Short, Chemoenzymatic Access to Cyctetryptomycin A and B

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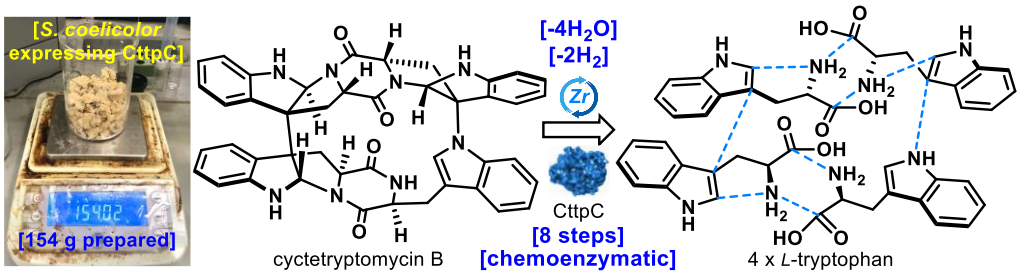
Supporting Information Placeholder

ABSTRACT: A concise and chemoenzymatic synthesis of cyctetryptomycin A and B (**1**-**2**) using a Zr-catalyst and CttpC is reported. The recent discovery of cyctetryptomycin A and B (**1**-**2**) and their intriguing neuroprotective activities necessitated scalable synthesis to facilitate structure-activity relationship studies. However, the synthesis of cyctetryptomycin A and B (**1**-**2**) solely by chemical reactions has proven to be impractical. Therefore, a novel approach using a short and chemoenzymatic strategy was developed to prepare cyctetryptomycin A and B (**1**-**2**) via Zr-catalyzed construction of two consecutive quaternary chiral carbons and CttpC-catalyzed direct oxidative coupling of the tryptophan moiety.



INTRODUCTION

Cyctetryptomycin A and B (**1**-**2**) (Figure 1) are tetrameric natural products that were isolated in 2021.[1] These intriguing compounds are characterized by the complex linkage of four naturally occurring tryptophans into an unprecedentedly complex macrocyclic molecule. These natural products possess neuroprotective activity and are expected to be effective in treating neurodegenerative diseases such as ALS (amyotrophic lateral sclerosis), Alzheimer's, and Parkinson's diseases.[1] However, the total synthesis of cyctetryptomycin A and B (**1**-**2**) has not yet been reported. In the present study, we developed a Zr-catalyzed scalable C3-C3 dimerization of tryptophan derivative and CttpC-catalyzed oxidative coupling of tryptophan and successfully prepared cyctetryptomycin A and B (**1**-**2**) in 8 steps. Our synthetic method is expected to contribute to the development of potential therapeutic agents for neurodegenerative diseases such as ALS, Alzheimer's, and Parkinson's diseases.

Polymerized natural products derived from tryptophan have long piqued the interest of researchers in the field of synthetic chemistry, due to not only their various potent biological activities but also due to their complex structures.[2] A number of dimerized natural products of tryptophan derivatives linked at the C3 position have been isolated, and their structures have been determined.[3] Several of these dimerized natural products have been successfully synthesized by several research groups.[4-10] Typical methods for linking the C3 positions of tryptophan derivatives include the utilization of Co,[5] Ni,[6] Cu,[7] Fe,[8] V_2O_5,[9] or hypervalent iodine reagents.[10] While these methods are useful for linking the C3 positions of tryptophan derivatives, some of the reaction conditions require more than one equivalent of metal reagents, strongly acidic conditions, expensive catalysts and ligands, and may present stereo- and regioselectivity issues when dimerizing tryptophan derivatives. These methods of dimerizing tryptophan derivatives pose difficulties in scale-up production, and therefore there is a need of an inexpensive and reproducible method of linking the C3 positions that is suitable for scalable synthesis.

Our retrosynthetic analysis is shown in Figure 1A. Cycletryptomycin A and B (**1**-**2**) have complex three-dimensional structures on a highly tryptophan-linked macrocycle. Therefore, in order to conduct a scalable synthesis of these natural products, it is important to develop a mild and scalable synthetic method that is inexpensive and does not require harsh conditions. For this reason, we carried out a Zr-catalyzed dimerization of tryptophan derivatives followed by the rapid construction of two diketopiperazines by an amide formation reaction. The key reactions in this synthetic pathway are the linking of C22 and C18' by macrocyclization of tetratryptomycin A (**3**) and the linking of the nitrogen on the indole to C18' to form cyctryptomycin A and B (**1**-**2**). We first attempted to synthesize cyctryptomycin A and B (**1**-**2**) by oxidative cyclization of tetratryptomycin A (**3**) as an initial screening (Figure 1B).[11] More than 100 oxidation conditions were applied, including NCS, NBS, NIS, $PhI(OAc)_2$, $PhI(OCOCF_3)_2$, $FeCl_3$, V_2O_5, $Mn(OAc)_3$, anodic oxidation,

photo-oxidation, pig's liver and chicken's liver as oxidants. However, no cyctryptomycin A and B (**1-2**) could be obtained from tetratryptomycin A (**3**). The inaccessibility of oxidative cyclization is anticipated to be attributed to the distance between the two tryptophan moieties, resulting from the distal position of C22 and C18' of tetratryptomycin A (**3**), which unfavorably impedes cyclization.

