nature portfolio

Peer Review File

Total Synthesis and Target Identification of Marine Cyclopiane Diterpenes

Corresponding Author: Professor hongdong hao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Hao and coworkers reported the asymmetric total synthesis of conidiogenones C, K and 12β-hydroxy conidiogenone C, and identification of Immunity-related GTPase family M protein 1 (IRGM1) as a cellular target. The synthesis started from Wieland-Miescher ketone and then transformed to an known compound 11, utilizing the Pauson-Kand reaction to establish a 6-5-5 ring system, a strategy reminiscent of the work conducted by Zhai (Angew. Chem., 2020, 59, 16475). The author further proceeded to construct the D-ring through a pivotal gold-catalyzed Nazarov cyclization reaction, extending the synthetic repertoire to 16 synthetic examples (line 144, table 2). It is imperative to note that this reaction is widely acknowledged in the literature as the Rautenstrauch rearrangement, as evidenced by the body of work from Toste (JACS, 2005, 127, 5802; Angew. Chem. Int. Ed, 2008, 47, 10110; OL, 2016, 18, 5058; JACS, 2005, 127, 5802). The synthesis applications of this rearrangement in natural products have undergone meticulous scrutiny (Angew. Chem., 2019, 131, 2512), encompassing comprehensive mechanistic and stereochemical inquiries (A. Iera, JACS, 2006, 128, 2434; Soriano, JOC, 2007, 72, 1443; JACS, 2007, 129, 9868). Consequently, the expansion of instances of this reaction within the context of the present study appears redundant and may potentially impart a misleading impression.

The methodology for introducing a methyl group at the C4 position in subsequent compounds aligns with established approaches by Tu (Angew. Chem., 2016, 55, 4456) and Han (Chem, 2023, 9, 1270). On a comprehensive scale, in comparison to the five previously published synthetic routes, the synthetic pathway herein predominantly derives inspiration from existing methodologies, offering a relatively modest contribution to novel chemistry.

Additionally, the investigation into the anti-inflammatory activity of the compound class, along with the identification of potential targets, reveals a notable weakness in activity. Although specific IC50 values are not explicitly provided, an approximate value of around 10uM is inferred from the data (line 166, figure 4 A). The inquiry arises as to whether further exploration of their target mechanisms is warranted, given the observed mediocre activity. The contextualization of these findings is imperative, considering the existence of numerous natural product molecules with analogous anti-inflammatory effects (online database: InflamNat: http://www.inflamnat.com; ref: J. Chem. Inf. Model. 2019, 59, 66). In summary, the overall chemical innovativeness of this study is deemed relatively modest, and the activity data for the natural products do not demonstrate exceptional efficacy. In light of these considerations, the referee has reached the consensus that this manuscript is not urgent for publication in Nat. Commun.

Reviewer #2

(Remarks to the Author)
Conidiogenones attracted the attention of the synthetic community as a new trophy synthetic target derived from a deep ocean sediment fungus and difficult to produce on scale by fermentation. The one notable biological activity reported was for conidiogenone C, with a cytotoxicity of 38 nM in a leukemina cell line (HL60), but no activity in other cell line. This may indeed be the product of a selective engagement of a target that uniquely drive HL60 replication. In addition, moderate antibiotic activity (8 microG/mL) was reported for a very closely related analogue, conidiogenone B. This is not impressive compared to the state-of-the-art and likely unactionable given the cytotoxicity of conidiogenone B or its immune modulation. The novelty of the target and biological activity motivated several synthetic chemistry groups to pursue total synthesis which have been published (Tu, Snyder, Zhai, Lee; refs 23-27). The synthesis of reported in the present manuscript is elegant and

