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Article

Biochemistry and Chemoinformatics Guided Classification of Hirsutane Sesquiterpenes Isolated from Mushroom

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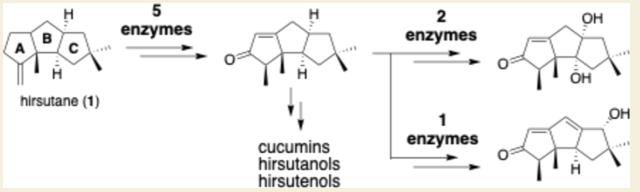
ABSTRACT: Hirsutanes are mushroom-derived sesquiterpenes with a characteristic 5-5-5 tricyclic ring skeleton. To date, more than 70 derivatives have been isolated from nature. In this study, we applied heterologous expression, in vitro enzymatic reactions, and biotransformation to characterize the function of nine enzymes involved in the synthesis of anhydroarthrosporone and six novel hirsutanes. The elucidated biosynthesis involves oxidative modifications of the A-ring followed by structural diversification of the B- and C-rings. Most importantly, biosynthetic pathways provide crucial insights into the classification and organization of isolated hirsutanes. We successfully classified 69 natural hirsutanes into three groups based on their A-ring modification patterns. Our classification covered 92% of the natural hirsutanes. A comprehensive understanding of their biosynthesis will provide opportunities to isolate structurally diverse hirsutanes using genetic engineering techniques.



KEYWORDS: *basidiomycota fungi, hirsutanes, heterologous expression, chemoinformatics*

INTRODUCTION

Sesquiterpenes are a major group of natural products found in mushrooms.[1] They have characteristic polycyclic structures such as protoilludane (skeleton: 5-6-4), hirsutane (5-5-5), illudane (5-6-3), and sterpurane (5-6-4), which are biosynthesized from reactive cationic intermediate protoilludyl cation.[2] Of particular importance is that each mushroom-derived sesquiterpene comprises a distinct family consisting of different oxidation patterns on its core skeleton.

Hirsutanes are a subgroup of linear triquinane sesquiterpenes characterized by a unique 5-5-5 tricyclic ring system (Figure 1).[3] To date, more than 70 hirsutanes have been reported to exhibit diverse biological activities, including cytotoxic, antimicrobial, and NO inhibitory activities. The characteristic bifunctional terpene synthase gene, Stehi1|52743, from *Stereum hirsutum* for the synthesis of hirsutane (**1**) was functionally characterized by in vitro enzymatic reaction.[4] This pioneering work identified putative biosynthetic gene cluster (BGC) consisting of 15 biosynthetic genes (Figures 1 and S1).[4] The BGC is characterized by different oxidative modification enzymes such as cytochrome P450, NAD-binding protein, and FAD-binding protein, which have been considered as key enzymes possibly involved in the oxidative modifications of **1** to synthesize natural hirsutanes, such as hirsutic acid C and hirustenols,[5,6] isolated from *S. hirsutum*. Given that the structural diversity of sesquiterpenes is constructed by a pathway branching strategy from a key intermediate, elucidation of the biosynthetic pathway of hirsutanes representing this group is important for understanding the structural diversification mechanism.

The *Aspergillus oryzae* expression system has been recognized as an effective method for examining the biosynthesis of natural products. We identified reliable expression loci in *A. oryzae* and improved the expression system by combining them with the CRISPR-Cas9 system.[7] The established hot spot-knock-in method has accelerated biosynthetic studies of fungal natural products including heterologous production and biosynthetic pathway elucidation.[8] Indeed, we have applied this expression system to elucidate the biosynthetic pathways of mushroom-derived terpenes, such as pleuromutilin,[9] erinacine,[7] and melleolide.[10] This established method was also effective for the global expression of mushroom terpene synthases.[11] The extensive heterologous expression of biosynthetic genes from five different fungi is another example of the importance of this expression system.[12] In this study, we applied the expression system, in vitro enzymatic reactions, and biotransformation experiments to characterize the function of nine enzymes in the synthesis of hirsutanes. Consequently, we elucidated the common A-ring modification processes of hirsutanes and the structural diversification mechanism by modifying the B,C-rings. With the elucidated biosynthetic pathway, we classified natural hirsutanes into three groups (G1−G3) based on their A-ring modification patterns.