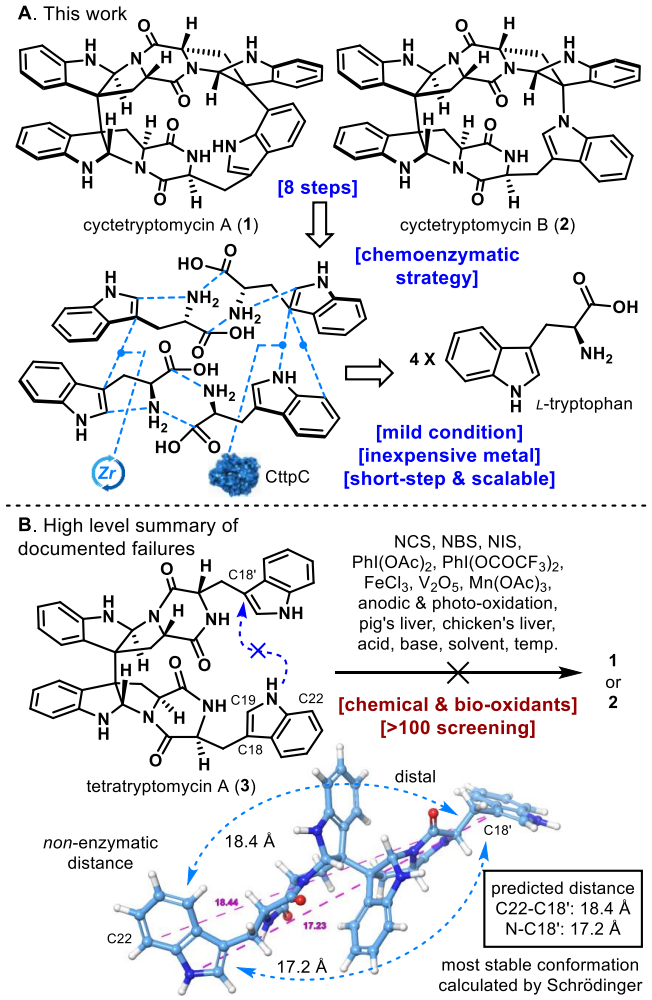


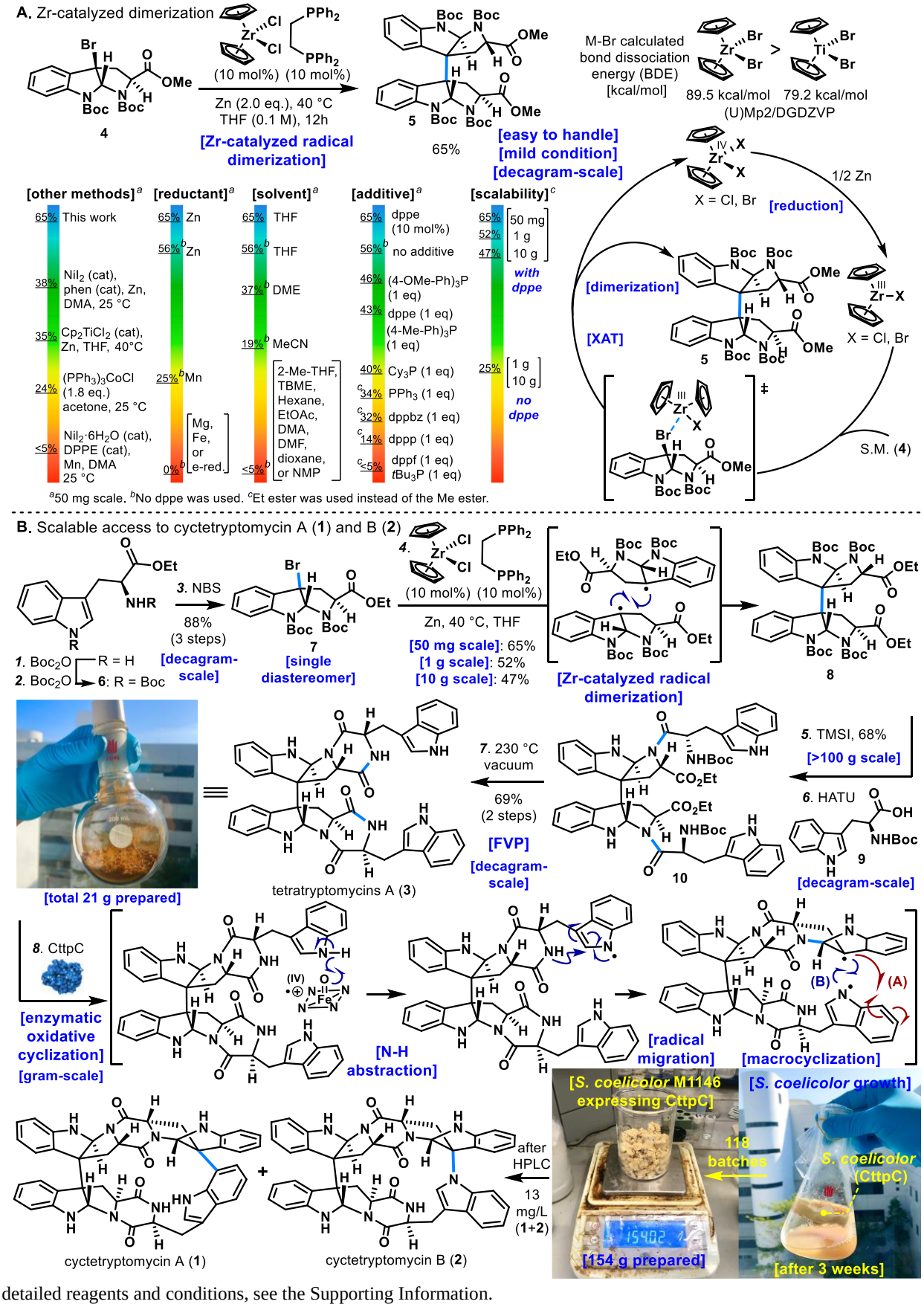
Figure 1. (A) A chemoenzymatic approach to the cyctetryptomycin A and B (**1-2**); (B) High level summary of documented failures.

