

Asymmetric Synthesis of Nodulones C & D by Chemoenzymatic Approach gives Insight into their Biosynthesis

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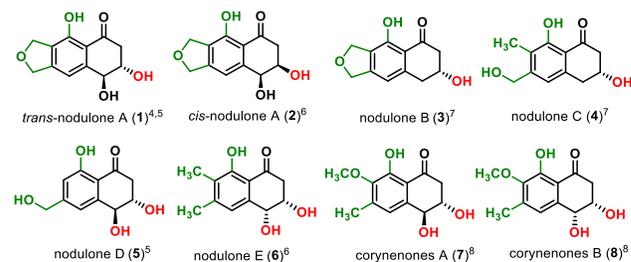
Supporting Information Placeholder

ABSTRACT: The first asymmetric total synthesis of fungal secondary metabolites, (*R*)-nodulone C (**4**) and *trans*-nodulone D (**5**) has been reported through the chemoenzymatic approach. The strategy utilizes NADPH-dependent naphthol reductases of *Magnaporthe grisea* for the reduction of putative biosynthetic substrates, synthesized non-enzymatically in multiple steps. A dihydronaphthalenone **32** and *cis*-nodulone D (**30**) has also been synthesized chemoenzymatically. The work implies for similar steps during the biosynthesis of nodulones and their analogs with the involvement of tetrahydroxynaphthalene reductase related enzyme(s).

Endophytic fungi are known to produce a large number of secondary metabolites with diverse structural features, which display a wide array of biological activities.¹⁻³ Among these, nodulones **1-6** belongs to a new class of bioactive polyketides produced by *Nodulisporium sp.*, a group of common endophytic fungi harboured by many plants (**Fig. 1**). Isofuranonaphthalenone **1**, a tricyclic compound has been isolated from *Nodulisporium sp.* A4 from *Aquilaria sinensis* and was mistakenly assigned *cis*-configuration.⁴ The stereochemistry was later found to be (*7S*, *8S*) based on the X-ray crystallographic analysis of **1**, isolated from *Daldinia eschscholtzii* and is named here as *trans*-nodulone A (**1**).⁵ It shows immunosuppressive activity and cytotoxicity against tumor cells SF-268.^{4,5} In addition, the *cis*-nodulone A (**2**) has also been isolated from *Nodulisporium sp.* of *Antidesma ghaesbilla* and was found to display antimycobacterial and antimalarial activities with IC50 values of 3.125 and 11.3 $\mu\text{g/ml}$, respectively.⁶ Related dihydronaphthalenones, nodulone B (**3**) and nodulone C (**4**) were isolated from *Daldinia eschscholtzii* associated with terrestrial orchid *Paphiopedilum exul* (Ridl.) Rolfe, collected from Chiang Mai, Thailand, and display moderate antimicrobial activity.⁷ Furthermore, the isolation of immunosuppressive and antimycobacterial ketodiols such as *trans*-nodulone D (**5**)⁵ and *cis*-nodulone E (**6**)⁶ from *D. eschscholtzii* and *cis*- as well as *trans*-corynenones A (**7** & **8**) from sponge derived *Corynespora cassicola* XS-2009017,⁸ suggests that all these metabolites are related and might have similar biogenesis. Moreover, the isolation of both *cis*- and *trans*-nodulones from nature suggests the formation of a monoreduced intermediate during their biosynthesis, which has not been isolated as yet. Although, detailed biosynthetic investigations supported by a chemoenzymatic synthesis of related metabolites produced during 1,8-dihydroxynaphthalene (DHN) melanin biosynthesis have been performed,⁹⁻¹¹ the biosynthesis of nodulones remained elusive.⁷ In addition, no synthesis has been reported so far for the preparation of chiral nodulones. It might be due to the presence of the chemically sensitive β -hydroxyketone group, which can undergo dehydration readily.¹²⁻¹³ This motivated us to envisaged a chemoenzymatic, biomimetic synthesis of nodulone A, B and C. The strategy relies on the synthesis of the putative biosynthetic

substrates, which on reduction with fungal naphthol reductases may give access to chiral nodulones. Such an approach will provide vital clues about the putative substrates, intermediates, and the enzymes involved in the biosynthesis of nodulones for the first time.