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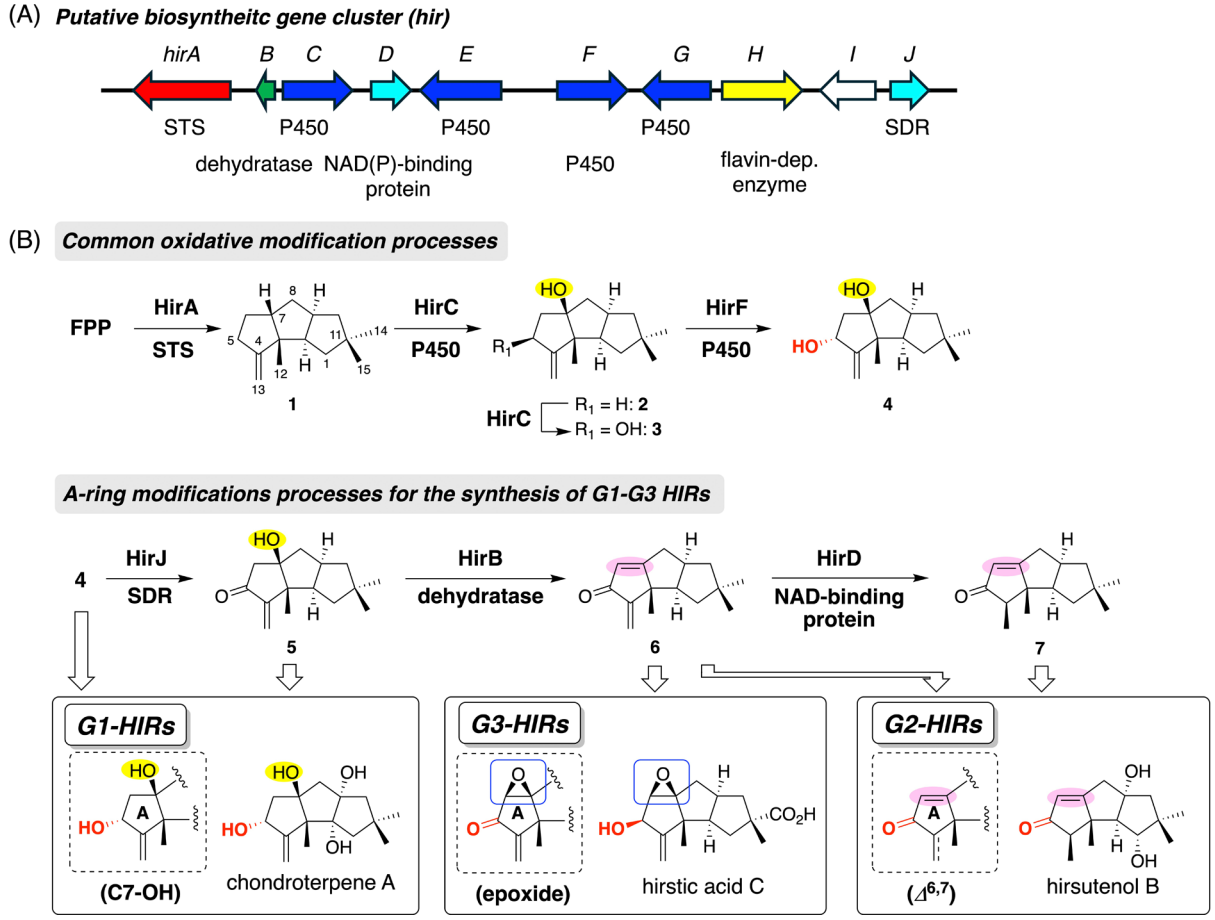

Figure 1. (A) Biosynthetic gene cluster of hirsutanes. (B) Proposed biosynthetic pathway of hirsutanes focusing on the common and A-ring modification processes. G1, G2, and G3-HIRs are three groups of natural hirsutanes defined in this paper. The natural hirsutanes classified in each group are summarized in Figure 5 and Supporting Information figures.

Notably, the classification covers the biosynthetic pathways of 92% of hirsutanes.

RESULTS AND DISCUSSION

S. hirsutum is a well-known mushroom that produces various types of hirsutane. For example, hirsutic acids and hirsutenols have been isolated from *S. hirsutum* (L515)[13] and MVS39 strains,[6] respectively. To elucidate the biosynthetic pathway of hirsutanes, we focused on the *S. hirsutum* MAFF4200200 strain, which has the same putative hirsutane biosynthetic genes as those reported for *S. hirsutum* FP-91666 SS1.[4] The putative biosynthetic gene cluster was defined as *hir* (Figures 1 and S1). Among these, nine genes were expressed under fermentation conditions (Figure S2). The expressed genes included a sesquiterpene synthase (STS, *hirA*), a dehydratase (*hirB*), four cytochrome P450s (*hirC*, *hirE*, *hirF*, and *hirG*), an NAD(P)-binding protein (*hirD*), a flavin-dependent enzyme (hirH), and a short-chain dehydrogenase/reductase (SDR, *hirJ*). In this study, we focused on the functions of these biosynthetic genes.

