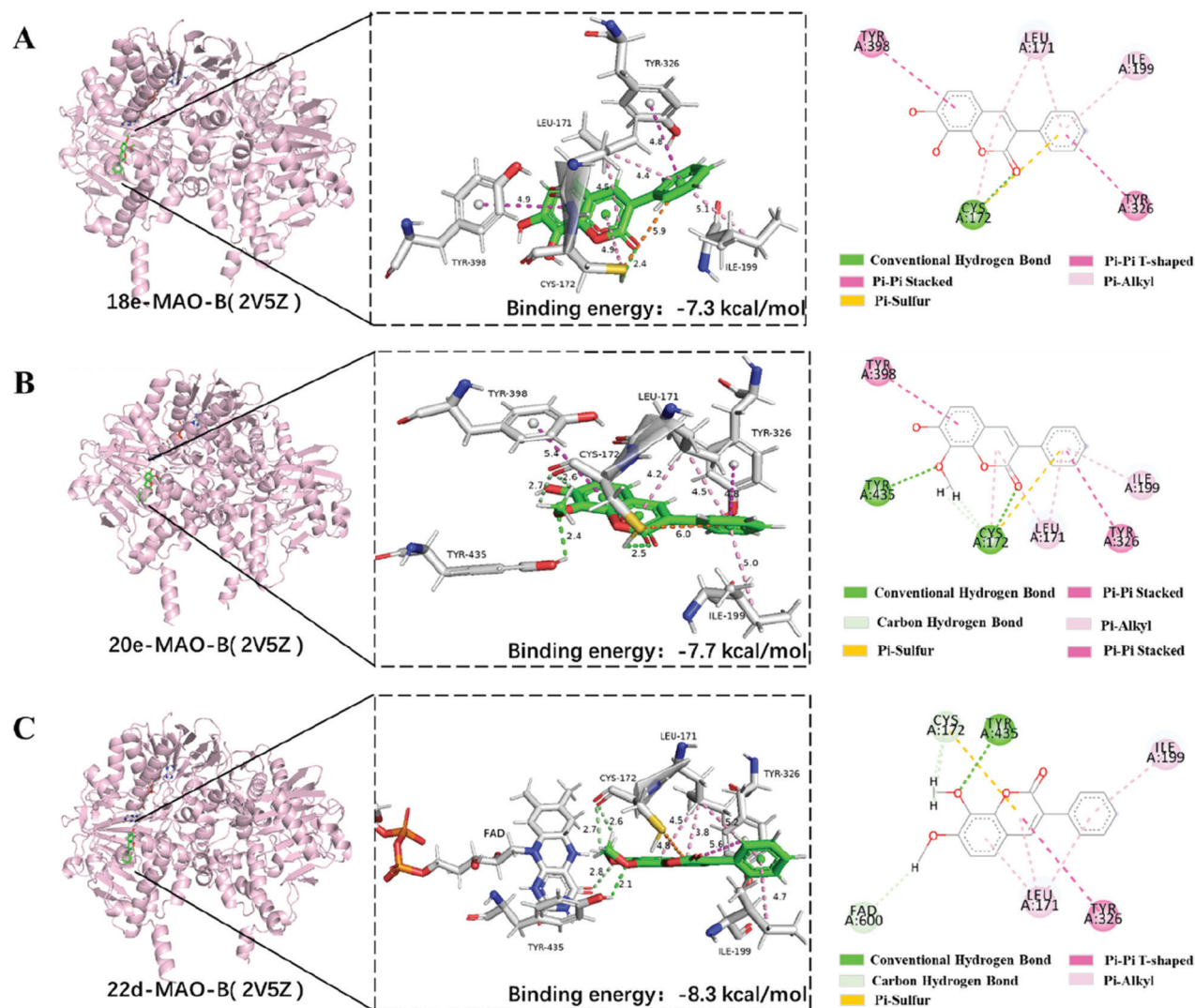


Figure 6 | Detailed view of the binding of 18e (A), 20e (B), and 22d (C) to the catalytic cavity of MAO-B and the interaction.

resulted in a more stable complex and increased inhibitory activity against MAO-A, consistent with the results of inhibitory activity assays.

Similarly, the detailed interaction modes of the 3-phenyl daphnetin, **18e**, and the methylated derivatives (**20e** and **22d**) with MAO-B are shown in Figure 6. The coumarin core and the 3-phenyl substituent of these compounds mainly interact with the MAO-B amino acid residues (Tyr-398, Leu-171, Ile-199, Try-326, and Cys-172). When the C-8 hydroxyl group of **18e** (Figure 6A) was replaced by the methoxy group in **20e** (Figure 6B), this methoxy group formed a hydrogen bond with the MAO-B residue (Tyr-435), resulting in more stable binding with binding energy changing from -7.3 kcal/mol to -7.7 kcal/mol. When both hydroxyl groups at positions 7 and 8 were replaced by methoxy in **22d** (Figure 6C), steric hindrance at the C-7 and C-8 positions increased, shifting

the overall position of the compound forward. This increase brought the phenyl group closer to the hydrophobic cavity, altering the hydrogen-bonding interaction between Cys-172 and the carbocyclic carbonyl of the coumarin core to interact with the methoxy group at C-7. Additionally, the hydrogen bonding of the methoxy group at C-8 with Fad-600 further enhanced binding strength. These interactions resulted in a binding energy as low as -8.3 kcal/mol for compound **22d** with MAO-B.