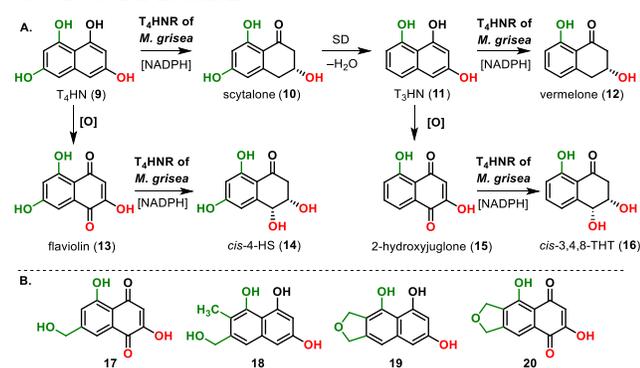
Figure 1. Various natural dihydronaphthalenones isolated from fungal sources.⁴⁻⁸



Tetrahydroxynaphthalene and trihydroxynaphthalene reductases (T₄HNR & T₃HNR) of plant pathogenic fungus *Magnaporthe grisea* are the two well-studied naphthol reductases involved in DHN-melanin biosynthesis.¹⁴⁻¹⁵ T₄HNR is known to catalyze the NADPH-dependent asymmetric reduction of 1,3,6,8-tetrahydroxynaphthalene (T₄HN, **9**) to (*R*)-scytalone (**10**) and T₃HNR catalyzes a similar reduction of 1,6,8-trihydroxynaphthalene (T₃HN, **11**) to (*R*)-vermelone (**12**) (**Scheme 1**). The extended melanin biosynthetic pathway involves the oxidation of T₄HN (**9**) and T₃HN (**11**) to flavolin (**13**) and 2-hydroxyjuglone (**15**), respectively.¹⁶⁻¹⁸ Both, **13** and **15** has been shown to be reduced by T₄HNR using NADPH to *cis*-4-hydroxyscytalone (**14**, 4-HS)¹² and *cis*-3,4,8-trihydroxytetralone (**16**, 3,4,8-THT)¹³ via a mono reduced intermediates (**Scheme 1A**).¹⁸ The combination of T₄HNR and NADPH-regeneration enzyme, glucose dehydrogenase (GDH), or T₃HNR and GDH results in the formation of *trans*-4-hydroxyscytalone, indicating the ability of GDH to reduce naphthalenone type compounds.¹⁹ Over the years, the two naphthol reductases have been shown to catalyze the reduction of several hydroxynaphthoquinones to *cis*-ketodiols,

leading to the biomimetic synthesis of several natural products and their analogs.^{9-11, 19}

Scheme 1. A. T₄HNR-catalyzed reduction of hydroxynaphthoquinones to *cis*- and *trans*-ketodiols. B. Proposed substrates for nodulones.



This led us to hypothesize that T₄HNR and/or T₃HNR can be used in a chemoenzymatic synthesis of chiral nodulones A, B and C. This would require, at first the chemical synthesis of putative hydroxynaphthalene and hydroxynaphthoquinone substrates. Considering the stereoselective reduction of the known substrates catalyzed by T₄HNR in particular,⁹⁻¹¹ we hypothesize that hydroxynaphthoquinone **17** and hydroxynaphthalene **18** may act as a substrate for nodulone D (**5**) and nodulone C (**4**), respectively. Similarly, **19** and its oxidized derivative **20** may act as putative substrates for the (bio)synthesis of nodulone B (**3**) *cis*-nodulone A (**2**), and *trans*-nodulone A (**1**) (Scheme 1B). Such an approach may unravel the steps involved in the biosynthesis of nodulones in general.

