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Asymmetric Synthesis of Sterically Hindered 1-Substituted Tetrahydro-β-carbolines Enabled by Imine Reductase: Enzyme Discovery, Protein Engineering, and Reaction Development

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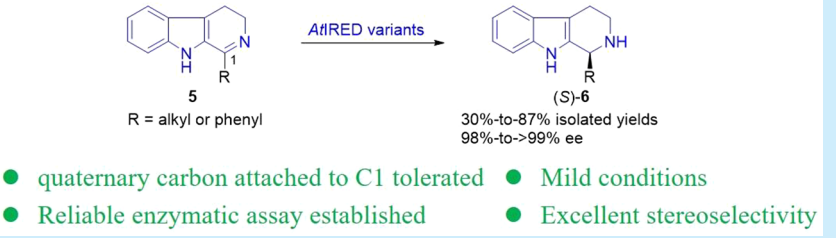

ABSTRACT: We report the discovery of a new imine reductase (IRED), named *At*IRED, by genome mining. Site-saturation mutagenesis on *At*IRED generated two single mutants M118′L and P120′G and the double mutant M118′L/P120′G with improved specific activity toward sterically hindered 1-substituted dihydro-β-carbolines. The synthetic potential of these engineered IREDs was showcased by the preparative-scale synthesis of nine chiral 1-substituted tetrahydro-β-carbolines (THβCs), including (*S*)-1-*t*-butyl-THβC and (*S*)-1-*t*-pentyl-THβC, in 30−87% isolated yields with excellent optical purities (98−99% ee).

Imine reductases (IREDs) are a class of enzymes capable of asymmetric reduction of prochiral imines to chiral amines.[1] As recognition of their great synthetic potential, both academic and industrial communities have rapidly paid attention to these enzymes over the past decade, ever since Mitsukura's first report on the discovery of two stereocomplementary IREDs originating from *Streptomyces* sp. GF3587 and *Streptomyces* sp. GF3546 in 2010.[2] Research across various topics, ranging from the identification of new IRED enzymes aided by modern bioinformatics tools and the established searchable databases,[3] to the improvement of IRED's catalytic properties using protein engineering,[4] to the pilot-scale synthesis of chiral amine pharmaceuticals using tailor-made IRED enzymes[5] is indicative of IREDs as a mature and sophisticated family of biocatalysts for chiral amine synthesis.

Tetrahydro-β-carboline (THβC) molecules with a substituent at the carbon-1 position are gaining increasing attention owing to their impressive pharmaceutical or biological significance (Figure 1). For instance, (+)-vincamine (**1**) and tadalafil (**2**) are clinically employed for the treatment of cerebrovascular disease and erectile dysfunction, respectively.[6] Furthermore, (+)-harmicine (**3**) and calligonine (**4**) are two natural alkaloids displaying anti-Leishmania activity and hypotensive effects, respectively.[7] Given their fascinating structural complexity and potent pharmaceutical/biological activities, substantial efforts have been devoted by the synthetic community to establish efficient, catalytic asymmetric synthesis of these molecules.[8−10] Within this context, our group recently achieved an enantioselective synthesis of (+)-vincamine (**1**) and its structural analogs (Scheme S1),[11] whereas the key tetracyclic lactam **S2** was afforded through a chiral iridium complex-catalyzed hydrogenation/lactamization cascade of a symmetrical dihydro-β-carboline (DHβC) diester **S1**, with THβC **S3** containing a quaternary carbon attached to C1 probably being the reaction intermediate. Although effective, this chemo-catalytic approach suffered from limitations, such as the use of expensive iridium metal and chiral ligands as well as the requirement of a high pressure of hydrogen gas.

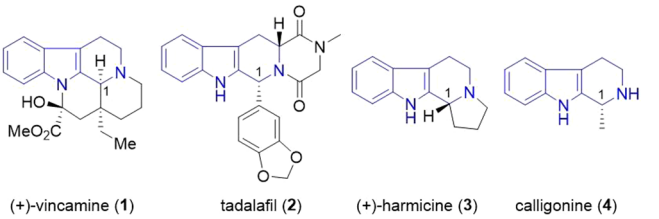

Figure 1. Representative examples containing a 1-substituted THβC skeleton.