Oxidative modification of the sesquiterpene core structure is frequently catalyzed by cytochrome P450s. These P450s may catalyze multistep reactions and, therefore, require careful analysis. The biosynthetic genes were initially cloned into expression vectors (pDP801, pDP201, pDP401, and pDPwA) and constructed the following transformants: AO-*hirA*, AO-*hirAC*, AO-*hirACF*, and AO-*hirACF*/*CPR*. Metabolite analysis of AO-*hirA* showed the production of **1**, whereas that of AO-*hirAC* showed the production of two compounds, **2** and **3** (Figure 2). ^{1}H- and ^{13}C NMR data of **2** and **3** were in good

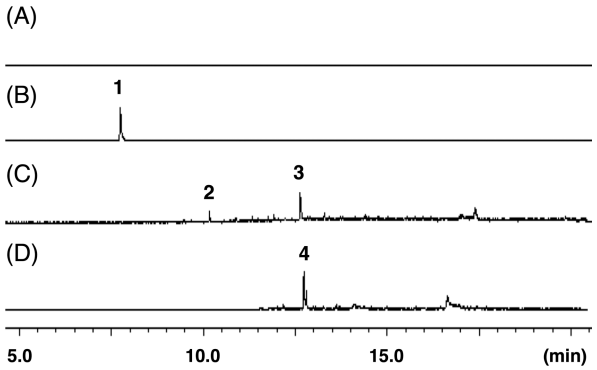

Figure 2. GC−MS profiles of the metabolites from (A) wild type strain (*m*/*z* 204), (B) AO-*hirA* (*m*/*z* 204), (C) AO-*hirAC* (*m*/*z* 204), (D) AO-*hirACF* (*m*/*z* 236).

agreement with those of the C-5 monohydroxylated and C-5/C-7 dihydroxylated products.[14] Further metabolite analysis revealed that AO-*hirACF* produced **4** as the major product (Figure 2), whose molecular weight (*m*/*z* 236.2 M^{+}) was the same as that of **3**. Extensive analysis of the 2D-NMR spectra showed that **4** has a *β*-hydroxy group at the C-5 position

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instead of α-hydroxy group found in **3**. The hydroxylation activities of HirC and HirF for the synthesis of **3** and **4** from **2** were further confirmed by biotransformation of **2** using AO-*hirC* and AO-*hirF* (Figure S3). These results showed that **2** is a branch point leading to **3** and **4**. The complete loss of the production of **3** in AO-*hirACF* under conditions where HirC and HirF compete indicated that HirF shows a much better hydroxylation activity than HirC. This is consistent with the previous reports that the pathway-specific enzymes show much better conversion than other downstream enzymes or enzymes from the host.[7,10]

We then constructed transformants, AO-*hirACFJB*, AO-*hirACFJBD*, and AO-*hirACFJBDEGH*, possessing the genes expressed in *S. hirsutam* to narrow down the candidate enzymes involved in subsequent modifications. However, no modification products were detected (Figure S4), which suggest that further modifications did not occur in *A. oryzae*. Therefore, we focused our attention on in vitro enzymatic reactions with recombinant proteins.

Initially, we focused on enzymatic reactions involving HirB, HirD, HirH, and HirJ. We cloned them into expression vectors, pMalc4E and pColdI, to prepare recombinant proteins (Figure S5). When recombinant HirJ was incubated with **4** in the presence of $NADP^+$, **4** was consumed and the new product **5** was detected as the sole product (Figures 3 and S6). The MS data (m/z 234.2 M^+) suggested that **5** is the oxidation product of **4** (m/z 236.2 M^+). The retention time of **5** was consistent with that of the corresponding ketone synthesized from **4** via TPAP oxidation (Figure S7). Incubation of **5** with HirB resulted in the formation of **6** (m/z 216.2 M^+), the molecular weight of which suggested that **6** was a dehydration product.