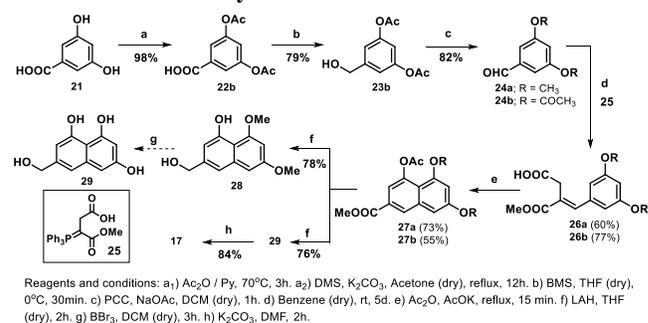
Result and Discussion

At first, we aimed to synthesize naphthoquinone **17** which might lead to the preparation of *trans*-nodulone D (**5**). For this, ylide **25** was obtained following a two-step synthesis from maleic anhydride and triphenylphosphine (see Supporting Information).²⁰ Then a Wittig reaction of 3,5-dimethoxy benzaldehyde (**24a**) with ylide **25** gave **26a** in 60% yield. The cyclization of **26a**, followed by aromatization in the presence of potassium acetate in boiling acetic anhydride gave **27a** in 73% yield.²¹ This is followed by lithium aluminium hydride reduction of ester to alcohol **28** in 78% yield. However, when **28** was treated with strong Lewis acid, BBr₃ to deprotect methoxy group, the desired product **29** was not obtained. Therefore, instead of OMe, we used OAc as protecting group which can be easily deprotected. To make 3,5-diacetoxy benzaldehyde **24b**, 3,5-dihydroxy benzoic acid (**21**) was used as a precursor. Protection of hydroxyl groups in acetic anhydride and a small amount of pyridine gave **22b** in 98% yield. Then **22b** was reduced to primary alcohol **23b** using borane dimethylsulfate (BMS) in 79% yield, followed by oxidation to aldehyde **24b** using potassium dichromate (PCC) in 82% yield. A Wittig reaction between **24b** and ylide **25** gave **26b** in 77% yield. This is followed by cyclization to obtain **27b** in 55% yield. Reduction of **27b** with LAH gave trihydroxynaphthalene **29**, which was oxidized in the presence of K₂CO₃ and dimethylformamide (DMF) to obtain desired compound **17** in 84% yield. The overall yield after 7 steps was found to be 16% (Scheme 2).

Next, **17** was used as a substrate for reduction with T₄HNR. For this purpose, the codon-optimized T₄HNR gene of *M. grisea* cloned into pET19b vector was overexpressed in *E. coli* BL21 (DE3) to obtain a cell-free extract of T₄HNR-his.¹⁰ **17** was then incubated with cell-free lysate of T₄HNR-his and NADPH (regenerated by glucose/glucose dehydrogenase system) in potassium phosphate

buffer (50mM, 1mM EDTA, 1mM DTT, pH 7.0) using 2-propanol as co-solvent under anoxic condition for 24 h.

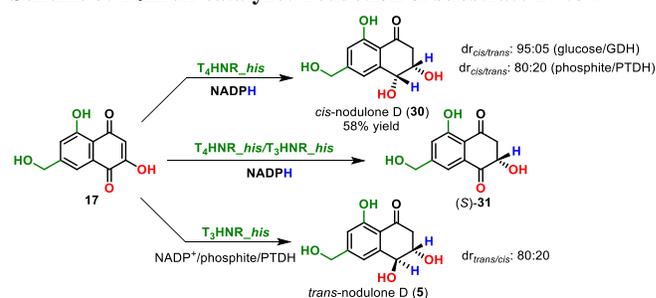
Scheme 2. Chemical synthesis of substrate 17



The ¹H NMR of the crude reaction mixture recorded in acetone-*d*₆ shows 62% conversion. The purification resulted in the isolation of the product in 58% yield, which was characterized using NMR spectroscopy and mass spectrometry and found to be *cis*-nodulone D (**30**) as a major diastereomer along with a small amount of *trans*-nodulone D (**5**). The dr_{cis/trans} was determined to be 95:5 (¹H NMR) (Scheme 3). The absolute configuration of the major diastereomer *cis*-nodulone C (**30**) was assigned by comparing the CD-spectra of the reported *cis*-3,4,8-trihydroxy-1-tetralone (**16**) synthesized by the reduction of 2-hydroxyjuglone catalyzed by T₄HNR (see Supporting Information).¹¹ Although, *cis*-nodulone D has not been isolated as yet from natural sources, we expect it to be a natural product considering the existence of other *cis*- as well as *trans* ketodiols.

