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Muta-mycosynthesis of Naphthalene Analogs

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Supporting Information

ABSTRACT: A mutasynthetic strategy is introduced for the mycosynthesis of naphthalene-based molecules (mutadalesols A−F) with directed substitution patterns and new frameworks by generating and using the Δ*pksTL* mutant strain of *Daldinia eschscholzii*. (±)-Mutadalesol A and its (+)-enantiomer are cytotoxic, and its (−)-enantiomer inhibits Toll-like receptor 5 (TLR5). The in-culture reactability of fungal oligoketide intermediates with 5-aminonaphthalen-1-ol (ANL) is demonstrated, shedding light on bioorthogonal accesses to unnatural molecule libraries valuable in drug discovery pipelines.

Owing to unique structures and potent bioactivities, natural products chemistry remains an attractive research topic of chemists, biologists, and pharmacologists.[1−3] As more and more natural products have been characterized, the probability of reisolating known compounds from different species keeps increasing. To cope with the situation, a mutasynthetic strategy provides an alternative access to new molecules from which new bioactivities can be screened.[4] *Daldinia eschscholzii* IFB-TL01, an ascomycete fungus residing in the mantis (*Tenodora aridifolia*) gut, is an efficient producer of immunosuppressive polyketides dalesconols A and B (Figure S1).[5] To solve the dalesconol supply issue, Snyder et al. have elegantly developed the total synthesis of dalesconols A and B,[6] and Shi's group reported an impressively concise access to the dalesconol framework.[7] To strengthen the fungal supply of dalesconols, we performed a genome-based biosynthetic investigation demonstrating that dalesconols A and B are constructed through the promiscuous coupling of different naphthol radicals through a concerted catalysis of polyketide synthase and laccase.[8] Laccases are a group of multicopper oxidases capable of oxidizing a broad range of substrates such as (poly)phenols and anilines.[9−11] Moreover, alkaloids usually have a higher hit rate for bioactive/lead molecules,[12] but *D. eschscholzii* seems to be a nonalkaloid producer.[13] This observation motivated us to develop a mutasynthetic approach for a library of naphthalene-based compounds, some of which may be the 'designed alkaloids'.

As presented in an early study, the wild type (WT) *D. eschscholzii* strain was subjected to gene deletion experiments to afford a mutant (Δ*pksTL*) strain which was proven to be deprived of naphthol biosynthesis governed by a kind of pentaketide synthase gene.[8] To enhance the novelty of possible products, the Δ*pksTL* strain was subsequently supplemented simultaneously with two "irregular" nonmicrobial chemicals, naphthalene-1,5-diol (NDL) and 5-aminonaphthalen-1-ol (ANL), which are structurally distinct from the WT strain-producible "regular" naphthol units (1,3,6,8-tetra-, 1,3,8-tri-, and 1,8-dihydroxynaphthalenes). Ascertaining their fungal acceptability, NDL and ANL were supplemented in the scaled-up culture of the Δ*pksTL* strain. Fractionation of the extract derived gave six new naphthalene-derived compounds named mutadalesols A−F (**1**−**6**). Their biosynthesis pathways were addressed or proposed, and the bioactivity was evaluated to demonstrate the chirality- and 8′-hydroxy-codependent activity of mutadalesol A (**1**); namely, enantiomer (+)-**1** was active against SW480 whereas (−)-**1** attenuated the flagellin-induced TNF-α production of RAW264.7 macrophages.

The Δ*pksTL* strain was cultivated for 10 days at 28 °C with an agitation of 120 rpm in a malt extract (ME) liquid medium,[5] with NDL and ANL supplemented both at 1.0 mM. The EtOAc extract (38 g) derived from the culture (40 L) was fractionated by column chromatographies over silica gel and Sephadex LH-20, followed by the semipreparative reversed-phase HPLC refinement, to afford mutadalesols A−F (**1**−**6**).