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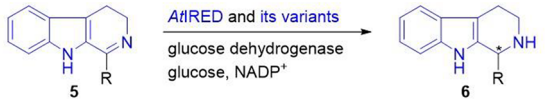
In principle, IRED-catalyzed reduction of **S1**, which contains a quaternary carbon attached to C1, could provide an alternative, environmentally benign access to **S2**. There were reports on the IRED-catalyzed reduction of 1-substituted DHβCs to THβCs when we initiated our study.[12] Very recently, the Qu group reported the efficient synthesis of 1-aryl-THβCs using engineered IRED enzymes,[13] while the Zhu group disclosed the IRED-catalyzed asymmetric synthesis of fused-ring THβCs.[14] To the best of our knowledge, IRED-catalyzed reduction of DHβCs bearing a quaternary carbon attached to C1 has not been reported until now. Herein, we report the discovery, engineering, and application of IREDs for the asymmetric synthesis of chiral, sterically hindered 1-substituted THβCs, including those containing a quaternary carbon (Scheme 1).

Scheme 1. Asymmetric Synthesis of Chiral, Sterically Hindered 1-Substituted THβCs 6 through *At*IRED- and Its Variants-Catalyzed Reduction of DHβCs 5

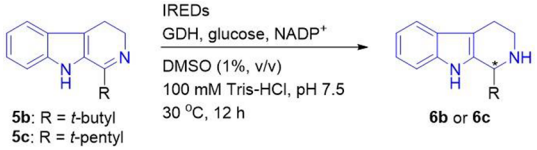


5a: R = methyl; **5b**: R = *t*-butyl; **5c**: R = *t*-pentyl; **5d**: R = *i*-propyl; **5e**: R = *n*-propyl; **5f**: R = *i*-butyl; **5g**: R = cyclopentyl; **5h**: R = cyclohexyl; **5i**: R = phenyl

Prompted by the recent report that six IREDs, namely IRED-G, IRED-I, IRED-J, IRED-K, IRED-L, and IRED-M, were shown to be able to reduce 1-methyl-DHβC (**5a**, Scheme 1 and Figure S1) to furnish (*S*)-1-methyl-THβC ((*S*)-**6a**, Scheme 1 and Figure S1) in high yields and excellent enantioselectivities,[12e] we were keen to find out whether these IREDs could reduce quaternary-carbon-containing DHβCs, namely 1-*tert*-butyl-DHβC (**5b**) and 1-*tert*-pentyl-DHβC (**5c**) as well. As depicted in Table S2, IRED-K, IRED-L, and IRED-M could transform **5b** to (*S*)-**6b** both in excellent conversions (≥99%) and enantioselectivities (97−99% ee) (Table S2, entries 4−6), while the three remaining IREDs yielded (*S*)-**6b** only in 40−73% ee (Table S2, entries 1−3). Next, the sterically more demanding substrate **5c** was subjected to IRED-K-, IRED-L-, and IRED-M-catalyzed reduction (Table S2, entries 7−9), with relatively lower conversions (44−70%) being observed.

In order to discover more efficient IRED toward the reduction of sterically bulky DHβCs **5b** and **5c**, a BLAST search was conducted by using the amino acid sequence of IRED-M as the template on account of this enzyme's good balance between catalytic activity and selectivity (Table S2, entries 7−9). Four candidate enzymes were chosen, and their sequence identity with IRED-M was in the range of 42−72%. The corresponding codon-optimized genes were synthesized and expressed in *E. coli* BL21 (DE3), with all four proteins being successfully obtained in a soluble form as indicated by the SDS−PAGE analysis (Figure S3). Although unsatisfactory results were seen for three out of the four enzymes mined in the reduction of **5b** and **5c** (Table 1, entries 2−4 and 6−8), gratifyingly, a new imine reductase originating from *Amycolatopsis thermoflava* and having 61% sequence identity with IRED-M, named *At*IRED hereafter, performed well in both reduction reactions. For example, complete conversion of **5b** to (*S*)-**6b** was realized along with perfect enantioselectivity (Table 1, entry 1). Moreover, 74% conversion and 93% ee were obtained in *At*IRED-catalyzed reduction of **5c** (Table 1, entry 5), both of which were slightly higher than those accomplished in IRED-M-catalyzed reduction of the same substrate. Nevertheless, it is worth pointing out that a significant amount of IRED lysate (corresponding to 100 g/L of wet cell weight) was used in the bioreduction reactions shown in Table 1, implying the catalytic activity of *At*IRED needed to be improved in order to render this enzyme suitable for the reduction of sterically demanding DHβCs in a practical sense.

