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Letter

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Elucidating Cysteine-Assisted Synthesis of Indirubin by a Flavin-Containing Monooxygenase

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ABSTRACT: Indirubin is a biologically active compound found in *Danggui Longhui Wan*, a traditional Chinese medicine for chronic myelocytic leukemia. In the biosynthesis of indirubin, the formation of indigo, a stereoisomer of indirubin, is a major side reaction. Recent finding suggested that cysteine supplementation shifts product selectivity from indigo to indirubin. Here, we disclose how cysteine is involved in enhancing the product selectivity in the biosynthesis of indirubin using a flavin monooxygenase from *Methylophaga aminisulfidivorans* (MaFMO). First, cysteine reacts with indoxyl to synthesize 2-cysteinylindoleninone, inhibiting dimerization of indoxyl. Second, the reducing power of cysteine allows FMO to additionally hydroxylate indoxyl toward isatin, overcoming the problem in biased distribution of two different precursors. Third, cysteine activates isatin to react with 2-cysteinylindoleninone to form indirubin. Based on this revealed mechanism, indirubin derivatives with different indole ring components were synthesized.

KEYWORDS: *indirubin, cysteine, flavin-containing monooxygenase, indoleninone, product selectivity*

For centuries, traditional Chinese Medicine (TCM) has shown its efficacy with low side effects in treating various diseases. Active compounds in TCM were discovered and their physiological roles have been gradually revealed,[1, 2] promoting safe usage of herbal medicine and development of the analogues of the active compounds. As one of the compounds successfully found in TCM, indirubin (**4a**) is known as an active compound found in *Danggui Longhui Wan*, which is used to treat chronic myelocytic leukemia.[3] Indirubin and its derivatives possess high affinity toward ATP-binding pockets of protein kinases involved in cell cycle regulation (e.g. CDKs),[3, 4] glycogen metabolism (e.g. GSK3β),[5] or Stat signaling (e.g. Src).[6] Owing to such multifaceted interactions, indirubin derivatives showed anti-proliferative and anti-tumor effects.[7, 8] They have shown promising efficacy and low adverse effects in human clinical trials[9, 10] and are currently being developed as potential agents for treating psoriasis or maintaining stem cell pluripotency.[10-12]

To overcome the drawbacks in indirubin production by plant extraction[13] or/and chemical synthesis,[14-16] microbial biosynthesis of indirubin has been attempted. A cytochrome P450,[17] flavin-containing monooxygenases,[13, 18] toluene monooxygenases[19, 20] and naphthalene dioxygenases[21, 22] showed their ability to synthesize indirubin (**4a**) from indole (**1a**) generated as a metabolite from tryptophan. However, undesired indigo (**5**) was mainly synthesized as byproduct by dimerization of indoxyl (**3a**), which became a major obstacle in the biosynthesis of **4a**. Recently, Han *et. al.*[23] showed high product selectivity toward indirubin by supplementing L-cysteine to *E. coli* expressing MaFMO (Figure 1A). However, the principle behind L-cysteine supplementation was not fully understood[19, 23] and the biosynthesis of indirubin derivatives has never been attempted. Here, we reveal the role of cysteine in the selectivity change from **5** to **4a**. Based on this mechanism, we aimed to design biocatalytic process for indirubin derivatives.

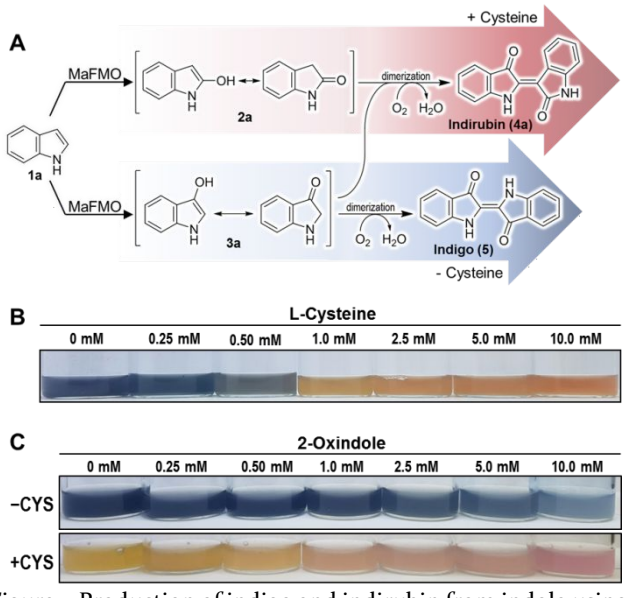


Figure 1. Production of indigo and indirubin from indole using *E.coli* expressing MaFMO. (A) Scheme of cysteine-assisted synthesis of indirubin. (B) Effect of L-cysteine addition on indirubin synthesis, 6 h. (C) Effect of **2a** supplementation on indirubin synthesis in the absence and presence of cysteine (5 mM), 6 h.