efficient. It makes use of Wieland-Miescher ketone to establish the key chirality of the quaternary center and use a Nazarov cyclization to close the fourth ring. All in all, the sequence is a pleasure to read but does not stand out far above previous syntheses. However, the authors go beyond previous work and investigate the mode of action of conidiogenone C, the most interesting member of the family. Disappointingly, the authors were enable to reproduce the reported cytotoxic activity reported on HL60 (ref 5). However, they observed an anti-inflammatory activity in RAW264.7 cells and use chemical proteomics to identify the putative target as IRGM1 from a list of 59 candidates. Since no photo crosslinker was used, this approach only works if the probe forms a covalent adduct with the target. The presence of the enone is likely key to this biological activity, as it is in many other terpenoids. Surprisingly, this is not mentioned in the manuscript, nor is there any mention or citation to previous studies aiming the exploit the covalent nature of enone in terpenoids. Ideally, the responsible nucleophile on IRGM1 would be identified.
In summary this is an impressive piece of work that contributes an alternative synthetic strategy to conidiogenones and more importantly, sheds light on its biological activity. It is regrettable that the most promising activity from conidiogenones (nM cytotoxicity again HL60) could not be replicated. The authors give very little prospective value to IRGM1-inhibition. What is the importance of an IRGM1-selective inhibitor? Given that IRGM1 is highly implicated in T-cell replication, could this explain the HL60 activity reported? It is not uncommon for immortalized cell lines to drift genetically. Are the cell lines tested in this report and in ref 5 identical? I would strongly encourage the authors to test conidiogenone C against a panel of cells that express IRGM1 (most leukemia cell lines). This part of the work is not fully matured.
Technical points:
The diastereoselectivity of C16 (46 -> 47) needs to be explained.
Gels in Fig 4 should have molecular weight markers shown.
The gel in Fig 4I is difficult to interpret. Why is the input protein impure? Why is there such a change in MW with the drug?
A western should be performed on the gels shown in Fig 4D to demonstrate that the most prominent band is indeed IRGM1.

Reviewer #3

(Remarks to the Author)
This manuscript from Hou, Li, Zhou, and co-workers describes the chemical synthesis of several members of the conidiogenone family of natural products as well as an initial biochemical identification identifying the molecular target of conidiogenone C which is responsible for its anti-inflammatory content. Collectively, these targets have been of significant synthetic interest from many groups throughout the world, with several syntheses accomplished to date via routes which are well-described and depicted within the opening paragraphs and figures of the manuscript. The present approach, while similar in length to several of these approaches, is entirely distinct, leveraging the lone all-carbon quaternary center within the Wieland-Miescher ketone to then forge all the remaining chiral centers (and three additional all-carbon quaternary centers) with relative stereocontrol. Of particular value is how the route contemplates the synthesis of the lowest ring of the targets (as drawn), one that allows far more facile access to differentially oxidized materials than previous approaches.
Overall, this referee is in strong support of publication of this manuscript in Nature: Communications. In truth, the substance of this paper, including its biological findings, would absolutely rate in the very top tier for this referee for the high standards within this journal (with arguably enough significant content to justify 1.5 papers if not 2). This referee has no substantive comments for improvement for these authors, and definitely believes the paper should not be truncated in any way as it is a complete and compelling story for a hot collection of targets in the natural product synthesis space. This work should be well received by the synthesis community at large.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The revised manuscript has addressed all the points that had been raised in the first evaluation. My only suggestion would be to include DOI10.1038/ncomms12470 as an additional reference or instead of ref 77 as the work more closely parallels the work flow of the present publication.
Given the that all the points have been addressed, I reiterate my enthusiasm for this impressive piece of work that contributes an alternative synthetic strategy to conidiogenones and more importantly, sheds important light on its biological activity and covalent interactome. This advance are worthy of publication in Nat. Commun. and will interest a broad readership and the interface of synthetic chemistry and chemical biology.

Point-by-point response letter (NCOMMS-23-57966)

Below are provided a point-by-point reply to all the comments raised by three expert reviewers. Note that all revisions and changes have been highlighted in yellow in the revised versions of manuscript.