The computational chemistry analysis of the most stable conformation of tetratryptomycin A (**3**) in fact revealed that the C22-C18' distance is 18.4 Å and the N-C18' distance is 17.2 Å (Figure 1B). These calculations also indicate a mutual distal position of the two tryptophan moieties in tetratryptomycin A (**3**). This significant distance was suggested to impede the macrocyclization of tetratryptomycin A (**3**), and over 100 macrocyclization conditions were investigated. However, none of the conditions yielded the desired cyctetryptomycin A and B (**1-2**), and the two tryptophan moieties of **3** were either transformed into oxindoles or halogenated as the major by-products. In 2021, we identified the CttpC enzyme as a key component for the oxidative macrocyclization of tetratryptomycin A (**3**) to produce cyctetryptomycin A and B (**1-2**). However, the biosynthesis of cyctetryptomycin A and B (**1-2**) could not be achieved on a large scale, and only a few milligrams were obtained.[1] Consequently, we devised a plan to develop an inexpensive, non-toxic, mild, and scalable Zr-catalyzed dimerization of tryptophan derivatives, followed by their enzymatic oxidative macrocyclization, to synthesize cyctetryptomycin A and B (**1-2**) in large quantities.[12] These compounds have the potential to serve as seed compounds for the treatment of neurodegenerative diseases such as ALS, Alzheimer's, and Parkinson's diseases.

RESULTS AND DISCUSSION

Initially, we focused on developing a scalable and mild dimerization method for the C3 position of tryptophan derivative **4** (Scheme 1A). As mentioned earlier, several methods have been reported for dimerizing the C3 position of tryptophan derivatives.[5-10] We first attempted the dimerization of **4** using 1.8 equivalent of $CoCl(PPh_3)_3$, as reported by Movassaghi *et al.*[5] Although the desired product **5** was obtained, only 24% isolated yield was achieved on a 50 mg scale. Next, we investigated the use of the Ni catalyst and the dppe ligand, as reported by Oguri *et al.*[6a] Although a trace amount of the desired compound **5** was obtained, the reaction was difficult to reproduce. The low yields observed under the Ni and Co conditions may be attributed to the sensitivity of these conditions to the purity and brand of the reagents and solvents, their hygroscopic nature, and the high sensitivity of the low-valent chemical species to oxygen atmosphere, rendering them challenging to handle. On the other hand, the dimerization using NiI_2 and 1,10-phenanthroline ligand, as reported by Peng *et al.*,[6b] resulted in the dimerized compound **5** in 38% isolated yield. Although dimerization reactions of **4** using existing methods such as $CoCl(PPh_3)_3$[5] and Ni-catalysts[6] are feasible, the delicate nature of the reaction makes adjustment and handling of reagents complicated, and the reproducibility of the desired product is poor. Moreover, in the case of $CoCl(PPh_3)_3$, more than one equivalent of the reagent is necessary since the reaction is not catalytic. Additionally, the use of Ni poses challenges due to the instability of its low valence state in the presence of oxygen. Given these limitations, we opted to explore a new, more dependable dimerization reaction that can be executed more effortlessly and economically on a decagram scale. After the screening of conditions, interestingly, we found that the use of a Cp_2TiCl_2 catalyst was successful in obtaining **5** in 35% isolated yield. The advantage of using a Cp_2TiCl_2 catalyst is that it is inexpensive, stable in air, does not absorb moisture, and requires no expensive ligands, making it suitable for scalable synthesis. On the other hand, in recent years, radical reactions using zirconium have been vigorously studied due to their mild reaction conditions and low toxicity.[13] Since zirconium has physical properties similar to those of titanium, we predicted the bond dissociation energies (BDE) of Ti-Br and Zr-Br bonds in Cp_2TiBr_2 and Cp_2ZrBr_2 using DFT calculations. The Ti-Br bond of Cp_2TiBr_2 was found to be 79.2 kcal/mol, while the Zr-Br bond of Cp_2ZrBr_2 was 89.5 kcal/mol. From the DFT calculation, it is evident that the Cp_2ZrBr_2 catalyst can easily produce radicals from **4** through single electron transfer. Indeed, the Cp_2ZrCl_2 catalyzed dimerization of **4** gave **5** in 65% isolated yield, which was attributed to the strong bond dissociation energy (BDE) of the Zr-Br bond. It is noteworthy to mention that through meticulous investigations of the reaction conditions, the use of dppe was crucial for this reaction.