First, to confirm the change in product selectivity by cysteine supplementation, **4a** production was monitored by varying the concentration of L-cysteine added to the whole-cell reaction system using *E. coli* expressing MaFMO (Figure 1B). 0.40 mM of **5** was produced in the whole-cell reaction without L-cysteine (Figure S1). The product selectivity shifted from **5** to **4a** when more than 1 equivalent of L-cysteine was added. As the L-cysteine concentration increased, the production of **4a** increased up to 0.12 mM when 5.0 mM of L-cysteine was added. In addition, to confirm an observation from the previous report that accumulation of 2-oxindole (**2a**) is a major factor of the selectivity shift[23], the effect of **2a** concentration toward **4a** production was monitored with or without 5.0 mM L-cysteine (Figure 1C). When **2a** was added without L-cysteine, production of **5** was still dominant. Only when L-cysteine was supplemented along with **2a**, the product selectivity was shifted toward **4a**. As the concentration of **2a** increased, production of 4a increased and saturated around 0.30 mM. At this step, we identified that L-cysteine inhibits the synthesis of **5** possibly protecting **3a** from autoxidation or dimerization.

To find which property of L-cysteine made the selectivity shift, production of indigoids with L-cysteine and other supplements including amino acids (L-alanine, L-valine, L-serine, L-methionine, S-methyl-L-cysteine, cystine, D-cysteine) and reducing agents (DTT, β-mercaptoethanol, hydroquinone, ascorbic acid) were compared *in vitro* (Figure S2-S4). Addition of L-cysteine, D-cysteine, DTT, or β-mercaptoethanol showed **4a** synthesis without **5,** but not with hydroquinone, ascorbic acid and other amino acids. Considering that all the effective compounds have thiol groups in common, we suspected that thiol groups can contribute to the selectivity shift. However, since hydroquinone and ascorbic acid also provide reducing power, but did not show the selectivity shift, it was inferred that the protection of **3a** against oxidation was not solely due to the reducing power by thiol groups.

Expecting a possible formation of the thio-intermediate formed with L-cysteine, LC-MS analysis was carried out (Figure 2A). When mixtures containing MaFMO, L-cysteine, and **1a** were carried out individually and together, a new peak at m/z 251 was detected only when all the three components were included. When **2a** or isatin (**7**) was added as a substrate, the peak was not detected. When other amino acids were supplemented to the reaction instead of L-cysteine, a mother peak with the same MS and MS/MS patterns was observed only when D-cysteine was supplemented (Figure S5). Further product identification showed that the intermediate is 2-cysteinylindoleninone (**6a**, Figure S6). When halogenated indoles (**1b**-**1d**) were applied instead of **1a**, new peaks with the corresponding mass differences were detected (Figure 2B&S7). The MS/MS patterns of the new peaks displayed isotopic patterns of the corresponding halogen derivatization, indicating that the peaks were derived from the addition of **1b**-**1d** (Figure S8). Here, we concluded that supplementation of L-cysteine resulted in the synthesis of **6a**, which effectively prevented the dimerization of **3a** into **5**.

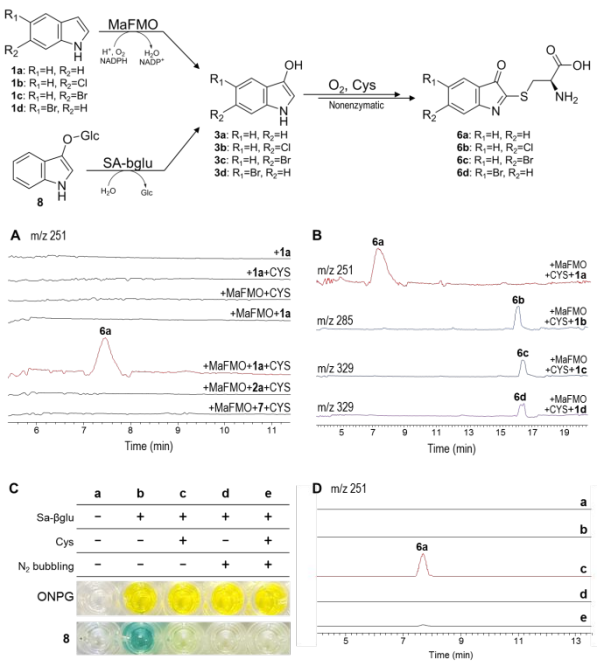


Figure 2. Synthetic scheme and analysis of **6a**. (A) Conditions required for **6a** synthesis, m/z = 251. (B) LC-MS analysis of **6a**-**6d**. Cysteine and oxidation requirement for (C) **5** and (D) **6a** synthesis. o-Nitrophenyl-β-glucopyranoside (ONPG) was used as an indicator to confirm the activity of Sa-βglu according to the conditions. The color change to yellow indicates that Sa-βglu is present in the active form.

We further verified whether cysteine supplementation shows the same effect on other biocatalytic reactions generating **3a**. First, we monitored **6a** synthesis using CYP102G4,[24] a cytochrome P450 that produces **5** from **1a**. Only when L-cysteine was supplemented into the CYP102G4 reaction system, synthesis of **6a** was also identified (Figure S9). Additionally, deglycosylation of indican (**8**) using β-glucosidase from *Sulfolobus acidocaldarius* (Sa-βglu) was used to generate **3a** (Figure 2C&2D). When Sa-βglu was treated with **8**, **5** was mainly produced. Again, cysteine addition prevented the blue dye formation enabling production of **6a**. When autoxidation was reduced by N_2 bubbling, neither **5** nor **6a** was synthesized. From the fact that indoxyl oxidation should be preceded for **6a** synthesis, it can be inferred that products from oxidized indoxyl, such as indolenine or indoxyl radical (Figure S10), are direct reactants for **6a** synthesis. Therefore, it was concluded that **6a** is synthesized between the reactive compound[25, 26] from autoxidation of **3a** and cysteine through a non-enzymatic process under oxidative conditions.