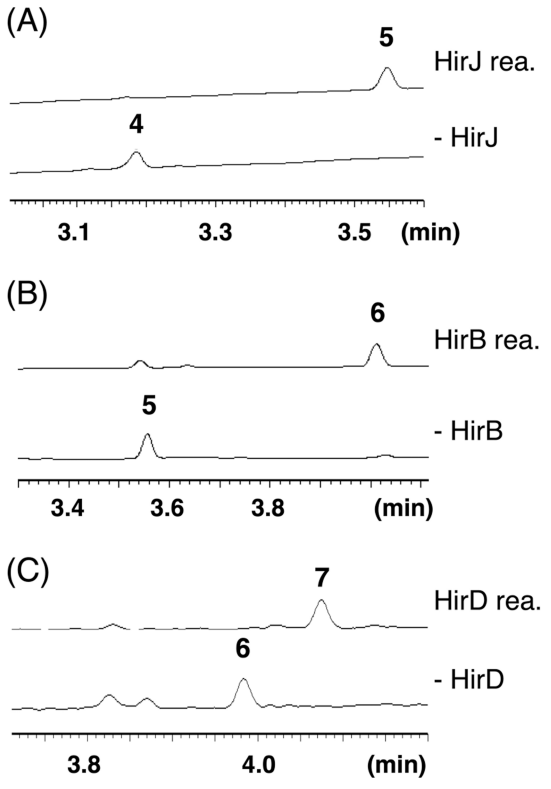

Figure 3. UPLC-MS profiles (200−400 nm) of the enzymatic reaction products. (A) HirJ reaction with **4**. (B) HirB reaction with **5**. (C) HirD reaction with **6**.

The retention time was consistent with that of dienone **6**, which was synthesized from **5** according to a literature procedure[14] (Figures 3 and S8). No cofactors were required for the dehydration reaction. We continued the in vitro enzymatic reaction with **6** and found that HirD catalyzed the reduction of **6** in the presence of NADPH to yield **7** (Figures 3 and S9). Large-scale enzymatic reaction allowed for the isolation of **7**. The ^{1}H- and ^{13}C NMR data showed that the olefinic protons derived from the exomethylene moiety disappeared, and a doublet methyl proton (Me-13, δ 1.04, doublet) was observed. Extensive 2D-NMR analysis confirmed the structure as shown in Figure 1. On the other hand, no products were detected in the HirH reaction. Through the in vitro enzymatic reactions described above, we have elucidated the modification pathways to afford the α,β-unsaturated ketone moiety found in **6** and **7**, which are common substructures frequently found in hirsutanes. Successful functional characterization of the recombinant proteins, HirJ, HirB, and HirD, suggested that HirJ is most likely inactive in *A. oryzae*.

Since compounds **6** and **7** were available for further investigation of the modification reactions, we focused on late-stage oxidative modifications. We focused on the functionally uncharacterized P450s, HirE and HirG. We constructed transformants, AO-*WT*, AO-*hirE*, and AO-*hirG*. The biotransformation of **6** with AO-*WT* showed the production of **7** as the sole product, demonstrating that the 1,4-reduction was also catalyzed by an inherent enzyme in *A. oryzae* (Figure S10). To avoid complications, we used **7** as the substrate for further biotransformation experiments. No product was detected in the metabolites when AO-*hirG* was used for the reaction. In contrast, new products **8A**, **9A**, and **9B** were detected in the metabolites of the biotransformation with AO-*hirE* (Figure 4). Based on MS analysis, we found that **8A** (m/z 234.2 M^+) was a simple oxidation product of **7** (m/z 218.2 M^+). The NMR data for **8A** were in good agreement with those of anhydroarthrosporone isolated from the arthroconidial fungus UAMH 4262.[15] The ^{1}H- and ^{13}C NMR data for **9A** were similar to those of **8A**, although an additional oxymethine proton (H-10, δ 3.53, singlet) was detected. Extensive 2D-NMR analysis showed that the hydroxylation occurs at the C-10 position. The NOE correlation between H-10 and Me-13 indicated that H-10 was located on the β-face. The molecular weight of **9B** (m/z 232.1 M^+) was 14 mass units higher than that of **7**. The ^{1}H NMR spectrum showed olefinic protons (H-6, δ 5.7, singlet; H-8, δ 6.39, doublet) and oxymethine proton (H-10, δ 4.20, singlet). Further 2D-NMR analysis allowed us to propose the structure shown in Figure 4. Large-scale fermentation allowed us to detect the minor product **8B**, which exhibited a UV−vis spectrum similar to that of **9B**. Although limited production prevented isolation of **8B**, **8B** was successfully converted to **9B** in the AO-*hirE* transformant (Figure S11). Taken together, the structure of **8B** was proposed to be an $\alpha,\beta,\gamma,\delta$-conjugated system. Conversion of **8B** to **9B** was confirmed by biotransformation with SC-*hirE* (host: *Saccharomyces cerevisiae*) (Figure S11). Surprisingly, the incubation of **6** with SC-*hirE* resulted in the production of exo-olefin analogs of **8A** and **8B**, thereby suggesting that HirE also recognizes **6** as a substrate to catalyze similar multistep oxidation reactions (Figure S12).