Reviewer comments:

Reviewer #1:

Hao and coworkers reported the asymmetric total synthesis of conidiogenones C, K and 12β-hydroxy conidiogenone C, and identification of Immunity-related GTPase family M protein 1 (IRGM1) as a cellular target. The synthesis started from Wieland-Miescher ketone and then transformed to an known compound 11, utilizing the Pauson-Kand reaction to establish a 6-5-5 ring system, a strategy reminiscent of the work conducted by Zhai (Angew. Chem., 2020, 59, 16475). The author further proceeded to construct the D-ring through a pivotal gold-catalyzed Nazarov cyclization reaction, extending the synthetic repertoire to 16 synthetic examples (line 144, table 2). It is imperative to note that this reaction is widely acknowledged in the literature as the Rautenstrauch rearrangement, as evidenced by the body of work from Toste (JACS, 2005, 127, 5802; Angew. Chem. Int. Ed, 2008, 47, 10110; OL, 2016, 18, 5058; JACS, 2005, 127, 5802). The synthesis applications of this rearrangement in natural products have undergone meticulous scrutiny (Angew. Chem., 2019, 131, 2512), encompassing comprehensive mechanistic and stereochemical inquiries (A. Iera, JACS, 2006, 128, 2434; Soriano, JOC, 2007, 72, 1443; JACS, 2007, 129, 9868). Consequently, the expansion of instances of this reaction within the context of the present study appears redundant and may potentially impart a misleading impression.

Response:

We are very grateful to you for the time and effort in reviewing this manuscript. We would like to thank you for the comments on the manuscript.

*In terms of our strategy of constructing the cyclopentenone (D ring), we respectfully disagree with the reviewer for his comments as "**the Rautenstrauch rearrangement**". In our synthesis, the pivotal step is "the gold-catalyzed 3,3-sigmatropic rearrangement and **Nazarov reaction**" which was original reported by Zhang and co-workers (ref 32 and 49), this efficient transformation utilizing enynyl esters as the corresponding precursor, and the cyclopentanone product would be obtained along with 1-2 contiguous stereocenters installed during the process.*

In terms of "the Rautenstrauch rearrangement" mentioned by the reviewer, in sharp contrast, the structures of corresponding precursor are totally different. As is witnessed in gold-catalyzed

*Nazarov cyclization by utilizing **enynyl acetates**, the gold-catalyzed Rautenstrauch rearrangement requires **1-ethynyl-2-propenyl acetates**, in line with the original report by Valentin Rautenstrauch (JOC 1984, 49, 950-952).*

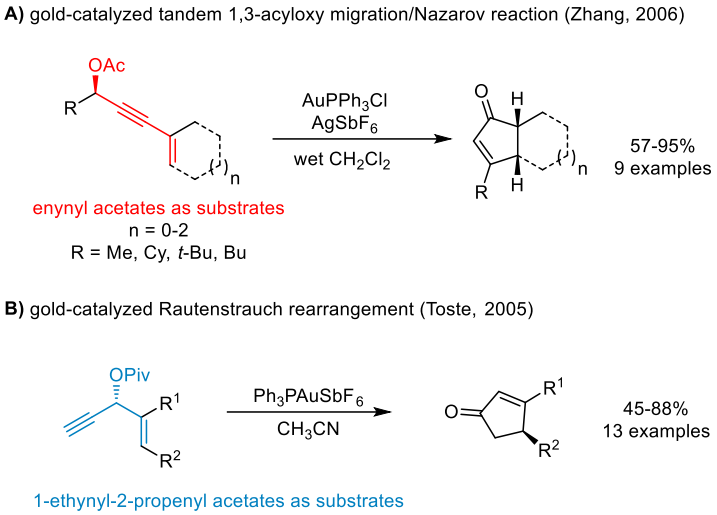

Fig. 1 gold-catalyzed rearrangements

The reviewer also provides several related references for methodology development, synthetic application and mechanistic study: "(JACS, 2005, 127, 5802; Angew. Chem. Int. Ed, 2008, 47, 10110; OL, 2016, 18, 5058; JACS, 2005, 127, 5802). The synthesis applications of this rearrangement in natural products have undergone meticulous scrutiny (Angew. Chem., 2019, 131, 2512), encompassing comprehensive mechanistic and stereochemical inquiries (A. Iera, JACS, 2006, 128, 2434; Soriano, JOC, 2007, 72, 1443; JACS, 2007, 129, 9868)"