So far, we identified **6a** as a stable intermediate and a potential precursor for ring B (Figure 3A). Since indirubin (**4a**) is composed of two different oxindole rings, we sought to identify the source of ring A for the cysteine-assisted **4a** synthesis by MaFMO. In the synthesis of **4a**, **2a** or **7** can be used to construct the ring A of **4a**, depending on its oxidation mechanism[22, 27-28]. To determine the involvement of each substance in cysteine-assisted **4a** synthesis, **4a** production was monitored during supplementation with either **2a** or **7**.

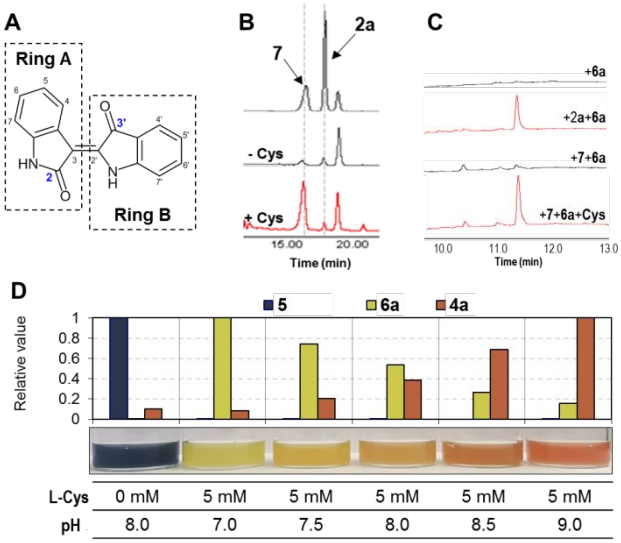
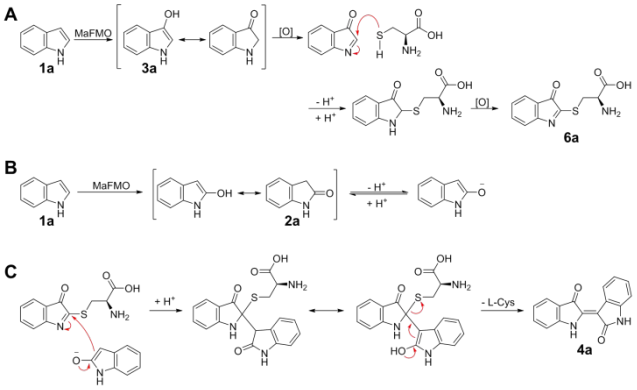


Figure 3. Reconstituted components and reaction conditions required for **4a** synthesis. (A) Structure of **4a** composed of two indole rings. (B) The product profile of **1a** oxidation by MaFMO with and without L-cysteine. (C) HPLC analysis for non-enzymatic reaction of **6a** with the indicated supplements. (D) pH dependency of **4a** and **6a** synthesis from **1a** using *E. coli* expressing MaFMO.

First, we identified the product profile of **1a** oxidation *in vitro*. **2a** was produced both with and without L-cysteine supplementation (Figure 3B& Figure S11-14). Interestingly, **7** production was increased with supplementation of L-cysteine, which was also observed *in vivo* (Figure S15). The profile revealed that both of the potential precursors for ring A are formed. Next, we confirmed that supplementation of either **2a** or **7** into **6a** shows increases in **4a** production in whole-cell reaction system (Figure 1C&S16). As a result, **2a** and **7** both work as precursors for the ring A. To clarify whether their dimerization to **4a** is a non-enzymatic reaction, the ring A precursors and **6a** were mixed and incubated at 30°C (Figure 3C). Addition of **2a** to **6a** showed synthesis of **4a,** but not the addition of **7**. Interestingly, **7** also led to the formation of **4a** when L-cysteine was supplemented. Therefore, we concluded that **2a** as well as **7** reacts with **6a** in a spontaneous reaction.

To further explore the mechanism, the pH dependency of the synthesis of **4a** was investigated. *In vivo* reactions were performed at pH values from 7 to 9 (Figure 3D). As the pH increased, remaining **6a** decreased and the production of **4a** increased. Further pH dependency was determined with **2a** or **7**. For **2a**, the spontaneous reaction with **6a** showed a similar tendency as the *in vivo* reaction, and **2a** derivatives with lower pKa showed faster **4a** synthesis (Figure S17). Additionally, when mixtures of **2a**, L-cysteine, and NaOH were carried out individually or together, no reactions were identified (Figure S18). Based on the pH dependency, we proposed a mechanism, inferring that deprotonated **2a** attacks the C2 of **6a** leading to the dimerization (Scheme 1). In the case of **7**, **4a** was synthesized when the pH was higher than 8.0 (Figure S19). This is consistent with previous research which **7** and indoleninone synthesize **4a** under basic condition with H_2S .[29] We detected intermediate peaks from the mixture of **7** and L-cysteine with MS (Figure S20), but additional evidence is required to suggest precise mechanism.