Scheme 1. (A) Zr-catalyzed dimerization; (B) Short, chemoenzymatic access to cyctetryptomycin A (**1**) and B (**2**).[a]



[a]For detailed reagents and conditions, see the Supporting Information.

Subsequently, the reducing agent for this reaction was investigated in detail (Scheme 1A). Fe, Mg, or electrochemical reduction conditions commonly employed for transition metal reduction resulted in meager conversion, and only trace amounts of **5** was obtained. Further investigations revealed that the use of Mn as the reducing agent afforded **5** in 25%. However, the most favorable outcome was achieved when Zn dust was employed as the reducing agent, providing an isolated yield of 65% on a 50 mg scale. The effects of solvent on this reaction were also explored. Solvents such as 2-Me-THF, TBME, hexane, EtOAc, DMA, DMF, 1,4-dioxane, and NMP proved to be ineffective. The dimerized compound **5** was obtained in a yield of 19% when MeCN was used, and in a yield of 37% when DME was employed. Further investigations revealed that THF as the solvent provided the highest yield (65%) of **5**.

Next, the influence of ligands was examined. The addition of ligands often accelerates the reaction greatly and is therefore a crucial factor to consider. Consequently, we evaluated several greatly, starting with bipyridine and phenanthroline ligands, but these were not effective. Subsequently, a variety of monodentate and bidentate phosphine ligands (tBu$_3$P, dppf, dppp, dppbz, Ph$_3$P, Cy$_3$P, (4-Me-Ph)$_3$P, (4-OMe-Ph)$_3$P, and dppe) were screened. Among these ligands, dppe (10 mol%) was the most effective ligand (yield of 65%) for this reaction. In fact, without dppe, the yield was 56% on a 50 mg scale. Interestingly, further investigations into the addition of dppe demonstrated that the effect of 10 mol% dppe was particularly significant on the gram and decagram scales. In fact, when **5** was synthesized using Cp_2ZrCl_2 catalyst and Zn dust without dppe, the yield was as low as 25% on 1 g and 10 g scales, and **5** could not be obtained reproducibly. However, when 10 mol% of dppe was utilized as a ligand, **5** was successfully obtained in isolated yields of 52% and 47% on 1 g and 10 g scales, respectively. Based on these findings, a synthetic route was established for the production of **5** by dimerization over Cp_2ZrCl_2 catalyst, Zn dust, and dppe in THF, providing the desired compound 5 on a decagram scale with good reproducibility. This dimerization approach can be applied to the synthesis of analogs and potential seed compounds on a decagram scale since it does not require strong acidic conditions or high temperatures and proceeds with mild and inexpensive reagents. The proposed reaction mechanism of this dimerization reaction is described below. Initially, the tetravalent catalyst is reduced by Zn dust to form trivalent Cp_2ZrCl. This highly active trivalent Cp_2ZrCl captures Br in compound **4** by single electron transfer, forming a strong and stable Zr-Br bond and generating a radical species at the benzylic position. The resulting radical above the benzylic position dimerizes to give the desired compound **5**.

