A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition Chemie

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201703932
Angew. Chem. 10.1002/ange.201703932

Link to VoR: http://dx.doi.org/10.1002/anie.201703932
http://dx.doi.org/10.1002/ange.201703932

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10.1002/anie.201703932

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Discovery of a calcium-dependent enzymatic cascade for the selective assembly of hapalindole-type alkaloids: On the biosynthetic origin of hapalindole U

Qin Zhu[a] and Xinyu Liu*[a]

Abstract: Hapalindole U (**4**) is a validated biosynthetic precursor to ambiguine alkaloids (*Angew. Chem. Int. Ed.* **2016**, *55*, 5780), of which biogenetic origin remains unknown. The recent discovery of AmbU4 (or FamC1) protein encoded in the ambiguine biosynthetic pathway (*J. Am. Chem. Soc.*, **2015**, *137*, 15366), an isomerocyclase that can rearrange and cyclize geranylated indolenine (**2**) to a previously unknown 12-*epi*-hapalindole U (**3**), raised the question whether **3** is a direct precursor to **4** or an artifact arising from the limited in vitro experiments. Here we report a systematic approach that led to the discovery of an unprecedented calcium-dependent AmbU1-AmbU4 enzymatic complex for the selective formation of **4**. This discovery refuted the intermediacy of **3** and bridged the missing links in the early-stage biosynthesis of ambiguines. This work further established the isomerocyclases involved in the biogenesis of hapalindole-type alkaloids as a new family of calcium-dependent enzymes, where the metal ions are shown critical for their enzymatic activities and selectivities.

The biosynthesis of hapalindole-type alkaloids, including hapalindoles, fischerindoles, ambiguines and welwitindolinones, has recently become a subject of intense investigations.[1-12] These studies, collectively directed towards deciphering the origin of structural complexities and diversities of hapalindole-type alkaloids, have thus far unraveled several layers of puzzles concerning their global biosynthetic diversifications (Figure S1). These include the identification of indolenine **2** derived from the enzymatic geranylation of *cis*-indolyl vinyl isonitrile **1** (Figure 1a), as the cryptic common biosynthetic precursor to all hapalindole-type alkaloids,[7] and establishing the enzymatic basis for halogenations in these alkaloids that account for both complexity and diversity generations.[4,6] More recently, a new family of proteins embedded in the hapalindole-type alkaloid biosynthetic pathways, originally named as U-proteins for their bioinformatically unknown nature,[1-3] were shown able to stereoselectively rearrange and cyclize **2** to three isomeric hapalindole-type alkaloids, typified by 12-*epi*-fischerindole U synthase WelU1, 12-*epi*-hapalindole C synthase WelU3 and 12-*epi*-hapalindole U synthase AmbU4.[10.11] As the enzymatic cyclizations by WelU1/WelU3/AmbU4 proteins are induced by the geranyl group rearrangement in **2**, these enzymes can be formally named as a family of isomerocyclases.[13] The identification and characterization of WelU1 and WelU3 isomerocyclases effectively connected the missing links in the early stage of the welwitindolinone biosynthetic pathway and provided the enzymatic basis for the altered hapalindole-type alkaloid structural diversities in two welwitindolinone producers.[11,12]

In contrast to the welwitindolinones, the enzymatic pathways for the assembly of ambiguine precursors remain incomplete. We recently showed that hapalindole U **4** is a biosynthetic precursor to hapalindole G **5** and related ambiguines in *Fischerella ambigua* UTEX1903 (Figures 1b and S2) by characterizing the pathway-specific prenyltransferase AmbP3 and halogenase AmbO5 (Figure 1c and S3).[6] However, the latest discovery of a thermostable AmbU4 protein in the cell lysate of *F. ambigua* UTEX1903 as a dedicated synthase for 12-*epi*-hapalindole U **3** (Figures 1b), a previously structurally unknown C12 epimer of **4**,[10] added a twist to the ongoing investigation of ambiguine biogenesis. The question immediately raised by this unexpected observation is whether **3** is a legitimate ambiguine pathway intermediate or it is an artifact due to the limited experimental investigations. Besides AmbU4, there are three AmbU homologs (U1-U3) encoded in the ambiguine pathway of *F. ambigua* UTEX1903 (Figures 1c and S4), of which functions remain unknown. Here we report the systematic in vitro characterization of all AmbU isomerocyclases that allowed us to conclusively establish the enzymatic basis for the selective formation of **4** and refuted the intermediacy of **3** in the biosynthesis of ambiguines. This effort further led to the discovery of calcium ion as a critical element in modulating the activities and selectivities of all isomerocyclases embedded in the biosynthetic pathways of hapalindole-type alkaloids.