Mutadalesol A (**1**), obtained as a brown crystal, was determined to have a molecular formula of $C_{20}H_{14}O_5$ according to the Na^+-liganded molecular ion at m/z 357.07329 ($C_{20}H_{14}O_5Na$ requires 357.07334) in its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). In the ^1H NMR spectrum of **1**, the coexistence of 1,4,8-trisubstituted naphthalene and 1,2,3-trisubstituted benzene nuclei was indicated by the eight clearly split signals at δ_H 6.85 (d, J = 7.8 Hz), 7.02 (d, J = 7.8 Hz), 7.08 (dd, J = 8.0, 0.8 Hz), 7.45 (t, J = 8.0 Hz), 7.84 (dd, J = 8.0, 0.8 Hz), 7.29 (dd, J = 8.0, 1.2 Hz), 7.47 (t, J = 8.0 Hz), and 7.55 (dd, J = 8.0, 1.2 Hz). In the upper field region of its ^1H NMR spectrum, a pair of methylene-originated double doublets was at δ_H 2.47 (J = 16.8, 12.4 Hz) and 2.91 (J = 16.8, 5.0 Hz), and an oxymethine at δ_H 5.23 (dd, J = 12.4, 5.0 Hz). Such a combination of splitting patterns and geminal coupling

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magnitude (J = 16.8 Hz) suggested that a 3-oxygenated propanoyl unit was most likely edited in a six-membered ring. These observations, along with the quaternary carbon at $\delta_{C\text{-}8'}$ 73.7 led to the proposed plane structure of **1**. The assumption was confirmed by the HMBC correlations of C-8′ with H-3 and H-6′, of H-7′ with C-4 and C-5, of C-5′ with H-4′ and H-6′, and of C-10′ with H-6 and reinforced by its single-crystal X-ray diffraction analysis (Figure 1). However, the crystal space group

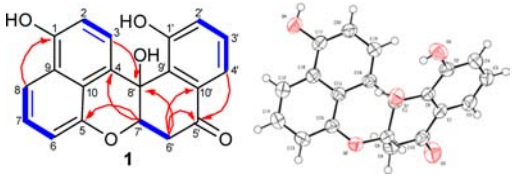

Figure 1. Key HMBC (red arrow) and ^{1}H−^{1}H COSY correlations (blue bond) of **1** (left) with its structure confirmed by the X-ray crystallographic analysis (right).

($P2(1)/n$) of **1** suggested that it was probably a racemic mixture. Subsequent chiral HPLC separation gave two enantiomers (+)-**1** and (−)-**1**, which were demonstrated to possess (7′*S*,8′*R*)- and (7′*R*,8′*S*)-configurations, respectively, by comparing their CD spectra with the ECD spectra predicted theoretically for all optional stereoisomers (Figure S3).

Mutadalesol B (**2**), an amorphous brown powder, was evidenced to have a molecular formula of $C_{20}H_{14}O_4$ (an oxygen atom less than that of **1**) from its Na^+-liganded molecular ion at m/z 341.07836 in its HR-ESI-MS ($C_{20}H_{14}O_4Na$ requires 341.07843). The ^{1}H and ^{13}C NMR signals of **2** were unambiguously assigned by the 2D NMR experiments (^{1}H−^{1}H COSY, HMBC and HMQC), showing collectively that it was an 8′-desoxy derivative of **1**. The chiral HPLC resolution of **2** afforded two enantiomers (+)-**2** and (−)-**2**, which were elucidated to have (7′*S*,8′*S*)- and (7′*R*,8′*R*)-configurations, respectively, by comparing their CD spectra with those computed for all optional stereoisomers (Figure S12).

Mutadalesol C (**3**) was shown to have a molecular formula of $C_{22}H_{17}NO_3$ by the protonated molecular ion at m/z 344.12808 in its HR-ESI-MS spectrum ($C_{22}H_{18}NO_3$ requires 344.12812). The ^{1}H NMR spectrum of **3** indicated the presence of a 1,5-disubstituted naphthalene moiety signaling at δ_H 7.60 (d, J = 7.8 Hz), 7.47 (t, J = 7.8 Hz), 8.01 (d, J = 7.8 Hz), 7.45 (d, J = 7.2 Hz), 7.35 (t, J = 7.2 Hz), and 6.93 (dd, J = 7.2, 0.6 Hz). A 2,5-disubstituted chromen-4-one ring was required by the proton signals at δ_H 6.29 (s), 6.77 (dd, J = 8.4, 1.2 Hz), 7.42(t, J = 8.4 Hz), and 6.81 (dd, J = 8.4, 1.2 Hz). Moreover, an (*E*)-1-propenyl group was indicated by the methyl signal at δ_H 1.98 (dd, J = 6.6, 1.8 Hz), which coupled to the two olefinic methine resonances at δ_H 6.88 (dd, J = 15.6, 7.2 Hz) and 6.40 (dd, J = 15.6, 1.8 Hz). The coexistence of these substructures was confirmed by the 2D NMR experiments, which accommodated the structure of **3** (Figure 2).