Our next objective was to establish a concise and chemoenzymatic approach to the synthesis of cyctryptomycin A and B (**1**-**2**) that possess neuroprotective activity with potential efficacy against neurodegenerative diseases such as ALS, Alzheimer's, and Parkinson's diseases. Our strategy involved with the mild and scalable Zr-catalyzed dimerization reaction to synthesize tetratryptomycin A (**3**) on a decagram scale. Notably, the synthetic route initiated a simple and cost-effective reaction (Scheme 1B). Specifically, **6** was synthesized by protecting the nitrogen atom on the indole and the primary amine with a Boc group, utilizing the natural form of *L*-tryptophan ethyl ester. Subsequently, **7** was synthesized as a single diastereomer by bromocyclization of **6** using NBS. The slow addition of NBS during bromocyclization of **6** was crucial for obtaining the single compound **7**, as a rapid addition resulted in inseparable diastereomeric mixtures. Compound **7** was then dimerized to **8** using a Cp_2ZrCl_2 catalyzed reaction on a decagram scale, driven by the formation of a strong and stable Zr-Br bond. Notably, the addition of dppe (10 mol%) was essential for the Cp_2ZrCl_2 catalyzed dimerization reaction, as it helped trap trace amounts of residual metal in the reagent and prevented reproducibility issues on both 1 g and 10 g scales, with yields decreasing to 25% without dppe. The resulting dimerized product **8** was simultaneously deprotected with TMSI to remove the four Boc groups, and the resulting Boc-protected *L*-tryptophan was condensed with HATU to yield **10**.[4, 9] Flash vacuum pyrolysis (FVP) was then employed to rapidly construct two diketopiperazines simultaneously in a single step, yielding a total of 21 g of tetratryptomycin A (**3**). The macrocyclization of tetratryptomycin A (**3**) by coupling the C22-C18' and N-C18' positions was a significant step towards a scalable synthesis of cyctryptomycin A and B (**1**-**2**).

According to the computational chemistry of the most stable conformation, the two tryptophan sites of tetratryptomycin A (**3**) are located a long distance from each other, making macrocyclization a challenging task. In fact, as mentioned above, more than 100 screenings employing chemical and bio-oxidation (e.g., chicken and pig liver) conditions did not result in macrocyclization, as the tryptophan moiety was oxidized to oxindole as a by-product and halogenated. On the other hand, the CttpC enzyme catalyzes the macrocyclization of tetratryptomycin A (**3**) to afford cyctryptomycin A and B (**1**-**2**).[1] Therefore, we investigated the conditions under which the specific gene for this essential enzyme, CttpC, could be introduced into *S. coelicolor* M1146 and cultivated in large quantities. After investigating various culture conditions, it was found that the most effective method was to place approximately 6 L of *S. coelicolor* M1146 culture medium containing the specific gene for CttpC and 3.54 g of tetratryptomycin A (**3**) in 118 Meyer flasks of 50 ml each and react at 28-30 °C (see the Supporting Information). This large-scale culture successfully grew 154 g of *S. coelicolor* M1146 expressing CttpC in one cycle (see the photo in Scheme 1B). Ensuring adequate oxygen was especially important for the scalable preparation of *S. coelicolor M1146*. Specifically, it was crucial to avoid the utilization of Meyer flasks (3 L or 5 L) that were excessively voluminous. In fact, after scrutinizing the conditions for mass culture, we endeavored to cultivate *S. coelicolor* M1146 in sizable Meyer flasks (3 L or 5 L), but the number of *S. coelicolor* M1146 colonies observed was insufficient and the cultivation of the organisms was unsuccessful. Furthermore, the inclusion of tetratryptomycin A (**3**) did not result in a reaction or any progress in macrocyclization. Since stirring efficiency is important for the culture of *S. coelicolor* M1146, it is likely that large Meyer flasks such as 3 L or 5 L were not sufficient to ensure oxygenation (see the Supporting Information). After careful examination of various culture conditions, it was interesting to note that many colonies of *S. coelicolor* M1146 were observed in relatively small Meyer flasks, such as 125 ml and 50 ml, and that the culture progressed well (Scheme 1B). Notably, this method proceeded well on a 3.5 g scale and finally led to the synthesis of cyctetryptomycin A and B (**1**-**2**) in 8 steps.

The putative reaction mechanism of the macrocyclization by CttpC can be described as follows. The tetravalent iron complex,