Table 1. Screening IREDs for the Stereoselective Reduction of Dihydro-β-carbolines 5b and 5c[a]



Entry	Enzyme	Substrate	Conv (%)[b]	ee (%)[b]
1	*At*IRED	**5b**	>99	>99 (*S*)
2	*Nt*IRED	**5b**	40	>99 (*S*)
3	*Es*IRED	**5b**	31	36 (*S*)
4	*Ss*IRED	**5b**	53	45 (*S*)
5	*At*IRED	**5c**	74	93 (*S*)
6	*Nt*IRED	**5c**	3	>99 (*S*)
7	*Es*IRED	**5c**	7	>99 (*R*)
8	*Ss*IRED	**5c**	12	46 (*S*)

[a]A reaction mixture (1 mL) composed of **5b** or **5c** (10 mM), glucose (20 mM), $NADP^+$ (1 mM), DMSO (1%, v/v), 100 g/L cell-free extract (CFE) (wet cell weight) of IREDs, and 30 g/L CFE (wet cell weight) of GDH in Tris−HCl buffer (100 mM, pH 7.5) was incubated at 30 °C with shaking at 200 rpm for 12 h. [b]Determined by chiral HPLC analysis.

To improve *At*IRED's catalytic activity, we focused on the amino acid residues located in the catalytic pocket, as numerous studies demonstrated that manipulation of these residues could significantly alter the catalytic properties of enzymes.[15] A homology model of *At*IRED was created with the SWISS-MODEL server, followed by energy minimization with Amber20. Upon docking (*S*)-**6b** to the built model, nine amino acid residues were found within 4 Å of (*S*)-**6b**, with two residues (S230 and M234) and seven residues (I117′, M118′, V119′, P120′, Y167′, L171′, and F174′) residing in subunits A and B, respectively (Figure S5). Notably, residue Y167′ is highly conserved among (*S*)-selective IREDs, and previous mutation of this residue to Ala or Phe resulted in IRED mutants of much diminished activity.[3a,12d,16] Hence, residue Y167′ and another conserved residue L171′ were excluded from the present mutagenesis study.[3a]

Activity assay and protein engineering of NAD(P)H-dependent enzymes such as ketoreductases and imine reductases commonly rely on photometrically monitoring the NAD(P)H consumption at 340 nm. Unfortunately, DHβCs like **5b** show strong absorption at 340 nm, even at concentrations as low as 0.2 mM. To solve this obstacle, a UV−vis scan ranging from 200 to 600 nm was conducted for substrate **5b**, racemic product standard **6b**, NADPH, and $NADP^+$ individually. By comparing the four spectra acquired (Figure S6), we considered 410 nm as suitable for our activity assays, as only **5b** exhibited strong absorption at this wavelength. In contrast, the other three components all showed negligible absorption. Accordingly, we determined the absorption of **5b** across a range of concentrations from

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0.25 to 2 mM. By plotting the absorption against the concentration (Figure S7), and adopting the Lambert−Beer law, the extinction coefficiency (ε) of compound **5b** at 410 nm could be determined as 1.484 mM^{-1} cm^{-1}.

With a reliable activity assay established, a semirational strategy was invoked. Specifically, all seven selected residues (S230, M234, I117′, M118′, V119′, P120′, and F174′) were mutated with the NNK codon degeneracy, and 94 transformants were screened for each mutated site so as to achieve 95% library coverage. Although no positive transformant was disclosed at sites S230, M234, I117′, V119′, or F174′, screening sites M118′ and P120′ afforded one hit each, with 4.2-fold and 2.3-fold increased specific activity relative to WT, respectively (16.0 and 8.8 mU/mg versus 3.8 mU/mg, Table 2). Sequencing showed these two variants carried mutations of M118′L and P120′G, respectively. The combination of these two mutation furnished a double mutant M118′L/P120′G, which showed a specific activity of 12.2 mU/mg toward **5b**.