Since the reduction of **17** might be a two-step process, we hypothesize the formation of a mono reduced intermediate during the enzymatic transformation. To detect such an intermediate, the transformation was performed only for 4 h. This indeed resulted in the isolation of a diketone intermediate **31**, which is characterized using NMR and HRMS (Scheme 3). Isolation of **31** indicates that T₄HNR reduces **17** to **31** using NADPH, which is then further reduced by either T₄HNR or by GDH to give *cis*-nodulone D as a major diastereomer along with *trans*-nodulone D. The control experiment, performed using phosphite and cell free lysate of PTDH²² for NADPH regeneration along with T₄HNR to rule out the role of GDH in the reduction of **31**, surprisingly, gave a mixture of *cis*- and *trans*-ketodiols with a dr_{cis/trans} of 80:20 (¹H NMR). The result suggests that if the reduction of **31** to *cis*-nodulone D, most probably catalyzed by T₄HNR is prevented, we may get *trans*-nodulone D as a major diastereomer.

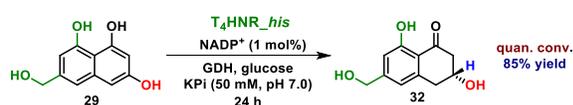
Scheme 3. T₄HNR-catalyzed reduction of substrate 17 to 5



Therefore, we used T₃HNR of *M. grisea* for the reduction instead of T₄HNR as it has been reported to give mono reduced product only with similar hydroxynaphthoquinone substrates.¹⁹ For this purpose, the codon-optimized gene of T₃HNR cloned into the pET-19b vector was expressed in *E. coli* BL21 (DE3) to finally obtain the crude lysate.¹⁹ The incubation of **17** with crude cell free lysate

of T₃HNR-his in potassium phosphate buffer (pH 7.0) and NADPH (regenerated using phosphite/phosphite dehydrogenase system), gave an inseparable mixture of *trans*- and *cis*-nodulone D in the *dr*_{trans/cis} ratio of 80:20 (determined from ¹H NMR spectra) with 16% isolated yield (**Scheme 3**). A similar transformation performed using NADPH (regenerated using glucose/glucose dehydrogenase system) under the same conditions does not result in the formation of any ketodiol product. The spectral data and sign of optical rotation of *trans*-nodulone D (**5**) match that reported in literature⁵, indicating it as same as the natural product (see Supporting Information). The configuration of *trans*-nodulone D was further confirmed by CD spectroscopy. Since PTDH shows activity towards the reduction of intermediate **31**, we have tested it with 1- and 2-tetralone as substrates, to confirm its ability to reduce a cyclic ketone. Although PTDH shows very low activity towards the reduction of 1-tetralone, we believe that PTDH might have some role in the reduction of **31** to *trans*-nodulone D. Further studies will be required to identify the exact role of PTDH in the reduction of **31**.

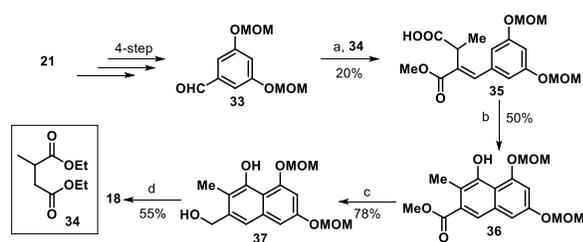
Scheme 4. A. T₄HNR-catalyzed reduction of substrate **29** to **32**



Considering the ability of T₄HNR to reduce hydroxy naphthalenes, we also subjected compound **29** for reduction. For this purpose, **29** was incubated with T₄HNR-his and NADPH (regenerated by glucose/GDH system) under the same condition as mentioned above. After 24 h, the reaction mixture shows the formation of dihydronaphthalenone **32** isolated in 85% isolated yield after purification (**Scheme 4**). The absolute configuration of **32** was assigned as (*R*) by comparison of its CD spectra with that of (*R*)-scytalone reported previously.²³ Although, no such natural product has been isolated as yet, we propose that **32** will be isolated in future and probably involve trihydroxynaphthalene **29** as substrate and T₄HNR related enzyme for reduction during its biosynthesis.