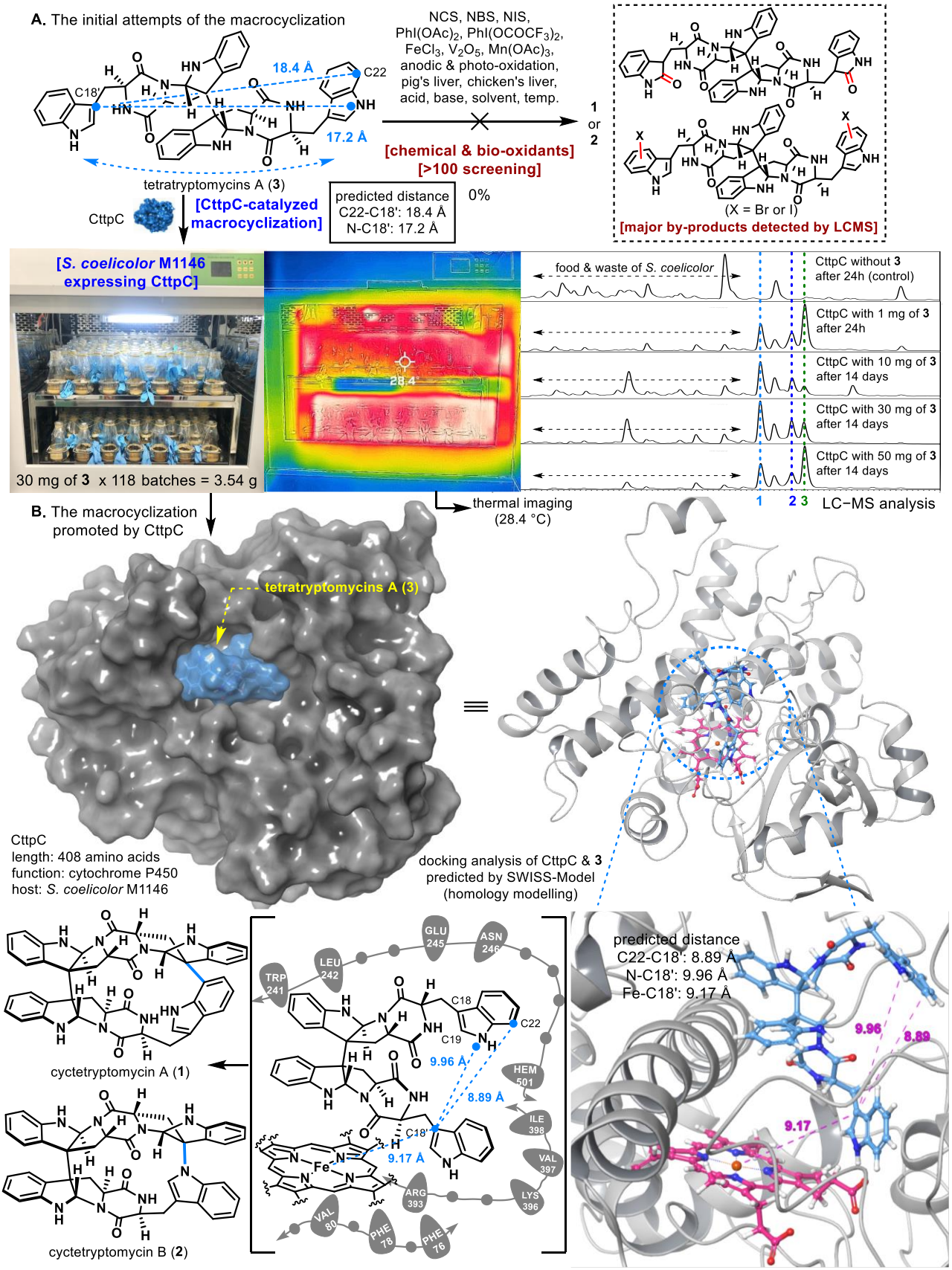
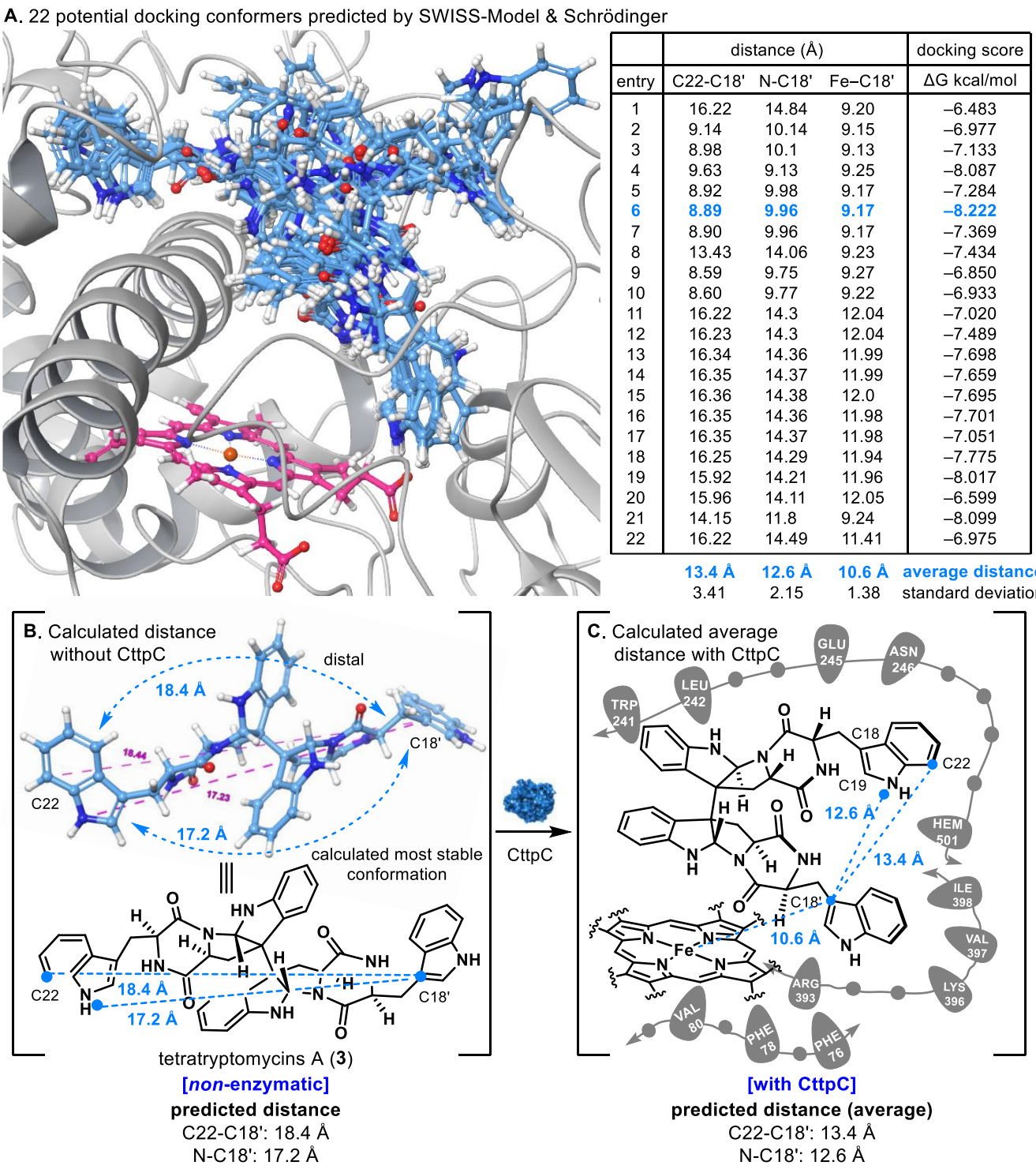