Mutadalesol D (**4**), isolated as a yellow gum, gave the protonated molecular ion at m/z 320.12816 in its HR-ESI-MS spectrum, corresponding to the molecular formula $C_{20}H_{17}NO_3$ ($C_{20}H_{18}NO_3$ requires 320.12812). As in the case of **3**, the ^{1}H, ^{13}C NMR, and DEPT data of **4** demonstrated the presence of a 1,5-disubstituted naphthalene group. Furthermore, an N-substituted 2-methyl-4-iminochroman-5-ol motif was suggested by the three aromatic proton signals (δ_H 6.65 (d, J = 8.0 Hz), 7.28 (t, J = 8.0 Hz), 6.45 (d, J = 8.0 Hz)) and the methylene resonances at δ_H

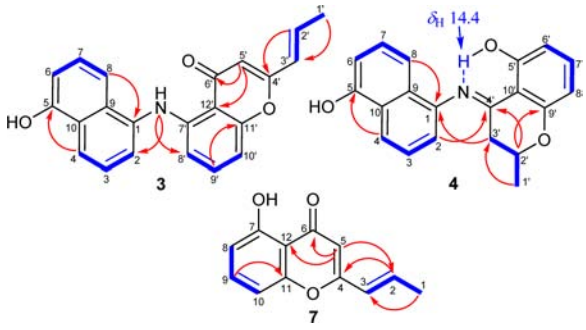

Figure 2. Key HMBC (red arrow) and ^{1}H−^{1}H COSY correlations (blue bond) of **3**, **4**, and **7**.

2.52 (dd, J = 16.8, 12.0 Hz) and 2.62 (dd, J = 16.8, 2.8 Hz), both being split by the methylated oxymethine group signaling at δ_H 4.25 (tq, J = 6.4, 2.8 Hz) and 1.35 (d, J = 6.4 Hz). With the recognized motifs, the structure of **4** was formulated according to its 2D NMR experiments (^{1}H−^{1}H COSY, HMBC, and HMQC) along with an exchangeable singlet at δ_H 14.4 of 5′-OH, which was shifted downfield owing to its H-bonding with the imine nitrogen (Figure 2). Chiral HPLC separation of **4** gave enantiomers (+)-**4** and (−)-**4**, which were demonstrated to possess (2′*S*)- and (2′*R*)-configurations respectively, by comparing their CD spectra with those calculated for all optional stereoisomers (Figure S27).

Mutadalesol E (**5**), isolated as an inseparable racemic mixture, was evidenced to have a molecular formula of $C_{18}H_{15}NO_3$ by its $[M + H]^+$ peak at m/z 294.11248 in its HR-ESI-MS spectrum ($C_{18}H_{16}NO_3$ requires 294.11247). A 1,2,5-trisubstituted naphthalene nucleus was indicated by the five aromatic protons in its ^{1}H NMR spectrum (Table S5). The presence of a tetraketide motif was required by two methylene signals at δ_H 2.75 (ddd, J = 16.0, 5.6, 1.6 Hz), 3.04 (dd, J = 16.0, 3.2 Hz), 3.36 (ddd, J = 16.8, 5.6, 1.6 Hz), and 3.53 (dd, J = 16.8, 3.2 Hz), all being split by the hydroxymethine group at δ_H 4.45 (dddd, J = 6.4, 3.2, 6.8, 3.6 Hz) and 5.24 (OH, d, J = 3.2 Hz). The magnitude of the two geminal couplings (16.8 and 16.0 Hz) highlighted that both bonded to polarized double bonds such as "C═O" or "C═N". This information, along with a methyl singlet at δ_H 3.0 (H-1′) and its HMBC correlation with C-2′ and C-3′, gave collectively the structure of **5** (Figure 3).

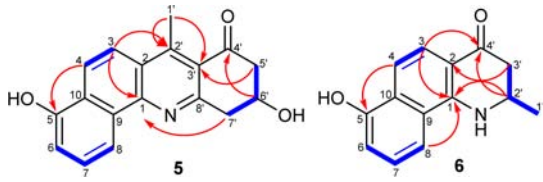

Figure 3. Key HMBC (red arrow) and ^{1}H−^{1}H COSY correlations (blue bond) of **5** and **6**.

The specific rotation (0°) indicated the racemic nature of **5**, which could not be separated with our chiral HPLC method presumably owing to the ready ability of **5** to undergo epimerization (Figure S41).