Next, we aimed to synthesize substrate **18** required for the preparation of nodulone C (**4**). For this purpose, 3,5-dihydroxy benzoic acid (**21**) was converted to MOM protected benzaldehyde **33** in 4-steps through protection, reduction and oxidation (see Supporting Information) (Scheme 5).²⁴ Then, aldehyde **33** was used in Stobbe condensation with methyl succinic ester **34** in the presence of NaH which lead to the isolation of acid **35** in 20% yield. **34** was synthesized by esterification of methyl succinic acid. Low yield in this reaction, was due to the Cannizzaro reaction²⁵ of aldehyde **33** which took place in presence of sodium hydride and resulted in the formation of side products, acid and alcohol (see supporting information).

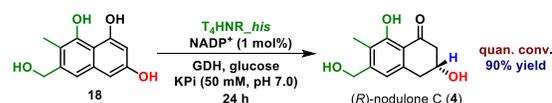
Scheme 5. Chemical synthesis of substrate **18**



Reagents and conditions: (a) NaH, Toluene (dry), cat. EtOH, rt, 1h. (b) Ac₂O, AcOK, reflux, 15 min. (c) LAH, THF(dry), 0°C, 1h. (d) MeOH, HCl(c), 60°C, 30 min.

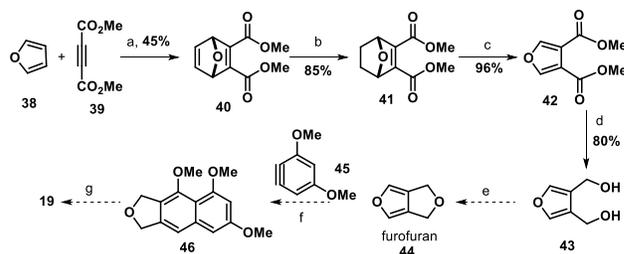
Despite optimization efforts, the yield of Stobbe condensation could not be improved further. Nevertheless, the cyclisation and aromatisation of **35** using potassium acetate in boiling acetic acid gave **36** in 50% isolated yield. Finally, the desired substrate **18** was obtained in a two-step process from **36**, involving reduction using LAH, followed by the deprotection of compound **37** by heating in MeOH in presence of acid (**Scheme 5**). Then, hydroxynaphthalene **18** was incubated with T₄HNR using NADPH (regenerated using glucose/glucose dehydrogenase as a co-factor in potassium phosphate buffer (50mM, 1mM EDTA, 1mM DTT, pH 7.0) for 24 h under anoxic conditions. After purification, we got the desired nodulone C (**4**) in 90% isolated yield (**Scheme 6**). **4** was characterized using NMR spectroscopy and mass spectrometry. The comparison of NMR data and optical rotation confirmed it to be identical to the naturally occurring nodulone C (**4**). Absolute configuration of the chiral centre was assigned by comparing the CD spectra of (*R*)-scytalone synthesized previously using T₄HNR.²³

Scheme 6. T₄HNR-catalyzed reduction of substrate **18** to **4**



Next, we also attempted the synthesis of the substrates **19** and **20** required for the preparation of furan containing nodulones A and B. For this, we designed a retrosynthetic strategy and according to which we first made ester **40** by [4+2] cycloaddition from furan (**38**) and dimethyl acetylenedicarboxylate (DMAD, **39**) in 45% yield (**Scheme 7**). Then, a single carbon-carbon double bond is reduced by H₂, Pd/C in 85% yield, followed by retro-Diels–Alder reaction to obtain **42** in 96% yield. The diester **42** was then reduced using LiAlH₄ in 80% yield to obtain **43**. Despite several attempts, we were unable to get furofuran **44** from **43**, which we plan to use for another Diels-Alder reaction with **45** to obtain **46**, which on deprotection might have given us the desired substrate **19** and **20** (**Scheme 7**).