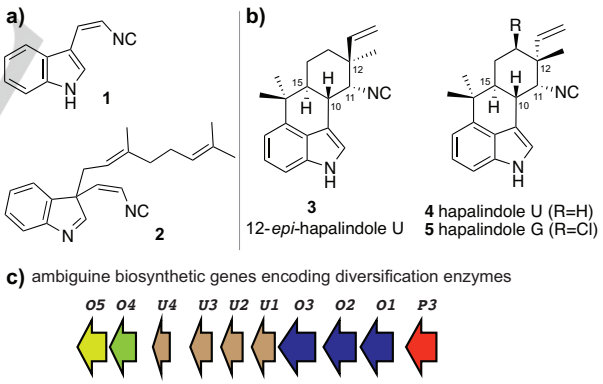

Figure 1. a) Structures of common biosynthetic precursors (**1** and **2**) to all hapalindole-type alkaloids; b) structures of previously identified hapalindole biosynthetic precursors (**3** and **4**) to ambiguines and hapalindole G (**5**) from the ambiguine producer *F. ambigua* UTEX1903; c) organization of ambiguine biosynthetic genes encoding diversification enzymes.

N-terminal His_7-tagged AmbU1, AmbU2, AmbU3 and AmbU4 proteins (Figure S4) devoid of the signal peptide sequences were procured by heterologous expression in *E. coli*. An initial glimpse on the enzymatic activity of AmbU protein complex was obtained by incubating stoichiometric amounts of AmbU1, AmbU2, AmbU3, AmbU4 (10 μM each) with **1** (1.0 mM) and GPP (1.5 mM) in the presence of $MgCl_2$ (20 mM) at pH=6.0. The enzymatic reaction was initiated by the addition of AmbP1 (10 μM) at 30 ºC and stopped by EtOAc extraction after 2 h. HPLC analysis of the crude extract revealed three discernible peaks eluted between 28 and 40 mins (Figure 2, HPLC trace a),

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of which UV spectra reassemble hapalindoles (Figure 2 inset and S5). Their structural identities were confirmed to be **4, 3** and hapalindole H **6** by either ^{1}H NMR analysis (Figures S6/7), or HPLC co-elution with the known compound (for **3**, *vide infra*).[11] The detection of **4** as one of the enzymatic products from **1** and GPP by the AmbU quartet and AmbP1 is distinctively different from what was recently observed with the cell lysate of *F. ambigua* UTEX1903,[10] or the purified AmbU4.[11] This suggests the enzymatic formation of **4**, an authentic pathway precursor to all ambiguines, might have been overlooked (Figure S8) and prompted us to conduct in-depth characterizations.

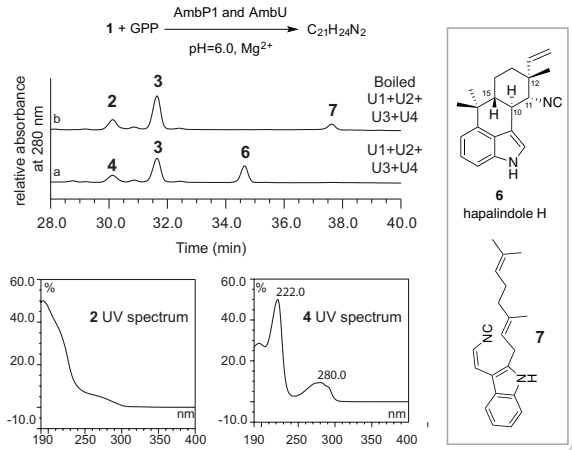

Figure 2. Initial characterizations of AmbU1-U2-U3-U4 protein mixture.