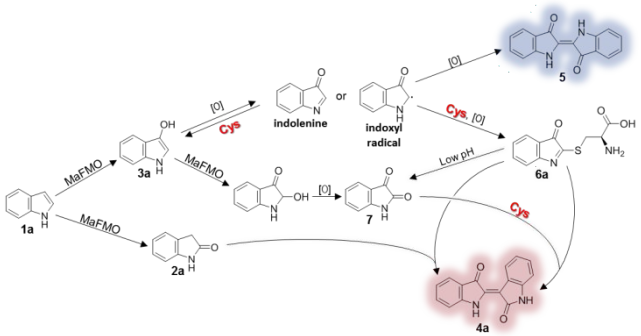


Scheme 1. Proposed mechanism for **4a** synthesis from **2a** and **6a**. A: synthesis of **6a** from **1a** oxidation by MaFMO. B: deprotonation of **2a**. C: dimerization of **2a** and **6a** to form **4a**.

Although we identified the sources of ring A, the increased production of **7** along with L-cysteine supplementation requires explanation. Unlike the synthesis of **2a** from **1a**, the production of **7** from **1a** requires two oxidation steps. Three possible pathways for the synthesis of **7** may be imagined: i) oxidation of **2a** at C3, ii) hydrolysis of **6a**, and iii) oxidation of **3a** at C2.

First, oxidation of **2a** was tested under various conditions. However, oxidation using MaFMO did not produce **7** from **2a** (Figure S21). Second, the hydrolysis of **6a** was monitored in solutions at different pH values and in the presence of cells. **6a** was hydrolyzed to **7** only when acidic pH was applied (Figure S22). However, at pH>7 (or in whole cells), insufficient hydrolysis was observed to account for formation of **7**. The third option is oxidation of **3a** at C2. **3a** was supplied by adding **8** and Sa-βglu into *in vitro* MaFMO oxidation. We detected only the synthesis of **7** when MaFMO and NADPH regeneration system were applied, and the synthesis of **4a** when L-cysteine was supplied additionally (Figure S23). Oxidation at C2 position of 3-methylindole supported the substrate specificity of MaFMO (Figure S24). The rigid docking simulation of **3a** and **2a** to MaFMO demonstrated that only **3a** is suitable for binding to MaFMO and being oxidized (Figure S25). Since cysteine could provide a reductive environment to delay the autoxidation of **3a**, we can infer that **3a** could have a longer lifetime and synthesis of **7** will increase by subsequent oxidation of **3a** by MaFMO.

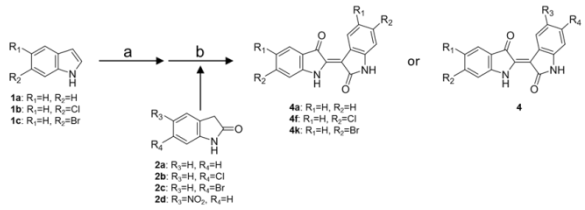
In Scheme 2, the cysteine-assisted synthesis of **4a** using MaFMO is summarized, whereby cysteine plays at least three roles. First, cysteine reacts with **3a** and forms **6a**, preventing formation of **5** and saving the ring B component for **4a** synthesis. Second, cysteine allowed further enzymatic oxidation of **3a** to **7** by MaFMO, but not to **5**. MaFMO predominantly produces **5** in the absence of cysteine,[23] indicating biased regiospecificity of MaFMO toward C3 of **1a** (Figure S26). Moreover, the increase of **4a** production by addition of **2a** showed that the ring A precursor is a limiting reagent (Figure 1C). The conversion of **3a** to **7** may have alleviated the uneven distribution between precursors for **4a** synthesis, enhancing **4a** production. Third, cysteine induced **7** to involve in non-enzymatic **4a** synthesis. In overall, cysteine roles were significant to achieve product selectivity shift from **5** to **4a**.



Scheme 2. Overall scheme of cysteine-assisted **4a** synthesis by MaFMO.

Finally, we applied this concept to synthesize various indirubin derivatives. Feeding combinations of **1a-1d** and **2a-2d** were applied (Table 1 & Figure S27-S30). The reaction resulted in producing the mixture of two **4**, one with the rings from **1** and the other with its ring A from **2** and its ring B from **1**. The yield was significantly affected by which **1** was added, possibly due to substrate specificity of MaFMO. Moreover, the yield slightly increased as **2** possessed the lower pKa value. In our knowledge, this is the first attempt to synthesize halogenated and nitrated **4** biologically. In addition, we further attempted the same strategy using CYP102G4 and observed the production of **4** determined by **2a** (Figure S31-S33). Therefore, it was confirmed that the strategy can be applied to other indoxyl-producing monooxygenases to synthesize diverse **4**.

Table 1. Synthesis of **4**, a: MaFMO, 200 rpm, 30ºC, 100 mM Tris-HCl pH 7. b: pH adjustment to 9 and addition of **2**.