Given that **8A** has an acidic H-8 hydrogen atom, which is located adjacent to the C-9 hydroxy group, **8A** has the potential to undergo dehydration to afford **8B**, as in the case of dehydration from **6** to **7** found in the A-ring modification.

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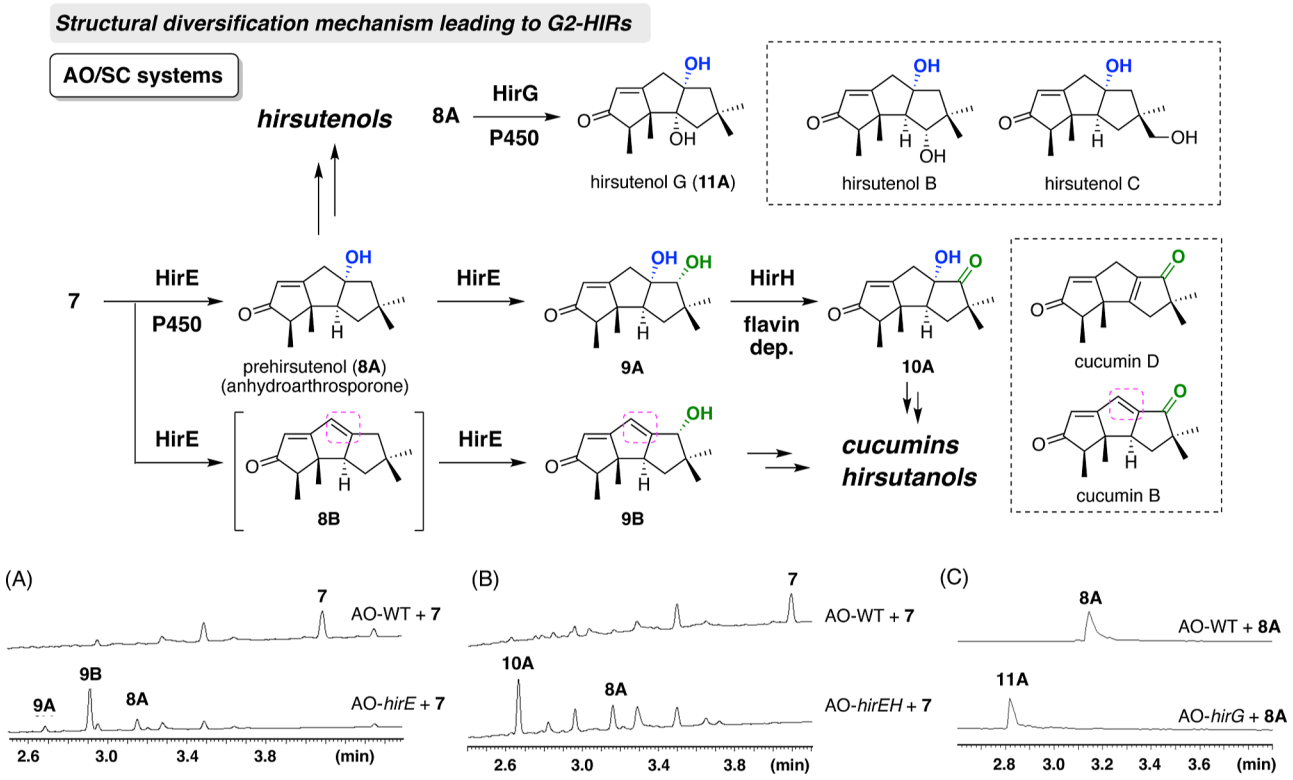

Figure 4. Structural diversification mechanism for the synthesis of Group 2 hirsutanes (G2-HIRs). UPLC-MS profiles of the biotransformation experiments. (A) AO-*hirE* reaction with **7** (200−400 nm) , (B) AO-*hirEH* reaction with **7** (200−400 nm), and (C) AO-*hirG* reaction with **8A** (EIC: *m*/*z* 235 (top), 251 (bottom)).