As AmbU4 was shown highly thermostable,[10] we first boiled the AmbU quartet and re-assessed their enzymatic activity with AmbP1. HPLC analysis (Figure 2, HPLC trace b) showed **3** became the sole detectable hapalindole along with the AmbP1 product **2** and its rearranged byproduct **7**, suggesting the formation of **4** requires the remaining AmbU protein(s). Under the HPLC condition we employed, **2** and **4** nearly co-elute, however they can be easily distinguished by their characteristic UV absorption spectra (Figure 2 insets). To trace the enzymatic origin of **4**, we first examined each individual AmbU protein. This analysis showed only AmbU3 and AmbU4 exhibited enzymatic activities (Figure 3, traces a-d). In contrast to AmbU4, AmbU3 is unselective and generated four isomeric hapalindole-type products (**4**, **8**, **9** and **6**) that were readily identifiable by UV and HRMS (Figure S9). While compound **9** was not obtainable in a pure form as it co-eluted with **6**, compound **8** was isolated and determined to be 12-*epi*-hapalindole C (Figure S10). This indicates AmbU3 is an active isomerocyclase but incapable of controlling the selectivity of all three elemental steps proposed for this enzymatic cascade (Figure S11). Next we examined the AmbU trio complex (Figure 3, traces e-h) by systematically omitting individual AmbU protein from the initially tested AmbU quartet (Figure 2). These assays showed the co-presence of AmbU2 and AmbU3 appeared strongly promoting the formation of **6** (Figure 3, traces f-g) and a small amount of **4** was detectable in all AmbU trio mixtures. Building on these observations, we proceeded further to examine all possible AmbU duo complexes (Figure 3, traces i-n). The combination of AmbU1 with AmbU2 or AmbU3 did not alter their respective inactive or unselective nature (Figure 3, traces i-j), however its

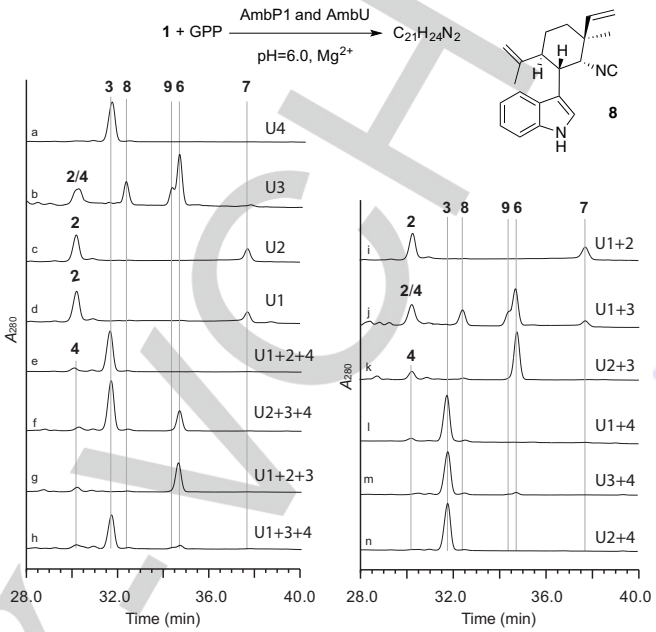

Figure 3. Enzymatic characterizations of individual AmbU protein, their 1-to-1 stoichiometric trio and duo complexes. Assays were conducted with **1** (1.0 mM), GPP (1.5 mM) in the presence of $MgCl_2$ (20 mM) at pH=6.0 and AmbU proteins (10 μM each). The enzymatic reaction was initiated by the addition of AmbP1 (10 μM) at 30 °C and stopped by EtOAc extraction after 2 h.

combination with AmbU4 yielded a small amount of **4**, ca. 3.5% to **3** (Figure 3, trace l). Interestingly, the addition of AmbU2 or AmbU3 to AmbU4 did not appear eroding the fidelity of AmbU4 (Figure 3, traces m-n), suggesting the detection of **4** from the AmbU1+AmbU4 assay is unlikely an artifact. In addition, as perceived from the AmbU trio assay, the combination of AmbU2 and AmbU3 robustly generated **6** with the concomitant formation of **4** (ca. 17% to **6**) (Figure 3, trace k).

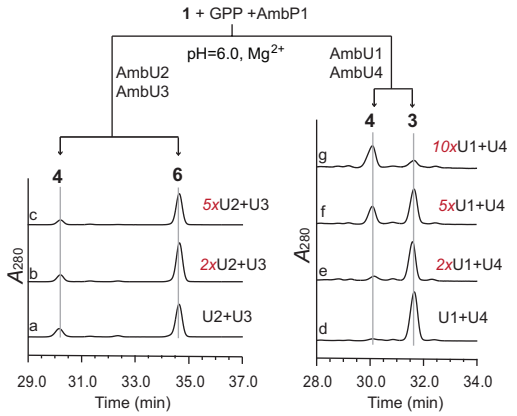

Figure 4. Effects of AmbU2 and AmbU1 stoichiometries in their respective AmbU2/AmbU3 and AmbU1/AmbU4 enzymatic duos for the selective formation of **6** and **4**.