Substrate		Product	R_1	R_2	R_3	R_4	MS→MS/MS	Yield (%)
1a	2a	4a	H	H	H	H	261→157	45.2±2.9
	2b	4b	H	H	H	Cl	295→191	39.0±3.9
	2c	4c	H	H	H	Br	341→237	40.5±2.7
	2d	4d	H	H	NO_2	H	306→202	40.6±6.8
1b	2a	4e	H	Cl	H	H	295→157	19.7±3.4
	2b	4f	H	Cl	H	Cl	329→191	25.7±2.2
	2c	4g	H	Cl	H	Br	375→235,237	24.1±2.5
	2d	4h	H	Cl	NO_2	H	340→202	22.7±5.9
1c	2a	4i	H	Br	H	H	339→157	4.6±2.3
	2b	4j	H	Br	H	Cl	375→191,193	4.9±1.6
	2c	4k	H	Br	H	Br	421→237	16.5±4.1
	2d	4l	H	Br	NO_2	H	384→186	5.3±2.6

To eliminate separation process for the mixtures, we produced **6** from **1** for ring B and separately added **2** for ring A (Figure S34). First, we performed *in vivo* production of **6** at pH 7.0, and removed **2** and **7** by extraction with organic solvents, avoiding production of **4**. The harvested water layer was supplemented with the selected **2** and the pH of the reaction mixture was adjusted to pH 9 to induce the production of **4**. As a result, the **4** with ring A from **2** and the ring B from **1** were successfully synthesized with high purity (Figure S35-S40 & Table S1). Finally, we were able to synthesize **4** with derivatization at the desired positions.

Biological synthesis of **4a** was found in various organisms[30-32]. However, the mechanism was not well understood and selective biosynthesis of **4a** could not be achieved due to autoxidation. Here, we successfully elucidated how cysteine benefits the synthesis of **4a.** Cysteine captured the reactive compounds and mediated the site-specific reactions. In particular, **6** can act as a stable donor of **3** and be incorporated into **2** or **7** to make **4** in a simple and defined manner [31, 33]. In this respect, there is a high potential that the biosynthetic method of **6** can be applied effectively in the synthesis of alkaloids or drug analogs with **3** as a substructure.

As demands for green chemistry increases, chemical synthesis is being replaced by innovative biological processes even for fine-chemicals. Synthesis of **4** was also achieved by enzymatic oxidation of **1** in the presence of cysteine, incorporation of **2** or **7**, and extraction using organic solvents. The derivatization can be achieved biologically by utilizing enzymes responsible for halogenation[34], nitration[35], and hydroxylation[36] of the indole ring. Moreover, the extraction step can be removed by utilizing indole oxidases highly specific for synthesis of **3** without overoxidation[37]. By incorporating and manipulating such enzymes, it will be possible to realize biosynthesis of **4** or its derivatives to be a fully sustainable process starting from abundant and safe materials.

ASSOCIATED CONTENT

AUTHOR INFORMATION

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Author Contributions

J. Kim carried out the experiment and interpreted the results. J. Kim took the lead in writing the manuscript with support from B-G Kim. P-G Lee, J. Lee and W Kroutil revised the manuscript and provided critical feedback. J. Lee performed NMR analysis. E-J Kim contributed to gene construction and culture system.

Notes

The authors declare no competing financial interests.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Experimental procedures; Figures S1-S40; Tables S1 (PDF).

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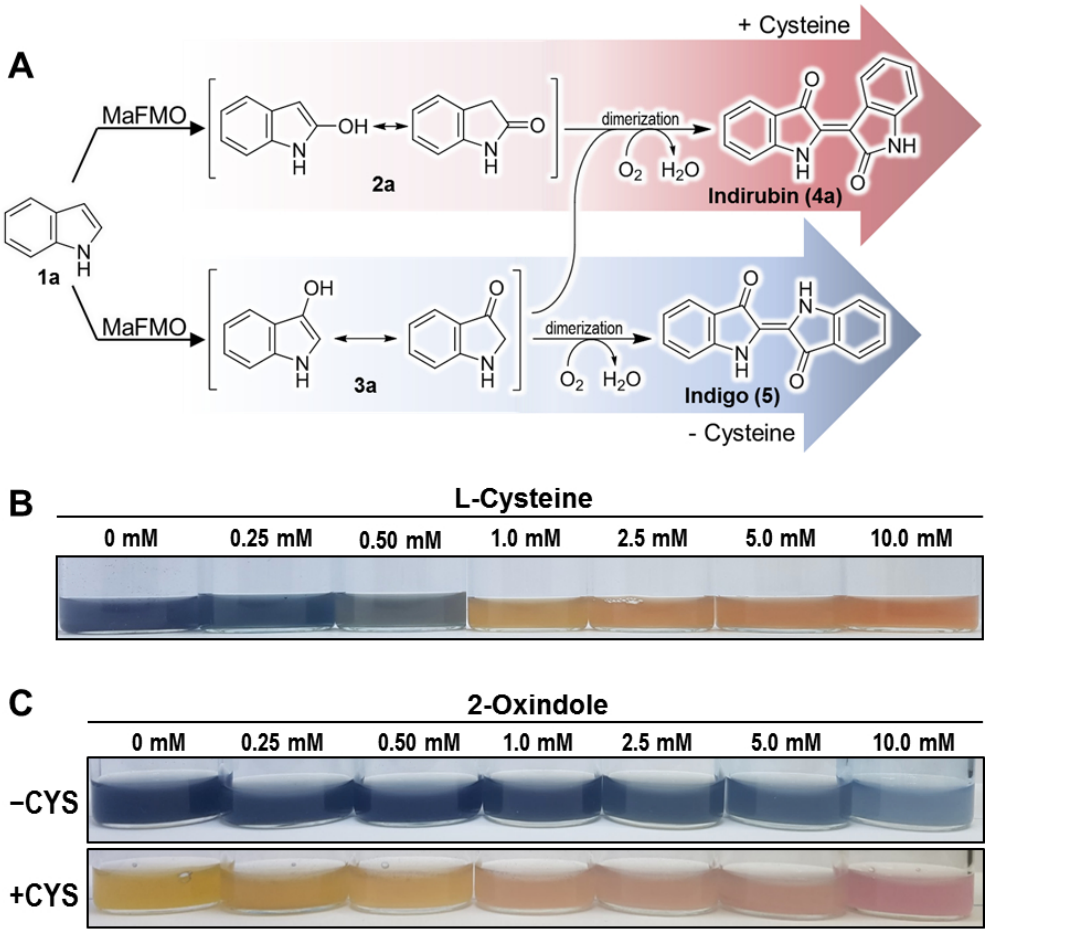