Considering the possibility that this dehydration reaction is catalyzed by endogenous enzymes from *A. oryzae*, we conducted a biotransformation of **8A** with AO-*hirE*. However, **8B** could not be detected under these conditions. Based on these results, we propose that **8A** and **8B** are independently biosynthesized from **7** by HirE (Scheme S1). The ferryl-oxo intermediate, known as compound I,[16] abstracts a hydrogen atom at the C-9 position to give a radical intermediate. This is a branching point that leads to the formation of **8A** and **8B**. Oxygen rebounds onto the C-9 radical to yield **8A**, whereas the abstraction of H-8 results in the formation of a double bond to afford **8B**. Once **8A** and **8B** are synthesized, α-hydroxylation occurs at the C-10 position to give **9A** and **9B**, respectively.

The functions of the remaining enzymes, HirG (a P450) and HirH (a flavin-dependent enzyme), were investigated using biotransformation experiments. Using **7** as the substrate for the HirE and HirH coupling reactions, a new product **10A** was observed (Figure 4). MS analysis showed that the molecular weight of **10A** (*m*/*z* 248.1 M^+) is 2 mass units less than that of **9A** (*m*/*z* 250.2 M^+). ^{13}C NMR data showed two carbonyl carbons (C-5, δ 210.0; C-10, δ 221.0). The structure was confirmed by 2D-NMR analysis as shown in Figure 4. Notably, **8B** and **9B** were not detected in this biotransformation, suggesting that the co-expression of HirE and HirH suppressed the oxidation pathway leading to **9B**. This result led us to hypothesize that an unexpected regulatory system is involved in the synthesis of structurally diverse hirsutanes. Biotransformation experiments with AO-*hirG* showed that **8A** was converted into **11A** (Figure 4). MS analysis showed that **11A** (*m*/*z* 250.2 M^+) was a hydroxylation product of **8A** (*m*/*z* 234.2 M^+), although no oxymethine proton was observed in the ^1H NMR spectrum, suggesting that the hydroxy groups were located on the quaternary carbon. The structure of **11A** was finally confirmed by 2D-NMR analysis. We named **11A** as hirsutenol G based on its structural similarity with other hirsutenols and the biological relationships described in the following paragraph.

Overall, we identified 11 hirsutanes through heterologous production, in vitro enzymatic reactions, and biotransformation (Figures 1 and 4). Among these, six compounds, **4**, **6**, **9A**, **9B**, **10A**, and **11A**, were novel hirsutanes. The biosynthetic pathways are shown in Figures 1 and 4. Early stage oxidative modifications occur at the A-ring. Two cytochrome P450s, HirC and HirF, mediate stepwise hydroxylation at the C-5 and C-7 positions, respectively, to afford diol **4** via **2**. Subsequently, three different types of modification enzymes, HirJ, HirB, and HirD, mediate the A-ring modifications to give α,β-unsaturated ketone **7**. **7** is a key intermediate that serves as the starting point for late-stage oxidative modification, thereby leading to structurally diverse hirsutanes. HirE recognizes **7** as a substrate and catalyzes multistep oxidations to yield both δ-hydroxy-α,β-unsaturated ketone **8A** and $\alpha,\beta,\gamma,\delta$-unsaturated ketone **8B** and their oxidation products, **9A** and **9B**. Coproduction of hydroxylated and dehydrated products has also been reported during the isolation of chondrosterins,[17] curcumins,[18] and hirsutanols/*ent*-gloeosteretriol.[19] Products **8A** and **9A** undergo further oxidation by HirG and HirH to yield **11A** and **10A**, respectively. A representative example of the functional analysis of a single enzyme involved in pathway branching is cytochrome P450 NodJ, which is involved in the biosynthesis of nodulisporic acid.[20] It should be noted that **8A** is probably a biosynthetic precursor of the hirsutenols B and C, which were isolated from their producer, *S. hirsutum* (Willd.: Fr.) S. F. Gray (Stereaceae).[6] Although HirG from *S. hirsutum*