The characterizations of individual AmbU protein as well as their 1-to-1 stoichiometric duos and trios indicate the formation of **4**, as observed in the initial examination of AmbU quartet, originates primarily from AmbU2/AmbU3 and AmbU1/AmbU4

duos. Although neither duo afforded **4** as a major product, these experiments clearly showed the isomerocyclase selectivity of AmbU4 or AmbU3 were altered when mixed with only one equivalent of AmbU1 or AmbU2. This suggested to us that varying the stoichiometry of AmbU proteins in each duo might lead to an altered production of **4**. To this end, we increased the ratio of AmbU2 and AmbU1 in their respective duos with AmbU3 and AmbU4 from 1:1 to 2:1 and 5:1 and analyzed their enzymatic products by HPLC (Figure 4). While increasing the AmbU2 stoichiometry to AmbU3 led to only marginal selectivity increase in the formation of **6** versus **4** (Figure 4, traces a-c), a nearly 14-fold selectivity enhancement for the formation of **4** versus **3** was observed when the ratio of AmbU1 to AmbU4 was increased to 5:1 (Figure 4, traces d-f). When this ratio was further increased to 10:1, **4** became the major enzymatic product (~2.5 times more than **3**, Figure 4, trace g), which translated to a >70-fold selectivity enhancement from the 1-to-1 stoichiometric AmbU1/AmbU4 duo.

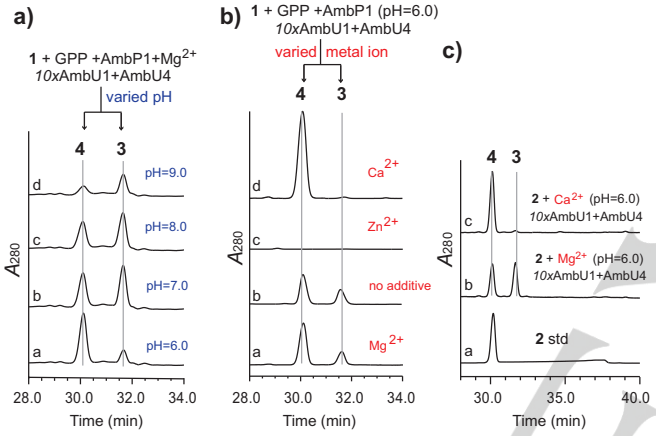

Figure 5. Effects of pH and divalent metal ions on 10xAmbU1-AmbU4 duo for the selective formation of **4**: the critical role of Ca^{2+} revealed

Encouraged by this observation, we sought to identify additional factor(s) for the AmbU1/AmbU4 enzymatic duo to achieve the selective formation of **4**. Initial examination of the pH effect revealed the selectivity of 10-to-1 stoichiometric AmbU1/AmbU4 (10xAmbU1-AmbU4) duo was optimal at pH=6.0 and deteriorated gradually to afford more **3** with the increased pH (Figure 5a). This observation mirrors our recent finding that the 12-*epi*-fischerindole U synthase WelU1 is most active at pH=6.0.[11] We next examined the influence of divalent ions as they have been found to influence the activity of AmbP1, the aromatic prenyltransferase that generates substrate **2** for the AmbU proteins (Figure 5b).[7] While the absence or presence of Mg^{2+} (20 mM) did not alter the selectivity of 10xAmbU1-AmbU4 duo (Figure 5b, traces a-b), the addition of Ca^{2+} (20 mM) led to a near-exclusive formation of **4** (>98% vs <2% for **3**) (Figure 5b, trace d). As a negative control, the addition of Zn^{2+} (20 mM), known to inhibit the activity of AmbP1,[7] completely abolished the formation of **4** and **3** (Figure 5b, trace c). The dramatic selectivity enhancement of 10xAmbU1-AmbU4 duo by the addition of Ca^{2+} was unexpected, in part because we have previously shown Ca^{2+} partially impeded the activity of AmbP1 at pH=8.0.[7] To ascertain the observed calcium effect, the enzymatic activity of 10xAmbU1-AmbU4 duo was directly assessed using **2** as its substrate at pH=6.0 (Figure 5c). This analysis convincingly showed that Ca^{2+}, but not Mg^{2+}, is critical for the selective generation of **4**. These results further indicate that the enzymatic reaction by 10xAmbU1-AmbU4 duo that converts **2** to **4** is kinetically more rapid than that of AmbP1 that generates **2** (Figure S12). These kinetic differences effectively diminished the nonenzymatic rearrangement from **2** to **7** (Figure S12), when AmbP1 is coupled to 10xAmbU1-AmbU4 duo with Ca^{2+} at pH=6.0, mirroring what was recently observed for WelU1.[11] In addition, we examined the calcium effect on the enzymatic selectivity of standalone AmbU4 and AmbU1 as well as 1xAmbU1-AmbU4, 2xAmbU1-AmbU4 and 5xAmbU1-AmbU4 duos (Figure S13). While the exogenous addition of Ca^{2+} did not alter the selectivity profile of AmbU4 or AmbU1, it enhanced the selectivity of AmbU1-AmbU4, 2xAmbU1-AmbU4 and 5xAmbU1-AmbU4 duos towards **4**, albeit the near-exclusive formation of **4** as shown by 10xAmbU1-AmbU4 was not achieved. These results collectively indicate that both the stoichiometry of AmbU1/AmbU4 and the presence of Ca^{2+} are critical for the selective formation of **4**.