Table 2. Specific Activity of Purified *At*IRED and Engineered Proteins toward DHβCs 5a−5i[a]

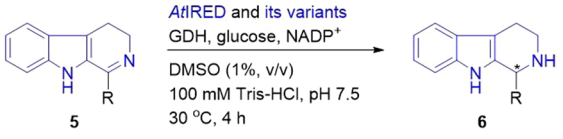
	*At*IRED	M118′L	P120′G	M118′L/P120′G
5a	25.3 ± 4.0	254.3 ± 17.2	107.8 ± 2.0	123.7 ± 7.4
5b	3.8 ± 0.7	16.0 ± 0.4	8.8 ± 0.7	12.2 ± 0.5
5c	1.4 ± 0.0	2.6 ± 0.2	3.6 ± 0.3	6.5 ± 0.2
5d	8.7 ± 0.6	35.1 ± 14.2	21.5 ± 0.6	23.9 ± 3.0
5e	22.4 ± 0.1	17.5 ± 3.4	18.8 ± 3.4	14.3 ± 2.8
5f	27.2 ± 1.3	178.0 ± 9.8	36.4 ± 2.3	24.2 ± 0.5
5g	14.3 ± 0.5	57.7 ± 16.0	24.3 ± 0.8	18.1 ± 1.5
5h	1.9 ± 0.6	3.1 ± 1.2	14.1 ± 2.8	7.8 ± 0.6
5i	2.0 ± 0.1	1.3 ± 0.6	2.6 ± 0.2	1.3 ± 0.2

[a]A standard assay mixture (200 μL) composed of 2 mM of **5a−5i**, 2 mM of NADPH, and 2 μL of DMSO (1%, v/v) in Tris−HCl buffer (100 mM, pH 7.5) was initiated at 30 °C by the addition of an appropriate amount of purified *At*IRED or its variants. The unit of specific activity was mU/mg.

To assess the substrate scope of these enzyme variants, seven additional DHβCs (**5a**, **5d−5i**) with varying substituents at C1 were chemically prepared (Scheme 1 and Figure S1). As for **5b**, we then determined the extinction coefficiency (ε) of them and **5c** at 410 nm (Figure S7). Next, the specific activities of *At*IRED and the three enzyme variants toward these eight 1-substituted-DHβCs were determined (Table 2). Notably, for all of the substrates except 1-*n*-propyl-DHβC (**5e**), at least one of the three variants exhibited elevated specific activity than WT. In fact, all the three variants displayed higher activity relative to WT for 1-methyl-DHβC (**5a**), 1-*t*-pentyl-DHβC (**5c**), 1-*i*-propyl-DHβC (**5d**), 1-cyclopentyl-DHβC (**5g**), and 1-cyclohexyl-DHβC (**5h**) (Table 2). Among the three mutant enzymes, variant M118′L was usually the best-performing one. Compared with WT, M118′L possessed 4-fold to 10-fold increased specific activities toward **5a**, **5d**, 1-*i*-butyl-DHβC (**5f**), and **5g**. In particular, specific activities of 254.3 and 178.0 mU/mg were detected for M118′L-catalyzed reduction of **5a** and **5f**, respectively.