### 3. CONCLUSION

In summary, 79 natural and synthetic coumarin derivatives with different substituents were designed and synthesized. The MAO inhibitory activities of these compounds were assayed using a fluorescence-based high throughput screening method and the potential

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structure–inhibition relationships of coumarin derivatives were summarized and discussed. The results indicated that the position of the phenyl group is critical for coumarin selectivity towards MAOs. Specifically, a phenyl group at the C-3 position enhances binding to MAO-B, while a phenyl group at the C-4 position is more favorable for MAO-A. Moreover, methylation of phenyl daphnetin enhanced the interactions with MAOs amino acid residues, resulting in more stable binding and increased inhibitory activity toward MAOs but did not significantly affect subtype selectivity between MAO-A and MAO-B. Furthermore, the mechanisms underlying inhibition of these phenyl coumarin derivatives were elucidated through enzymatic kinetics analysis and molecular docking simulations. These findings provided new insight into the interactions between coumarins and MAOs, significantly contributing to the development of coumarin-based MAO inhibitors.

## 4. MATERIALS AND METHODS

### 4.1. Reagents and materials

It has been reported that MR2 [38], in which the amino group is linked to resorufin, is a fluorescent probe substrate for MAOs and is based on a tandem amine oxidation/ $\beta$ -elimination mechanism. The substrate was synthesized according to the synthetic route [41–43]. Toloxatone, selegiline, potassium dihydrogen phosphate, potassium phosphate dibasic, phosphoric acid, dimethyl sulfoxide, acetonitrile, and recombinant human MAO-A and MAO-B were purchased from Sigma-Aldrich (St. Louis, MO, USA). To investigate the specific inhibition of different coumarin derivatives on MAO-A and MAO-B, different series of coumarin derivatives were synthesized.

### 4.2 Enzyme assays

The activity of MAOs was investigated using the non-selective substrate, MR2, as a probe. The MR2 oxidative deamination reaction was determined by the methods published previously with a slight modification [38]. MAO activity was measured using MR2 as a substrate and at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. MAO-A activity was measured at a substrate concentration of 0–20  $\mu$ M for 40 min and MAO-B activity was measured at a substrate concentration of 0–10  $\mu$ M for 30 min. The reaction rate was expressed as a change in concentration per minute.

### 4.3 Analysis of inhibitory activities

The inhibitory activities of the synthesized and purified compounds were investigated using MAO-A and MAO-B. The incubation system (total volume of 100  $\mu$ L) involved mixing various components, including 50 mM PB buffer (pH 7.4), and MR2 with or without coumarin derivatives (0–200  $\mu$ M), as previously described. The concentrations of MR2 were 7 and 2  $\mu$ M for MAO-A and MAO-B,

respectively, and the protein concentrations were 5 and 3  $\mu$ g/mL for the MAO isoforms. The mixture was incubated at 37°C for 60 min and all reactions were terminated by adding an equal volume of ice-cold acetonitrile. Measure fluorescence values were determined using a microplate reader. Toloxatone and selegiline were used as positive inhibitors for MAO-A and MAO-B, respectively.

### 4.4. Inhibition kinetic assay

The MAO-A and MAO-B inhibition kinetics were studied for compounds with an  $IC_{50} < 10$   $\mu$ M. Inhibition kinetic experiments were performed with 5 concentrations of compounds (0–20  $\mu$ M) and 4 concentrations of MR2 substrates (0.5–14  $\mu$ M). Inhibitory patterns and  $K_i$  values were determined using Lineweaver-Burk plots and Dixon plots. Toloxatone and selegiline were used as positive inhibitors for MAO-A and MAO-B, respectively.

### 4.5 3D-QSAR model building

3D-QSAR models were built using the corresponding package of Drug Discovery Studio. Discovery Studio 2016 was used to build the partial least squares model. Electrostatic potential and steric effect probes were used for the energy lattice point calculation, small molecular compounds were stacked using the parent nucleus, the experimentally obtained  $pIC_{50}$  was added to the stacked molecule set form, and the 3D QSAR models were built setting input ligands as the training set for screening, input test ligands as the test set, activity property as the experimentally determined  $pIC_{50}$ , and the rest of the values were kept as a default.

### 4.6 Molecular docking

The molecular docking process was performed using Discovery Studio (BIOVIA Discovery Studio 2016, Dassault Systèmes, San Diego, CA, USA). The docking protocol was performed with the hMAO-A (2Z5X) and hMAO-B (2V5Z) X-ray crystal structure downloaded from the Protein Data Bank (<https://www.rcsb.org/>). Small molecule compounds were drawn using ChemDraw 20.0 software and converted to the corresponding 3D structures by Chem3D, which are used as ligand libraries for molecular docking. The CHARMM 40.1 force field was used to represent the protein and ligand structures. Docking simulations were performed using a standard CDOCKER protocol. The rigid poses were placed into the active site of MAO. The interaction force of the compound ligands with the highest CDOCKER score was analyzed at the protein binding site.

### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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