Figure 2. (A) Optimization of the macrocyclization catalyzed by CttpC; (B) Docking analysis of CttpC and tetratryptomycin A (**3**) using the SWISS-Model.



	distance (Å)			docking score
entry	C22-C18'	N-C18'	Fe–C18'	ΔG kcal/mol
1	16.22	14.84	9.20	–6.483
2	9.14	10.14	9.15	–6.977
3	8.98	10.1	9.13	–7.133
4	9.63	9.13	9.25	–8.087
5	8.92	9.98	9.17	–7.284
6	**8.89**	**9.96**	**9.17**	**–8.222**
7	8.90	9.96	9.17	–7.369
8	13.43	14.06	9.23	–7.434
9	8.59	9.75	9.27	–6.850
10	8.60	9.77	9.22	–6.933
11	16.22	14.3	12.04	–7.020
12	16.23	14.3	12.04	–7.489
13	16.34	14.36	11.99	–7.698
14	16.35	14.37	11.99	–7.659
15	16.36	14.38	12.0	–7.695
16	16.35	14.36	11.98	–7.701
17	16.35	14.37	11.98	–7.051
18	16.25	14.29	11.94	–7.775
19	15.92	14.21	11.96	–8.017
20	15.96	14.11	12.05	–6.599
21	14.15	11.8	9.24	–8.099
22	16.22	14.49	11.41	–6.975
	13.4 Å	**12.6 Å**	**10.6 Å**	**average distance**
	3.41	2.15	1.38	standard deviation

Figure 3. (A) 22 potential docking conformers predicted by SWISS-Model & Schrödinger; (B) Calculated distance without CttpC; (C) Calculated average distance with CttpC.

consisting of a heme moiety and iron, causes *N*-H abstraction of tetratryptomycin A (**3**), leading to the formation of radicals at the nitrogen indole. Subsequently, radical migration generates radicals at the C3 position of tryptophan. The nitrogen atom of diketopiperazine nucleophilically attacks the simultaneously formed imine, resulting in the formation of pyrroloindoline. The radical on the C3 of the tryptophan then couples with the radical on the nitrogen atom of the other tryptophan to form cyctetryptomycin B (**2**). On the other hand, it is assumed that the radical on the C3 of the tryptophan leads to the formation of cyctetryptomycin A (**1**) by radical addition to the C7 position of the other tryptophan.

A detailed discussion of further scale-up of macrocyclization of tetratryptomycin A (**3**) with CttpC is described below (Figure 2A). The macrocyclization process was monitored by LC-MS while tetratryptomy-cin A (**3**) was catalyzed by CttpC. The findings revealed that upon adding 1 mg of tetratryptomycin A (**3**) to 50 ml of *S. coelicolor* M1146 culture expressing CttpC in a Meyer flask, cyctryptomycin A (**1**) was the major product obtained, while cyctryptomycin B (**2**) was the minor product after a 24-hour incubation. Further experiments were conducted on a 10 mg scale, revealing that after 14 days, the starting material **3** was almost depleted, and cyctryptomycin A (**1**) was the major product obtained, with cyctryptomycin B (**2**) being the minor product. To further increase the scale, macrocyclization was performed using 30 mg of tetratryptomycin A (**3**), and after 14 days, the starting material **3** was observed, and although the reaction was not completed, the desired compounds cyctryptomycin A (**1**) and cyctryptomycin B (**2**) were obtained in the similar ratio on 10 mg scale. Conversely, on 50 mg scale, the concentration of **3** may have been too high, and after 14 days, a significant amount of starting material **3** remained, resulting in a poor conversion rate of the reaction. Based on these results, it is evident that the most efficient method was to prepare a 50 ml *S. coelicolor* M1146 culture in a Meyer flask and add 30 mg of tetratrypto-mycin A (**3**) for macrocyclization, using CttpC as a catalyst. It is worth mentioning that macrocyclization can be performed on a 3.54 g (= 30 mg × 118 batches) scale of tetratryptomycin A (**3**) in one cycle by simultaneously stirring 118 Meyer flasks in an incubator during the scale-up. The temperature in the incubator was also investigated, and it became clear that it is crucial to maintain the temperature between 28 °C and 30 °C. Conversely, if the temperature exceeds 35 °C, the growth of *S. coelicolor* M1146 expressing CttpC was inhibited.