Figure 1. Production of indigo and indirubin from indole using E.coli expressing MaFMO. (A) Scheme of cysteine-assisted synthesis of indirubin. (B) Effect of L-cysteine addition on indirubin synthesis, 6 h. (C) Effect of 2a supplementation on indirubin synthesis in the absence and presence of cysteine (5 mM), 6 h.

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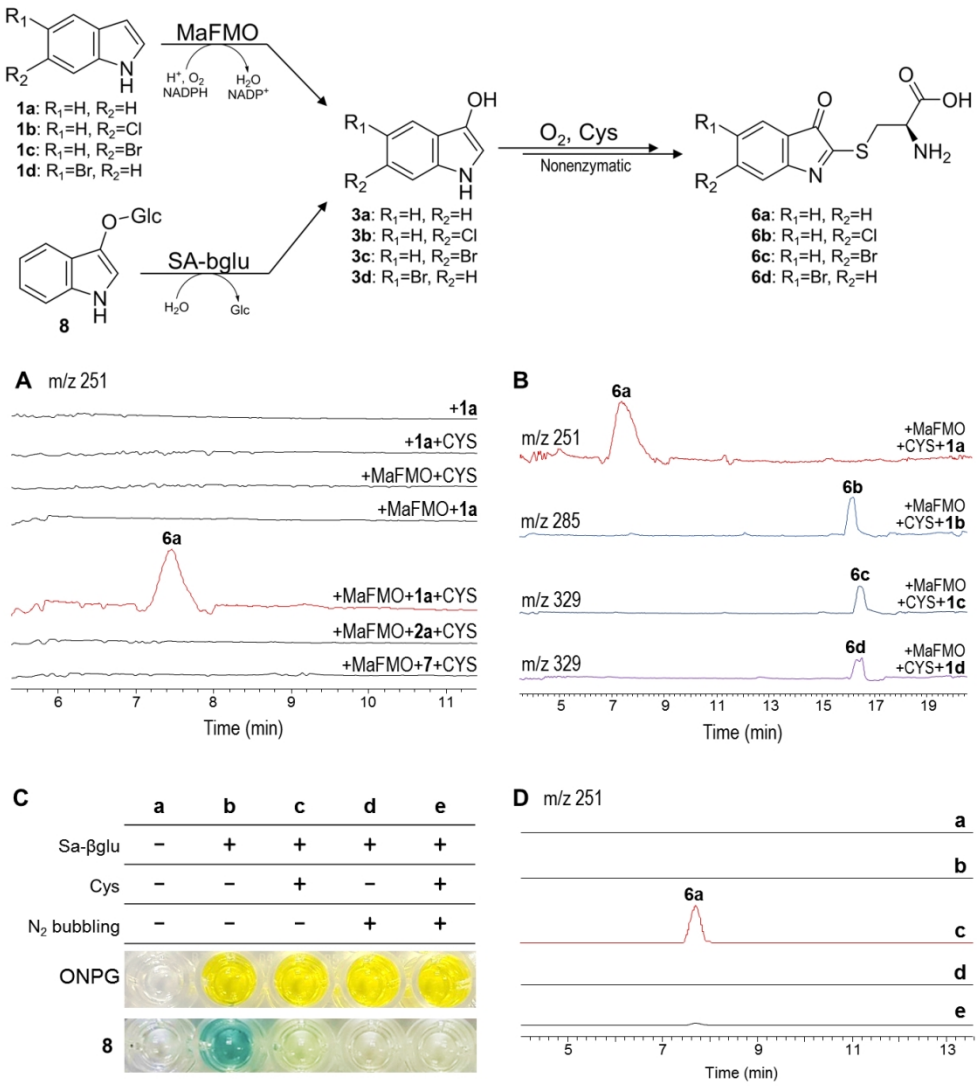

Figure 2. Synthetic scheme and analysis of 6a. (A) Conditions required for 6a synthesis, m/z = 251. (B) LC-MS analysis of 6a-6d. Cysteine and oxidation requirement for (C) 5 and (D) 6a synthesis. o-Nitrophenyl-β-glucopyranoside (ONPG) was used as an indicator to confirm the activity of Sa-βglu according to the conditions. The color change to yellow indicates that Sa-βglu is present in the active form.