Mutadalesol F (**6**), an amorphous yellow powder, was demonstrated to have a molecular formula of $C_{14}H_{13}NO_2$ from the protonated molecular ion at m/z 228.10187 in its HR-ESI-MS spectrum ($C_{14}H_{14}NO_2$ requires 228.10191). In its ^{1}H NMR spectrum, the five aromatic hydrogen proton signals suggested a

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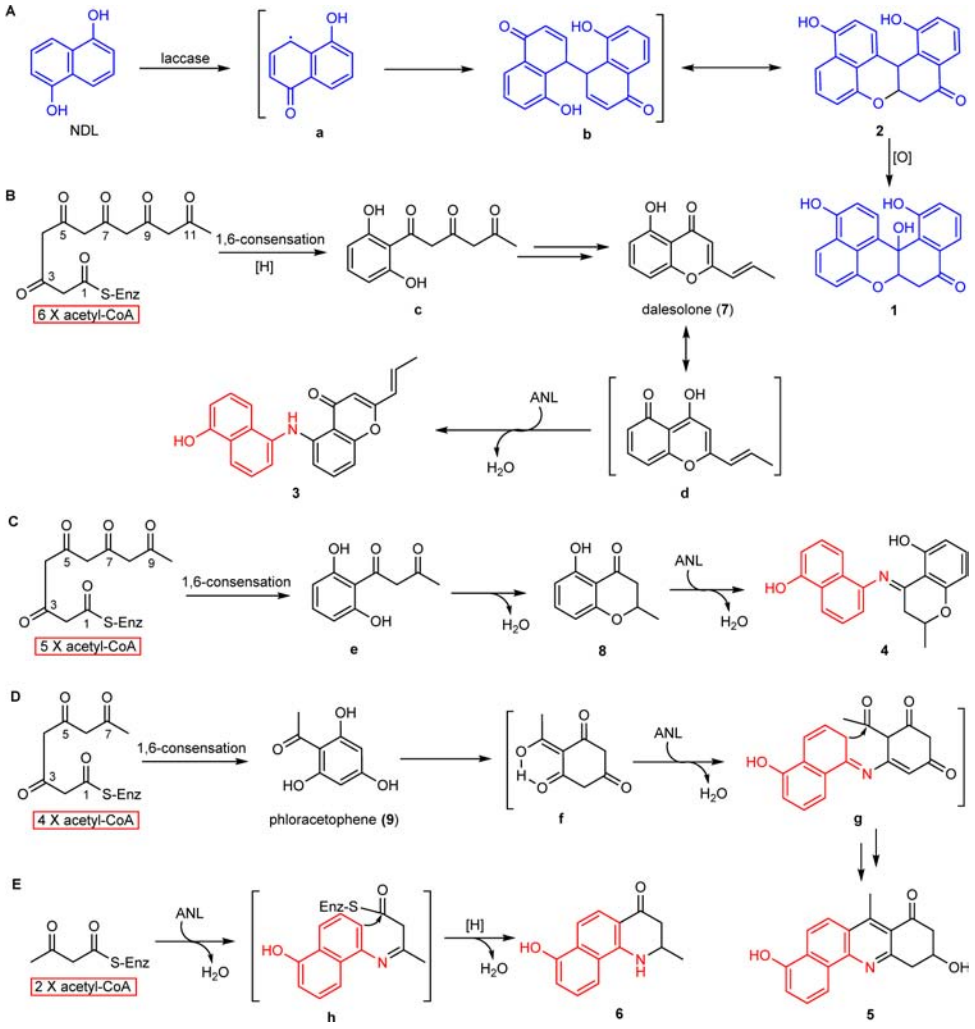
Scheme 1. Possible Biosynthetic Pathway of Mutadalesols A–F (1–6)



1,2,5-trisubstituted naphthalene nucleus as in the case of **5**, and a 3-substituted *n*-butanoyl group was indicated by the signals at δ_H 1.46 (d, J = 6.4 Hz), 3.91 (dqd, J = 6.4, 6.4, 4.0 Hz), 2.43 (dd, J = 15.6, 6.4 Hz), and 2.60 (dd, J = 15.6, 4.0 Hz), and at $\delta_{C=O}$ 192.5. The HMBC correlations of H-2′ to C-1 and C-4′ and of H-3′ to C-2 gave collectively the structure of **6** (Figure 3). The chiral HPLC separation of **6** afforded two enantiomers (+)-**6** and (−)-**6**, which were shown to have (2′*R*)- and (2′*S*)-configurations, respectively, by comparing their CD spectra with those predicted for all optional stereoisomers (Figure S43).