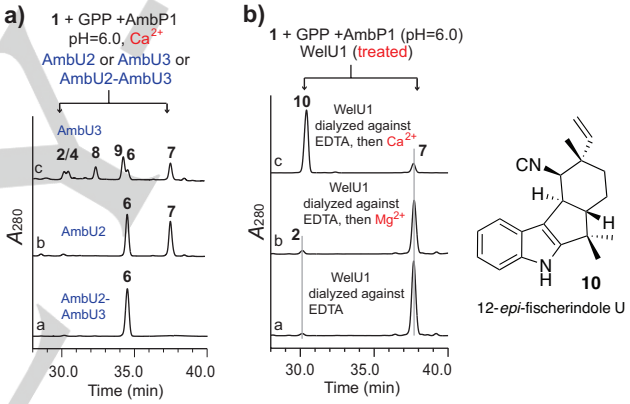

Figure 6. The calcium effect on the selectivity of AmbU2-AmbU3 duo and the activity of AmbU2 and WelU1.

Analogous to what was observed for the 10xAmbU1-AmbU4 duo, the addition of Ca^{2+} (20 mM) to the AmbU2-AmbU3 duo also enhanced its diastereoselectivity for the generation of **6** relative to **4** from a ratio of ~5:1 to >98:2 (Figure 4 trace a vs. Figure 6a trace a). Furthermore, in the presence of Ca^{2+}, AmbU2 alone gained activity and was able to generate **6** selectively with the concomitant formation of **7** (Figure 3 trace c vs. Figure 6a trace b), whereas the selectivity profile of AmbU3 in generating hapalindoles was unaltered (Figure 3 trace b vs. Figure 6a trace c). The critical role of Ca^{2+} in modulating the enzymatic selectivity of 10xAmbU1-AmbU4 and AmbU2-AmbU3 duos as well as the activity of AmbU2 prompted us to trace its biochemical basis. Routine BLAST-P search (ref 14) showed the U-type isomerocyclases encoded in the hapalindole-type alkaloid biosynthetic pathways had no homology to any functionally characterized proteins (Figure S14). However, bioinformatics analysis using HHpred (ref 15) for remote protein homology detection and structure prediction revealed they are weakly related to a broad family of polysaccharide hydrolases, which are known to naturally bound to calcium (Figure S15).

Sequence alignment of all characterized AmbU and WelU proteins revealed that the calcium binding residues as observed in the related polysaccharide hydrolases are well conserved (Figure S16). These bioinformatical data along with the calcium effect observed for the 10xAmbU1-AmbU4, AmbU2-AmbU3 duos and standalone AmbU2 led us to hypothesize that the entire family of U-type isomerocyclases encoded in the hapalindole-type alkaloid biosynthetic pathways are calcium-dependent.