The macrocyclization of tetratryptomycin A (**3**) was efficiently facilitated by the CttpC enzyme, enabling the rapid synthesis of tetratryptomycin A and B (**1**-**2**) in a concise manner. Despite an investigation of over 100 chemical and bio-oxidants, as previously mentioned, the macrocyclization of tetratryptomycin A (**3**) did not occur under conditions other than those involving CttpC. This noteworthy fact prompted an exploration of the reaction mechanism for the macrocyclization of tetratryptomycin A (**3**). To this end, a docking analysis was conducted between CttpC, whose amino acid sequence had already been elucidated, and tetratryptomycin A (**3**) (Figure 2B). In a comprehensive docking analysis, it is intriguing to note that significant changes in the conformation of tetratryptomycin A (**3**) were observed in the presence of CttpC (see the Supporting Information). Specifically, the predicted result with the best docking score revealed that the interatomic distance between C22-C18', which is involved in the macrocyclization of tetratryptomycin A (**3**), is computationally predicted to be 8.89 Å in the presence of CttpC, whereas in the absence of CttpC, the interatomic distance between C22-C18' in the most stable conformation of **3** is predicted to be 18.4 Å. Therefore, the computational results suggest that the presence of CttpC reduces the interatomic distance between C22-C18' of **3** by approximately half. This is also observed for the interatomic distance between N-C18' of **3**. Specifically, in the absence of CttpC, the interatomic distance was 17.2 Å, while in the presence of CttpC, the distance was shortened to 9.96 Å, as shown by the calculations. Furthermore, the computational results also revealed that hemoglobin within the pocket of CttpC and **3** were in close proximity (Fe-C18': 9.17 Å).

However, it cannot be ruled out that the interatomic distances may be predicted to be short by chance when considering the results of a single docking analysis. Therefore, to exclude these coincidental predictions, we conducted further detailed docking analyses (Figure 3). Specifically, we calculated the average and standard deviation of the interatomic distances C22-C18', N-C18', and Fe-C18' for all 22 potential docking conformers derived from the docking analysis of **3** and CttpC in the known rational active site (Figure 3A). The average interatomic distances for **3** in the presence of CttpC were C22-C18': 13.4 Å, N-C18': 12.6 Å, and Fe-C18': 10.6 Å, with low standard deviations of C22-C18': 3.41, N-C18': 2.15, and Fe-C18': 1.38. These results indicate that the macrocyclization of **3** catalyzed by CttpC is crucial for the proper positioning of the two tryptophan moieties and hemoglobin, as evidenced by computational results and docking analysis.

CONCLUSION

In summary, this study enabled the 8-step synthesis of cyctetryptomycin A and B (**1**-**2**) through the dimerization of tryptophan derivatives using a newly developed Zr catalyst and macrocyclization employing CttpC. Previous reports have described similar reactions using Ni catalysts, $(PPh_3)_3CoCl$, V_2O_5, $CuCl_2$, and $PhI(OAc)_2$ reagents for the dimerization of tryptophan derivatives. However, some of these reaction conditions may be highly dependent on the brand, quality, and humidity of the location where the reaction is carried out, and implementation on a larger scale than a decagram was not realistic due to decreased yields. Additionally, some of the reaction conditions are stoichiometric rather than catalytic, making them extremely expensive and environmentally burdensome on larger scales. Furthermore, it is noteworthy to mention that while there are existing inexpensive methods available, some methods require highly acidic conditions, such as MsOH, which are not practically viable for large-scale synthesis due to safety concerns. In contrast, we provided herein a new option for the dimerization of tryptophan derivatives using an inexpensive, low-toxicity, mild condition, and non-hygroscopic Zr catalyst, which successfully synthesized 21 g of tetratryptomycin A (**3**). This method is applicable to not only tetratryptomycin A (**3**) but also many other bispyrrolidinoindoline-type natural products, offering a highly practical, reliable, and scalable technique that is not location-dependent. For the macrocyclization of tetratryptomycin A (**3**) using CttpC, we identified optimal reaction conditions on a gram scale, allowing for the 8-step synthesis of cyctetryptomycin A and B (**1**-**2**), which has not been reported previously. The significant advancements in genetic engineering techniques in recent years will likely enable the development of further optimized CttpC-related artificial proteins in the future.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures, analytical data (^{1}H and ^{13}C NMR, MS) for all new compounds as well as optimization tables. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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