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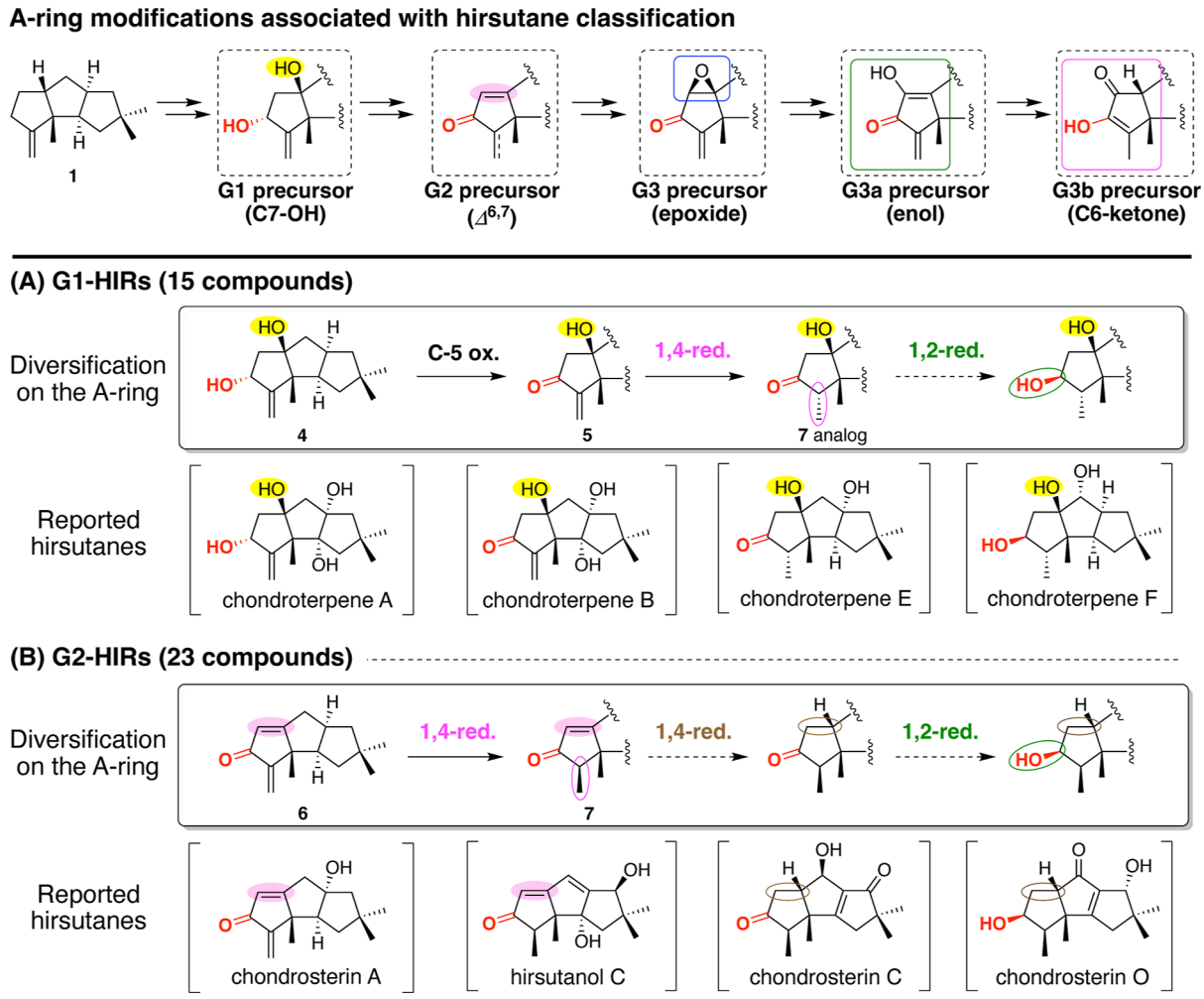

Figure 5. Classification and biological relationship of (A) group 1 hirsutanes (G1-HIRs) and (B) group 2 hirsutanes (G2-HIRs).

MAFF4200200 catalyzed the C-2 hydroxylation, the possible hydroxylations at the C-1 and C-15 for the synthesis of hirsutenols B and C could be catalyzed by HirG-related P450s, reflecting the slight difference in their amino acid sequence. Another possible explanation for this contradiction is that putative P450 genes may be located at the different loci in the genome of *S. hirsutum* MAFF4200200, although further experimental support is required to probe these hypotheses.