During the previous characterization of 12-*epi*-fischerindole U synthase WelU1,[11] we have shown that the addition of Ca^{2+} or EDTA to the WelU1 assay mixture immediately prior to initiating the enzymatic reaction had little effect on its activity. We reasoned this conundrum was due to the presence of prebound Ca^{2+} in the heterologously purified WelU1 that might not be readily sequestered by EDTA. ICP-MS analysis of purified WelU1 confirmed the presence of Ca^{2+}. Furthermore, when we pre-dialyzed the WelU1 protein against EDTA (20 mM) for 1 h, its enzymatic activity was completely abolished (Figure 6b, trace a). The diminished activity was readily rescued by further dialyzing the EDTA-treated WelU1 against Ca^{2+} (20 mM) for 1 h (Figure 6b, trace c), but not against Mg^{2+} (20 mM) (Figure 6b, trace b), confirming the enzymatic activity of WelU1 is calcium-dependent. Analogously, the enzymatic activities of the standalone 12-*epi*-hapalindole C synthase WelU3, 12-*epi*-hapalindole U synthase AmbU4 as well as 10xAmbU1-U4 and AmbU2-AmbU3 duos were shown completely abolished upon dialyzing against EDTA but rescuable by re-dialyzing against Ca^{2+} (Figure S17). Collectively, these results validate that the activities and selectivities of U-type isomerocyclases depend on the presence of calcium.

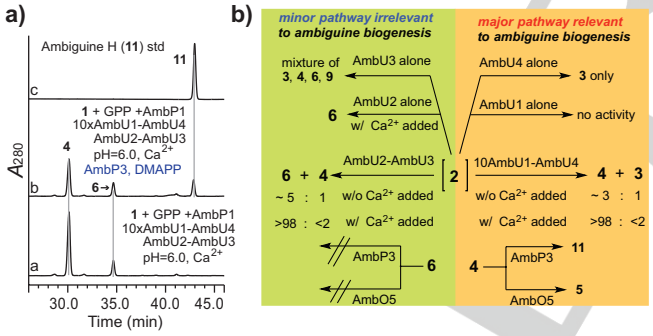

Figure 7. a) Contrast and compare the biosynthetic relevancy of **4** and **6** with regards to the assembly of ambiguine H (**11**), b) Summary of the enzymatic basis for the early stage structural diversifications in the biogenesis of ambiguines. All U-type proteins are most active at pH=6.0. The activity and selectivity profiles are based AmbU proteins purified heterologously from *E. coli* without EDTA treatment. The activity and selectivity profiles of standalone AmbU1, AmbU4 and AmbU4 were unaltered with the exogenous addition of Ca^{2+}, whereas AmbU2 was inactive without exogenous Ca^{2+}.

Having revealed the enzymatic basis for the selective formation of **4** and **6**, we sought to further examine and compare their biosynthetic relevancy to the ambiguine assemblies. Although **6** has been isolated from two hapalindole-type alkaloid producers including the ambiguine producer *F. ambigua* UTEX1903 (Figure S2), [16,17] its chlorinated or prenylated derivatives are not known, indicating it is a pathway outlier. When the 10xAmbU1-U4 and AmbU2-AmbU3 duos are mixed in a 1:1 ratio and coupled with AmbP1, we observed the preferential formation of **4** to **6** (Figure 7a, trace a). When this enzymatically generated mixture of **4** and **6** was further treated with AmbP3 and DMAPP, only **4** was processed to ambiguine H **11** (Figure 7a, traces b-c, for the structure of **11**, see Figure S2). In addition, when **6** was treated with the pathway-specific halogenase AmbO5,[6] no enzymatic conversion was observed (Figure S18). These results thus conclusively demonstrate **6** is irrelevant to the biogenesis of ambiguines (Figure 7b).

In summary, intrigued by the biosynthetic origin of **4** and the conundrum presented by the recent discovery of its C-12 epimer (**3**) synthase AmbU4, we systematically reconstituted all AmbU-type isomerocyclases encoded in the ambiguine pathway. This endeavor led to the discovery of two calcium-dependent enzymatic cascades for the selective formation of **4** by 10xAmbU1-U4 and **6** by AmbU2-U3 or AmbU2 (Figure 6b). The mechanism for their formations from **2** can be formulated as proceeding through a Cope rearrangement via a boat transition state, followed by a stereodivergent aza-Prins cyclization and a regioselective carbocation deposition to the C4 indole (Figure S19). Notably, in the presence of Ca^{2+}, the 10xAmbU1 *completely altered* the initial Cope rearrangement selectivity of AmbU4, which otherwise is a dedicated synthase for **3** in vitro (Figure 6b, right panel). AmbU2, on the other hand, is a dedicated synthase for **6** in the presence of Ca^{2+}, and its activity can be further enhanced by AmbU3, an unselective isomerocyclase that generates four isomeric hapalindoles (**4**, **6**, **8**, **9**).