The paper (JACS, 2005, 127, 5802) is the first paper on gold-catalyzed Rautenstrauch rearrangement reported by Toste, with improvement later by Lautens (OL, 2016, 18, 5058). And The paper "Angew. Chem. Int. Ed, 2008, 47, 10110" is the related "homo Rautenstrauch rearrangement", with mechanistic studies (A. Iera, JACS, 2006, 128, 2434; Soriano, JOC, 2007, 72, 1443; JACS, 2007, 129, 9868).

For the paper (Angew. Chem., 2019, 131, 2512), which we have been cited as ref 35, described the total synthesis of Merochlorin A by Carreira and co-workers through gold-catalyzed Nazarov cyclization.

To conclude, based on the abovementioned information, it's very inappropriate and inattentive to described our strategy as "the Rautenstrauch rearrangement", as gold-catalyzed Nazarov cyclization and Rautenstrauch rearrangement are actually two different types of reactions en route to the formation of cyclopentenone.

"Consequently, the expansion of instances of this reaction within the context of the present study appears redundant and may potentially impart a misleading impression."

Prior to our efforts, only one synthetic application of the gold-catalyzed Nazarov cyclization reported by Carreira (ref 35). During our total synthesis investigation, Trost reported their total synthesis of Kadcoccinic Acid A Trimethyl Ester (ref 36) and found the stereochemistry of the enynyl acetate dictates regioisomeric cyclopentenone formation, as seen in Fig. 2:

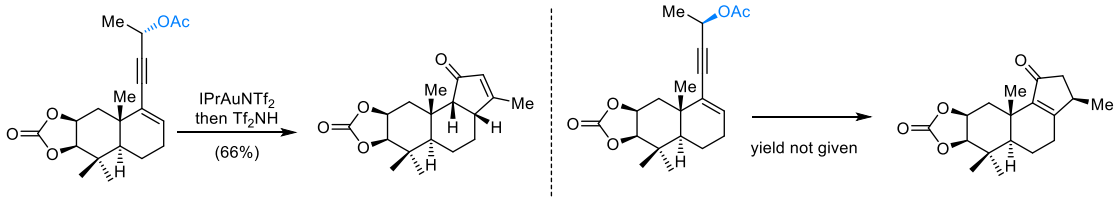

Fig. 2 regioisomeric cyclopentenone formation by Trost

*One thing we want to clarify, that according to Trost's seminal report (ref 36), the two diastereomers of enynyl acetate led to different cyclopentenone products. However, in our case, after subjecting our substrates **13** and **23** into optimal reaction conditions reported by Zhang and Liu (ref 49), to our delight, we observed an unusual chirality transfer which overcome the innate substrate control, and the stereochemistry of the newly-formed two contiguous stereocenters could be switched by simply using two diastereoisomers of enynyl acetate precursors (Table 1). By further exploration of a small series of complex substrates shown in Table 2 of the manuscript, we thereby concluded that a chirality-transfer gold-catalyzed Nazarov cyclization could indeed occur in multiple complex settings.*

Overall, we respectfully disagreed with the reviewer, and to the best of our knowledge, the scope described herein stands as the first demonstration of this reaction in various complex settings and thereby would undoubtedly serve as an inspiration to the synthetic community worldwide.

The methodology for introducing a methyl group at the C4 position in subsequent compounds aligns with established approaches by Tu (Angew. Chem., 2016, 55, 4456) and Han (Chem, 2023, 9, 1270). On a comprehensive scale, in comparison to the five previously published synthetic routes, the synthetic pathway herein predominantly derives inspiration from existing methodologies, offering a relatively modest contribution to novel chemistry.