Mutadalesols A–F (**1**–**6**) were tested for antitumor and anti-inflammatory activities that might be of potential significance in managing some life-threatening diseases.[2] (±)-Mutadalesol A ((±)-**1**) and its enantiomer (+)-**1** were shown to inhibit the growth of the human colon carcinoma SW480 cell line, with the IC_{50} values of 6.74 and 8.39 μM, which were comparable to that (3.19 μM) of doxorubicin coassayed as a positive reference. However, compounds (−)-**1** and **2**–**6** showed no cytotoxicity to the cell line at 10 μM. The data suggested the preliminary structure–activity relationships. The cytotoxicity of **1** is most likely chirality sensitive and dependent on the presence of the 8′-hydroxy group as deduced from the inactivity of **2**. Compounds **1**–**6** were also evaluated for the inhibitory effect on Toll-like receptor 5 (TLR5) by assessing the flagellin-induced TNF-α production in RAW264.7 macrophages. (−)-Mutadalesol A ((−)-**1**) displayed at 10 μM an inhibition rate of 30.64% which was comparable to that (42.34%) of dexamethasone, a prescribed anti-inflammatory drug coassessed as a positive control (Table S8).

Motivated by the bioactivity of mutadalesol A (**1**), we expanded our curiosity to its mycosynthetic pathway. The structural feature of **1** and **2** suggested that they could be generated from the laccase-catalyzed dimerization of naphthalene-1,5-diol (NDL) supplemented in the culture of Δ*pksTL* mutant of *D. eschscholzii*. To confirm the involvement of laccase in generating **1** and **2**, the *D. eschscholzii* was codeprived of the polyketide synthase and laccase genes, and the obtained Δ*pks*Δ*lac*TL mutant strain was unable to produce any of **1** and **2** upon its equal NDL exposure (see above and Figure S58). Ascertaining the key role of laccase in the formation of **1** and **2**, we were curious about whether **1** and **2** were constructed via radical couplings. Thus, the Δ*pksTL* mutant strain supplemented with NDL at 1 mM was cultured with separate exposures to hydrophilic free radical scavengers $MgSO_4$ (2.5 g/L) and vitamin C (30 mg/L).[8,14,15] After 10 days of cultivation, none of **1** and **2** could be detected by LC-MS in the EtOAc extracts derived from the cultures supplemented separately with the free radical scavengers (Figure S58). This observation could only be explained by assuming that both were produced via the radical coupling like dalesconols A and B.[8] Although inactive in the two

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bioassays, mutadalesols C−F (**3**−**6**) seem mycosynthetically interesting since they were most likely generated from the condensation of 5-aminonaphthalen-1-ol (ANL) with fungal hexa-, penta-, tetra-, and diketides (Scheme 1), respectively. All oligoketides except for diketides have been characterized from the culture of the wild-type strain of *D. eschscholzii*.[13] The proposal was reinforced by our identification of the shunt oligoketides **7**−**9** (Scheme 1). Compound **7**, named here dalesolone, is a new natural product (Figure 2). Products **8** and **9** were identified as 2,3-dihydro-5-hydroxy-2-methyl-4*H*-1-benzopyran-4-one[16] and phloracetophene[17] (Figure S59). The detection of **7**−**9** in the culture of the Δ*pksTL* mutant is not surprising in view of more than 20 polyketide synthase genes in the fungal genome of *D. eschscholzii*.[8] In particular, the characterization of **6** highlighted the fact that ANL compounded in culture with the fungal diketide, an early stage smaller intermediate in the fungal polyketide assembly line. To our knowledge, such an in-culture condensation of exogenous chemicals with fungal oligoketides was not described before. This observation may be of value for the bioorthogonal synthesis of new compounds by hybridization of appropriate exochemicals with arrays of biosynthetic intermediates.

In summary, we have obtained the Δ*pksTL* mutant strain of *D. eschscholzii*, which enables the mutasynthesis of six new naphthalene derivatives, mutadalesols A−F (**1**−**6**), with directed substitution patterns and/or new frameworks. (±)-Mutadalesol A and its (+)-enantiomer are cytotoxic, and its (−)-enantiomer is a TLR5 inhibitor. The chirality and 8′-hydroxy codependent bioactivity of mutadalesol A (**1**) was disclosed in conjunction with the clarification of its mycosynthetic pathway. Mutadalesols C−F (**3**−**6**) highlighted collectively the in-culture reactability of fungal oligoketide intermediates with properly structured exochemicals. This observation may lighten the bioorthogonal access to new unnatural molecules required for drug discovery efforts.

■ ASSOCIATED CONTENT

Supporting Information

Complete description of methods, additional tables, and figures, including full NMR data and crystallographic file (CIF). 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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