The unprecedented calcium-dependency of the 10xAmbU1-U4, AmbU2-U3 duos and standalone AmbU2 led us to re-evaluate and re-define the isomerocyclase proteins encoded in the hapalindole-type biosynthetic pathways as a new type of calcium-dependent enzymes. To the best of our knowledge, these U-type isomerocyclases represent the only known calcium-dependent isomerase or cyclase involved in the biogenesis of microbial secondary metabolites. While the elucidation of the mechanism underlying how calcium modulates the activities and selectivities of these isomerocyclases and their complexes remain underway, the studies presented here effectively bridged the missing links and refuted the intermediacy of **3** in the early stage biosynthesis of ambiguines (Figure 6b). The identification of the 10xAmbU1-U4 enzymatic complex as the dedicated hapalindole U synthase also allowed to establish **4** as the *bona fide* biosynthetic intermediate for the assembly of hapalindole A, the founding member of hapalindole-type alkaloids,[18] based on the co-occurrence of AmbU1-AmbU4 coding genes in its biosynthetic gene cluster sequenced from *Fischerella muscicola* UTEX LB1829 (Figure S20).[19,20] This corroborates with our recent observation that an enzyme-mediated tertiary carbon epimerization at the C-10 center of **4** and **5** is operant for the stereodivergent biogenesis of the hapalindole-type alkaloids in the same organism. [21]

In addition, the disclosure on the in vitro enzymatic assembly of **4**, an on-pathway biogenetic intermediate to ambiguines and related hapalindole alkaloids, that requires a 10-to-1 mixture of AmbU1 and AmbU4 in the presence of calcium contrasts a

recent claim, emerged during the preparation of this manuscript,[22] that states the structural diversifications from **2** in the biosynthesis of hapalindole-type alkaloids is controlled by the homo- or hetero-dimeric complex of the U-type isomerocyclases. We reasoned this over-interpretation is likely due to the failure to appreciate that **6** is a pathway outlier in the biogenesis of ambiguines and related hapalindole alkaloids. As the in vitro enzymatic reconstitution remains the only viable method to dissect the biochemical basis for the biosynthesis of hapalindole-type alkaloids, we caution for the pitfalls in the interpretation of these in vitro data without thoroughly correlating them with the observed structural diversity in the known hapalindole-type alkaloid producer.

Acknowledgements

We thank University of Pittsburgh and National Institute of Health for supporting this work.

Keywords: alkaloid • biosynthesis • calcium • enzyme • cascade

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[22] S. Li, A. N. Lowell, S. A. Newmister, F. Yu, R. M. Williams, D. H. Sherman, *Nat Chem Biol* **2017**, doi:10.1038/nchembio.2327. The authors of this paper also reported that both AmbU3 and AmbU2 had no observable activity but were active when combined and gave the exclusive formation of **6** at pH=7.8 in the *absence* of Ca^{2+}.

10.1002/anie.201703932

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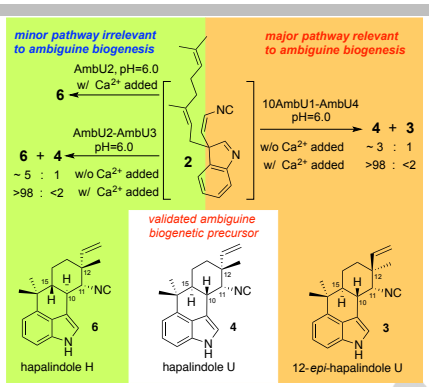
COMMUNICATION

Calcium is the key! The pursuit of the enzymatic basis for hapalindole U (**4**), a validated biogenetic precursor for the ambiguine alkaloids, led to the discovery of an unprecedented calcium-dependent isomerocyclase complex for its selective assembly. This discovery allowed to establish the isomerocyclases involved in the hapalindole-type alkaloid biogenesis as a new family of calcium-dependent enzymes.



*Qin Zhu and Xinyu Liu**

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Discovery of a calcium-dependent enzymatic cascade for the selective assembly of hapalindole-type alkaloids: On the biosynthetic origin of hapalindole U