We next investigated the reduction of DHβCs **5a−5i** catalyzed by *At*IRED and the three enzyme variants at an analytical scale. It is worth mentioning that the amount of IREDs utilized for the reduction of **5a**, **5b**, **5d**, **5e**, **5f**, and **5g** in this part of study (Table 3) was much lower than that for the reduction of **5b** in the above enzyme-screening section shown in Table 1 (5 g/L versus 100 g/L of wet cell weight) because we intended to keep the reaction conversions mostly below certain levels, thereby making the comparison of the enzymes' performance feasible and meaningful. On the other hand, the same amount of enzymes as that used in Table 1 was employed for the reduction of **5c**, **5h**, and **5i** (Table 3) since WT and mutant enzymes exhibited inferior specific activity toward these substrates (Table 2). Several useful conclusions could be drawn. First and foremost, at least one of the three mutant enzymes provided higher reaction conversion than WT for all nine substrates. In essence, in the case of reducing **5a**, **5b**, **5c**, **5d**, and **5g**, all three variants outperformed WT. Second, for five out of the nine substrates, namely **5c**, **5f**, **5g**, **5h**, and 1-

Table 3. Stereoselective Reduction of 5 Catalyzed by *At*IRED and Its Enzyme Variants in Analytical Scale[a]



	*At*IRED		M118′L		P120′G		M118′L/P120′G	
Substrate	Conv (%)[b]	ee (%)[b]	Conv (%)[b]	ee (%)[b]	Conv (%)[b]	ee (%)[b]	Conv (%)[b]	ee (%)[b]
5a	77.8	>99 (*S*)	83.1	>99 (*S*)	89.3	>99 (*S*)	85.8	>99 (*S*)
5b	5.4	91 (*S*)	18.3	93 (*S*)	18.4	98 (*S*)	23.5	98 (*S*)
5c[c]	37.5	>99 (*S*)	65.6	>99 (*S*)	83.2	>99 (*S*)	84.0	>99 (*S*)
5d	22.6	97 (*S*)	50.8	98 (*S*)	46.3	98 (*S*)	60.0	99 (*S*)
5e	90.9	>99 (*S*)	95.6	99 (*S*)	94.9	96 (*S*)	82.4	64 (*S*)
5f	66.9	96 (*S*)	73.8	97 (*S*)	67.4	95 (*S*)	29.0	78 (*S*)
5g	47.7	>99 (*S*)	62.5	98 (*S*)	55.1	98 (*S*)	54.0	98 (*S*)
5h[c]	59.3	98 (*S*)	51.8	>99 (*S*)	99.4	>99 (*S*)	87.0	>99 (*S*)
5i[c]	13.9	>99 (*S*)	12.9	>99 (*S*)	42.0	>99 (*S*)	11.7	>99 (*S*)

[a]A reaction mixture (1 mL) composed of **5** (10 mM), glucose (20 mM), $NADP^+$ (1 mM), DMSO (1%, v/v), 5 g/L CFE (wet cell weight) of IREDs, and 15 g/L CFE (wet cell weight) of GDH in Tris−HCl buffer (100 mM, pH 7.5) was incubated at 30 °C with shaking at 200 rpm for 4 h. [b]Determined by chiral HPLC analysis. The absolute configuration of known compounds **6a**, **6b**, and **6d−6i** were assigned by comparing the elution order in chiral HPLC with known data, while the absolute configuration of unknown compound **6c** was assigned in analogy. [c]100 and 30 g/L CFE (wet cell weight) for IREDs and GDH.

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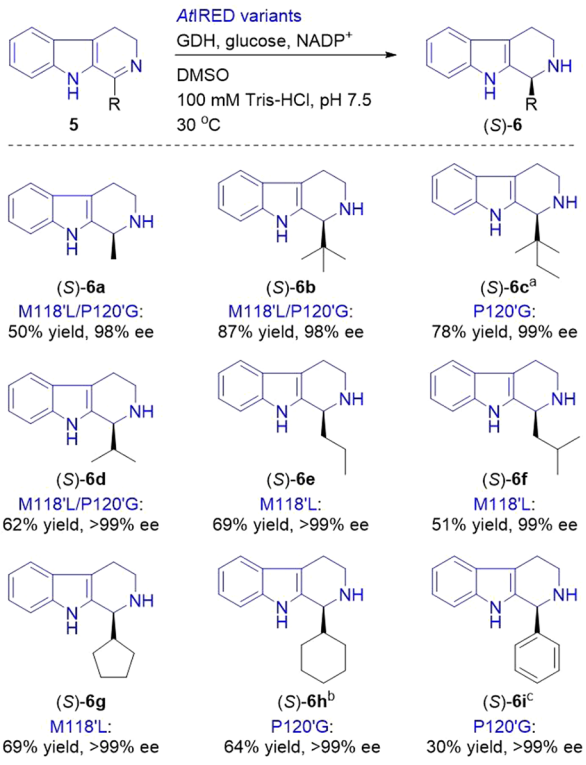
phenyl-DHβC (**5i**), the enzyme variant showing the highest specific activity was also the one that gave the highest reaction conversion. In contrast, for the remaining four DHβCs **5a**, **5b**, **5d**, and **5e**, the most active enzyme identified via the activity assay was not the best performing one in the reduction reaction. For instance, the double mutant M118′L/P120′G displayed lower specific activity toward **5b** than the single mutant M118′L (12.2 mU/mg versus 16.0 mU/mg), but it afforded the best reaction conversion (23.5%). Third, (*S*)-configured 1-substituted THβCs with excellent optical purity (≥95% ee) were obtained in most cases.