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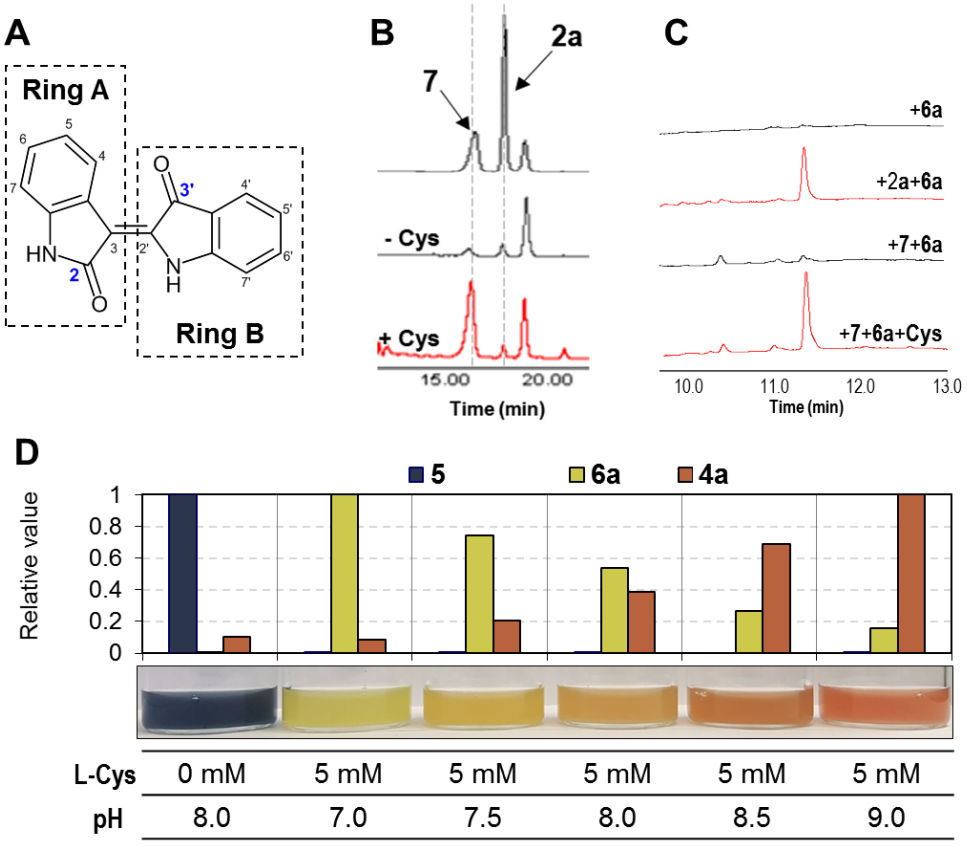
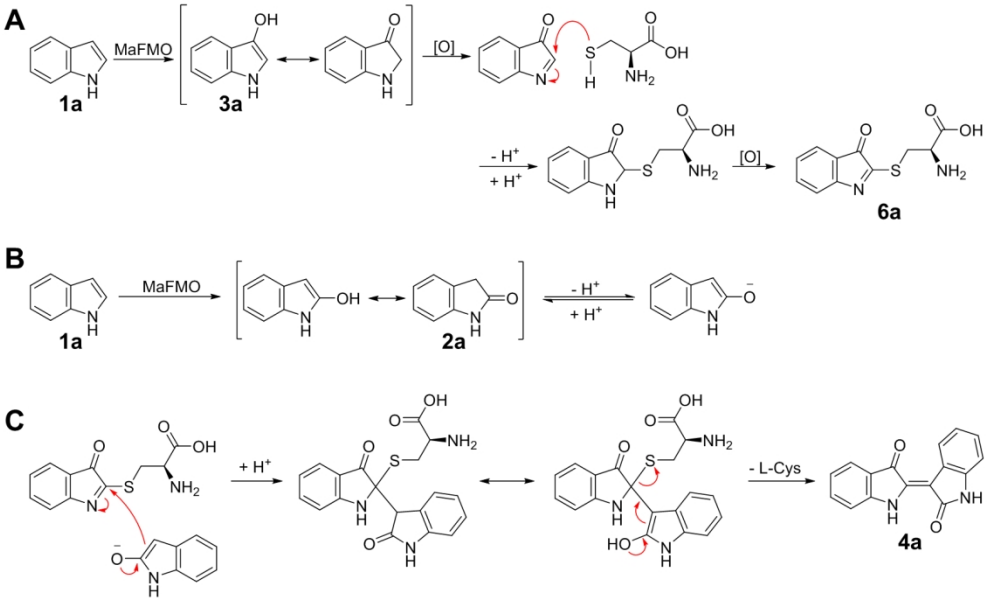