The biosynthetic pathways elucidated in this study provide crucial insights into the classification and organization of isolated hirsutanes. The key point is that the modification reactions of A-ring precede those of B- and C-rings. Therefore, we classified natural hirsutanes into three groups based on their A-ring modification patterns (Figures 5, S13 and S14). Group 1 hirsutanes (G1-HIRs) share oxygen functional groups at the C-5 and C-7 positions on the A-ring, as in the case of **4** (Figures 5 and S14). The degree of oxidation at the C-5 and C-13 positions varies from one hirsutane to another. The modification reactions were explained by C-5 alcohol oxidation, C-13 reduction, and C-5 reduction. The first two reactions are similar to those catalyzed by HirJ and HirD. The isolation of chondroterpene A (C-5/C-13: α-OH, exo-olefin), chondroterpene B (ketone, exo-olefin), chondroterpene E (ketone, methyl), and chondroterpene F (β-OH, methyl) from the same strain, *Chondrostereum* sp.,[21] provided circumstantial evidence for this biosynthetic hypothesis. Furthermore, B- and C-ring modifications afford **15** structurally diverse hirsutanes. The Group 2 hirsutanes (G2-HIRs) are derivatives of **6** with an α,β-unsaturated moiety on the A-ring (Figures 5 and S14). In contrast to G1-HIRs, the dehydration process at the A-ring catalyzed by HirB is essential to synthesize the α,β-unsaturated ketone moiety, which is a suitable substrate for subsequent 1,4- and 1,2-reductions. Co-isolation of chondrosterin A, chondrosterin C, and chondrosterin O from the same strain, *Chondrostereum* sp.,[17] supports the proposed modification process, although the biosynthetic enzymes catalyzing 1,4- and 1,2-reductions have not been characterized. Subsequent modification of the B,C-rings yielded 20 hirsutanes. Similar structural features were found in chlorinated derivatives such as chlorostereone. Therefore, they were classified into this group. Group 3 hirsutanes (G3-HIRs) have an epoxide moiety on the A-ring (Figures S13 and S14). From a synthetic point of view, it is reasonable to assume that this epoxide could be synthesized by the epoxidation of the corresponding α,β-unsaturated ketone. Unlike G2-HIRs, G3-HIRs share an exo-olefin moiety, which suggest that **6** is a key branching point for both G2-HIRs and G3-HIRs. There are 16 hirsutanes in this group, including characteristic dimerization products such as xeromphalinone E.[22] The remaining hirsutanes reported in literature are derivatives of G3-HIRs. Therefore, we classified them as Group 3a-HIRs and Group 3b-HIRs (Figures S13 and S14). G3a-HIRs can be synthesized via an epoxide opening

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reaction. Subsequent keto−enol tautomerization, followed by 1,4-reduction, may afford the characteristic structure of G3b-HIRs, although this proposal requires experimental support. Overall, we successfully classified 69 hirsutanes (92% of the reported 75 hirsutanes) into three major groups, while focusing on the A-ring modification patterns.

Gloeostereum incarnatum is known to produce both G1-HIR such as gloeosteretriol (hirsutanol F) and G2-HIRs such as hirsutanol A, hirsutanol C, and incarnal.[23−25] The genome data of *G. incarnatum* showed the presence of a homologous biosynthetic gene cluster (BGC) with *hir* (Figure S15),[26,27] supporting the close relationship for the synthesis of G1- and G2-HIRs described above. On the other hand, to the best of our knowledge, there are no reports on the BGC involved in the synthesis of G1- and G3-HIRs. Nonetheless, we found putative BGCs with the A-ring modification genes, *hirC* and *hirF*, located adjacent to uncharacterized biosynthetic genes in the public database (Figure S15). These biosynthetic genes are candidates for the synthesis of G1- and G3-HIRs. The accumulation of genomic data provides opportunities to characterize the group specific modification enzymes involved in the biosynthesis of structurally diverse G1- and G3-HIRs.

CONCLUSION

In summary, we applied heterologous expression, in vitro enzymatic reactions, and biotransformation experiments to characterize the function of nine genes expressed in *S. hirsutum* MAFF4200200 strain, which produces hirsutenols based on this study. The unexpected problem of the oxidation product **4** not being converted within the heterologous host was solved by an in vitro enzymatic reaction using the recombinant enzymes HirJ, HirB, and HirD. Successful functional characterization of each enzyme enabled us to elucidate the A-ring modification processes. The subsequent late-stage conversions to compounds **9B**, **10A**, and **11A** were determined by biotransformation experiments. Most importantly, we classified natural hirsutanes into three groups, G1-G3 HIRs, based on the elucidated biosynthetic pathways. In particular, the oxidative modification patterns of the A-ring are important for classification. Our classification covers 92% of the natural hirsutanes. Their proposed biosynthetic pathways provide clear pathway-specific modifications for the synthesis of uncharacterized G1- and G3-HIRs, enabling the identification of key modification enzymes from public databases.

To date, many sesquiterpenes have been isolated from mushrooms. Recent biosynthetic studies have focused on specific sesquiterpenes. In contrast, the present study on hirsutanes demonstrated the importance of an integrated approach utilizing accumulated chemical data and elucidated the biosynthetic pathway to understand the complex structural diversification mechanism of a group of sesquiterpenes. Since promiscuous modification enzymes play a key role in diversification during late-stage biosynthesis, the present approach may be important for understanding the structural diversification of other mushroom-derived sesquiterpenes.[28]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00983.

Experimental procedures, UPLC-MS data, GC−MS data, and NMR data (PDF)

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Notes

The authors declare no competing financial interest.

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