Finally, a preparative-scale (0.5 mmol) reduction reaction was conducted to further showcase the application potential of our developed method (Scheme 2), with the suitable IRED

Scheme 2. Preparative-Scale Synthesis of Chiral Tetrahydro-β-carbolines 6 Catalyzed by the *At*IRED Variants*



*A reaction mixture (50 mL) composed of **5** (10 mM), glucose (20 mM), $NADP^+$ (1 mM), DMSO (1%, v/v), 20 g/L CFE (wet cell weight) of IREDs, and 15 g/L CFE (wet cell weight) of GDH in Tris−HCl buffer (100 mM, pH 7.5) was stirred at 30 °C and 500 rpm. Isolated yields are given. [a]100 and 30 g/L of CFE (wet cell weight) for IREDs and GDH. [b]120 and 30 g/L of CFE (wet cell weight) for IREDs and GDH. DMSO (2%, v/v). [c]160 and 30 g/L of CFE (wet cell weight) for IREDs and GDH. DMSO (5%, v/v).

identified for each DHβC substrate based on the results of the above analytical reactions utilized in most cases. Consistent with the analytical reactions, larger amount of enzymes was used for the bioreduction of the three challenging DHβC substrates **5c**, **5h**, and **5i**, compared to that for the other six substrates. Gratifyingly, all nine DHβC compounds except 1-phenyl-DHβC (**5i**) were completely reduced under the reaction conditions employed, affording the corresponding chiral THβCs in 50−87% isolated yields. Remarkably, all nine tetrahydro-β-carbolines thus synthesized exhibited outstanding optical purity (98−99% ee).

In conclusion, a new imine reductase, *At*IRED, was discovered by genome mining. Aided by our established, reliable enzymatic activity assay, seven amino acid residues located in the putative catalytic pocket of *At*IRED were probed using site-saturation mutagenesis, resulting in two single mutants M118′L and P120′G and the derived double mutant M118′L/P120′G with improved specific activity. After evaluating the specific activity and the catalytic performance of *At*IRED and the three enzyme variants toward nine DHβCs with varying substituents at C1 in the analytic scale reactions, the best IRED enzyme for each DHβC substrate was identified. Finally, the synthetic applicability of the developed *At*IRED variants was underscored by the stereoselective synthesis of nine chiral 1-substituted THβCs ((*S*)-**6a** to (*S*)-**6i**) in 30−87% isolated yields with excellent enantioselectivities (98−99% ee). To the best of our knowledge, this work represents the first study on the IRED-catalyzed reduction of DHβCs bearing a quaternary carbon attached to C1, with (*S*)-1-*t*-butyl-THβC ((*S*)-**6b**) and (*S*)-1-*t*-pentyl-THβC ((*S*)-**6c**) being synthesized in high yields and excellent optical purities (87% and 78% yields, 98% and 99% ee). Further protein engineering based on the currently developed *At*IRED variants is ongoing, aiming to identify an effective IRED enzyme for the chemo-enzymatic synthesis of (+)-vincamine and related alkaloids.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.3c00147.

Experimental procedure, spectral data (PDF)

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Notes

The authors declare no competing financial interest.

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