Scheme 7. Attempt to synthesize substrate **19** & **20**

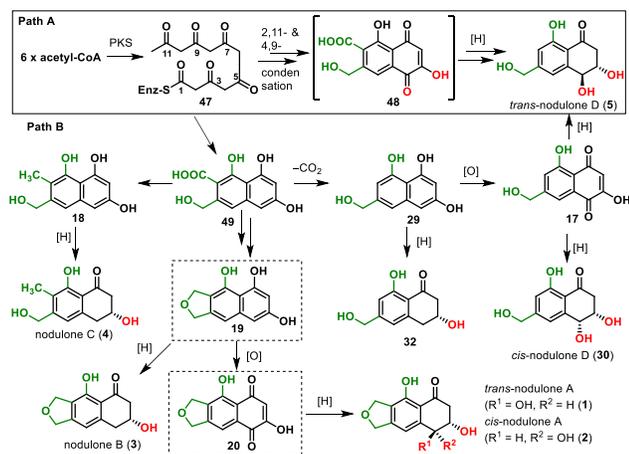


Reagents and conditions: (a) 100°C, 18h. (b) 10% Pd/C, H₂, 6h. (c) 175°C, 1h. (d) LiAlH₄, Ether (dry), 0°C - rt, 1h. (e) MnO₂, Triethylsilane, TFA, DCM (-5°C to rt)

Nevertheless, based on the earlier studies on related natural products and our chemoenzymatic synthesis of nodulone C and D presented here, we proposed the following biosynthetic pathway for the formation of nodulones. In previous work,⁷ *trans*-nodulone D was proposed to be formed by the decarboxylation followed by reduction of hydroxynaphthoquinone **39**, formed by the cyclization of a linear polyketide **47** (**Path A**, **Scheme 7**). Alternatively, we propose the formation of compound **49** by the regioselective cyclization, which on decarboxylation will give substrate **29**, which will be reduced to give compound **32**, which is yet to be isolated from natural sources (**Path B**, **Scheme 7**). We propose, that **29** on oxidation will form hydroxynaphthoquinone **17**, similar to the oxidation of T₄HNR to flaviolin. This will then undergo reduction

catalyzed by T₄HNR related enzyme to form either *cis*-nodulone D (30), which is not yet isolated or *trans*-nodulone (5) which is a known natural product. Furthermore, 49 may also form 18 by reduction of carboxylic acid, which on reduction by naphthol reductase related enzyme(s) will form nodulone C (4).

Scheme 8. Proposed biosynthesis of nodulones A-D



Although the putative substrates 19 and 20 could not be synthesized and tested with the reductase as yet, we propose that reduction and cyclization of 49 will form furano naphthalene substrate, which on reduction catalyzed by T₄HNR related enzyme will form nodulone B (3) and on oxidation will give hydroxynaphthoquinone 20 which on reduction by the enzyme will lead to the formation of *cis*- as well as *trans*-nodulone A (1 & 2). This needs to be validated in future by the reduction of substrate 20 using a naphthol reductase.

In summary, we report here the first asymmetric synthesis of (*R*)-nodulone C (4) and *trans*-nodulone D (5) using a chemoenzymatic approach. Although we could not synthesize the furan naphthalene substrate proposed for the preparation of *cis*- and *trans*-nodulone A, we speculate that their biosynthesis would involve a similar strategy for its formation. In addition, we have obtained *cis*-nodulone D (30) and dihydronaphthalenone 32 after the enzymatic reduction, which we propose to be natural products and may be isolated in future. Our results further corroborate the broad substrate scope shown by T₄HNR and T₃HNR and their involvement in the biosynthesis of several fungal metabolites. Finally, based on the chemoenzymatic approach a probable biosynthetic pathway B has been proposed for the formation of various nodulones, which further shows the ability of nature to create molecular diversity through branching achieved through oxidation and reduction reactions during biosynthesis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Protein expression and purification, experimental details, characterization data, copies of NMR spectra, and CD-spectra (PDF).

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Notes

The authors declare no competing financial interest.

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