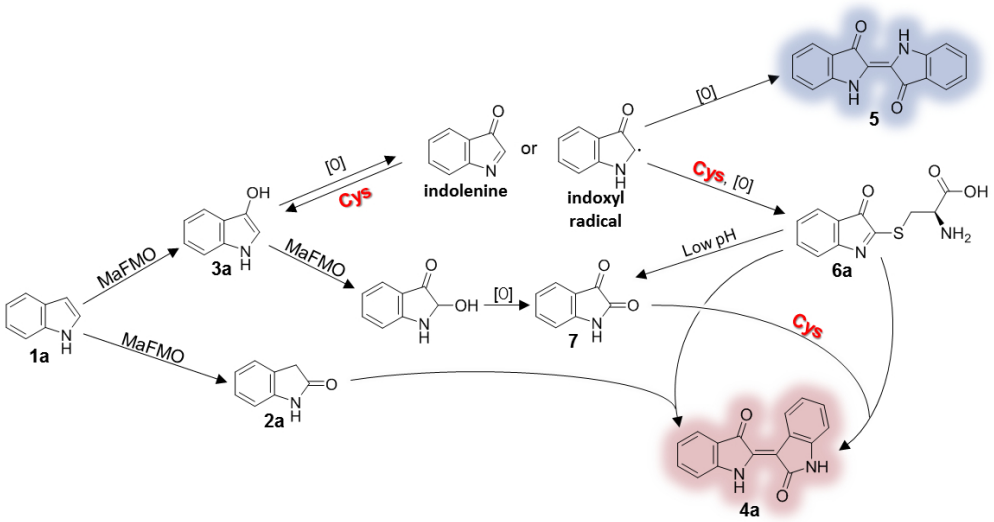
Figure 3. Reconstituted components and reaction conditions required for 4a synthesis. (A) Structure of 4a composed of two indole rings. (B) The product profile of 1a oxidation by MaFMO with and without L-cysteine. (C) HPLC analysis for non-enzymatic reaction of 6a with the indicated supplements. (D) pH dependency of 4a and 6a synthesis from 1a using E. coli expressing MaFMO.

277x236mm (96 x 96 DPI)



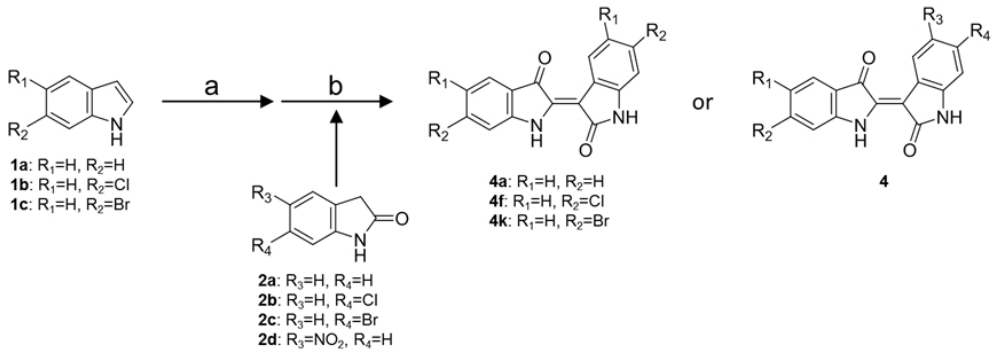
Scheme 1. Proposed mechanism for 4a synthesis from 2a and 6a. A: synthesis of 6a from 1a oxidation by MaFMO. B: deprotonation of 2a. C: dimerization of 2a and 6a to form 4a.

460x279mm (96 x 96 DPI)



Scheme 2. Overall scheme of cysteine-assisted 4a synthesis by MaFMO.

306x169mm (96 x 96 DPI)



Substrate		Product	R_1	R_2	R_3	R_4	MS→MS/MS	Yield (%)
1a	2a	4a	H	H	H	H	261→157	45.2±2.9
	2b	4b	H	H	H	Cl	295→191	39.0±3.9
	2c	4c	H	H	H	Br	341→237	40.5±2.7
	2d	4d	H	H	NO_2	H	306→202	40.6±6.8
1b	2a	4e	H	Cl	H	H	295→157	19.7±3.4
	2b	4f	H	Cl	H	Cl	329→191	25.7±2.2
	2c	4g	H	Cl	H	Br	375→235,237	24.1±2.5
	2d	4h	H	Cl	NO_2	H	340→202	22.7±5.9
1c	2a	4i	H	Br	H	H	339→157	4.6±2.3
	2b	4j	H	Br	H	Cl	375→191,193	4.9±1.6
	2c	4k	H	Br	H	Br	421→237	16.5±4.1
	2d	4l	H	Br	NO_2	H	384→186	5.3±2.6

Table 1. Synthesis of 4, a: MaFMO, 200 rpm, 30ºC, 100 mM Tris-HCl pH 7. b: pH adjustment to 9 and addition of 2.

230x199mm (96 x 96 DPI)