*We are very grateful to the reviewer for the insightful comments on the manuscript. Previously, there are **four** reported total synthesis papers. However, in our manuscript, we not only reported the total synthesis of three natural products with highly oxygenated functionalities of the congested cyclopentane D ring through stereocontrolled Nazarov cyclization but also revealed immunity-related GTPase family M protein 1 (IRGM1) as a key cellular target of conidiogenone C responsible for its anti-inflammatory activity. This piece of work will be of great interest to the general readership of Nature Communications.*

Additionally, the investigation into the anti-inflammatory activity of the compound class, along with the identification of potential targets, reveals a notable weakness in activity. Although specific IC50 values are not explicitly provided, an approximate value of around 10uM is inferred from the data (line 166, figure 4 A). The inquiry arises as to whether further exploration of their target mechanisms is warranted, given the observed mediocre activity. The contextualization of these findings is imperative, considering the existence of numerous natural product molecules with analogous anti-inflammatory effects (online database: InflamNat: http://www.inflamnat.com; ref: J. Chem. Inf. Model. 2019, 59, 66). In summary, the overall chemical innovativeness of this study is deemed relatively modest, and the activity data for the natural products do not demonstrate exceptional efficacy. In light of these considerations, the referee has reached the consensus that this manuscript is not urgent for publication in Nat. Commun.

The reviewer is partially right in terms of the moderate anti-inflammatory activity of the Cyclopiane class. However, IC_{50} value is one thing, but not the whole thing. One of the main goals for the identification of the cellular targets of bioactive natural products is to provide an irreplaceable approach for the identification the small-molecule modulator of the biologically significant protein of interest. As witnessed in a recent seminal example reported by Adibekian, Dai and co-workers (JACS 2021, 143, 4379), together they identified the previously undruggable oncoprotein BRAT1 as the cellular target of moderate cytotoxic Curcusone diterpenes (IC_{50} reached to 20 μM). And prior to our studies, immunity-related GTPase family M protein 1 (IRGM1) has been identified as a master suppressor of type I interferonopathy (ref 70-73), however, the potential therapeutical value of IRGM1 for the treatment of a series of autoimmune diseases (IBD, Psoriasis, SLE, etc.) has been hampered by the absence of small-molecule modulators. In our case, we discovered conidiogenone C as the very first small-molecule activator of IRGM1, which shed light on the further investigation of IRGM1 biology and pharmacology.

Reviewer #2:

Conidiogenones attracted the attention of the synthetic community as a new trophy synthetic target derived from a deep ocean sediment fungus and difficult to produce on scale by fermentation. The one notable biological activity reported was for conidiogenone C, with a cytotoxicity of 38 nM in a leukemina cell line (HL60), but no activity in other cell line. This may indeed be the product of a selective engagement of a target that uniquely drive HL60 replication. In addition, moderate antibiotic activity (8 microG/mL) was reported for a very closely related analogue, conidiogenone B. This is not impressive compared to the state-of-the-art and likely unactionable given the cytotoxicity of conidiogenone B or its immune modulation. The novelty of the target and biological activity motivated several synthetic chemistry groups to pursue total synthesis which have been published (Tu, Snyder, Zhai, Lee; refs 23-27). The synthesis of reported in the present manuscript is elegant and efficient. It makes use of Wieland-Miescher ketone to establish the key chirality of the quaternary center and use a Nazarov cyclization to close the fourth ring. All in all, the sequence is a pleasure to read but does not stand out far above previous syntheses. However, the authors go beyond previous work and investigate the mode of action of conidiogenone C, the most interesting member of the family. Disappointingly, the authors were enable to reproduce the reported cytotoxic activity reported on HL60 (ref 5). However, they observed an anti-inflammatory activity in RAW264.7 cells and use chemical proteomics to identify the putative target as IRGM1 from a list of 59 candidates. Since no photo crosslinker was used, this approach only works if the probe forms a covalent adduct with the target. The presence of the enone is likely key to this biological activity, as it is in many other terpenoids. Surprisingly, this is not mentioned in the manuscript, nor is there any mention or citation to previous studies aiming the exploit the covalent nature of enone in terpenoids.

The reviewer is correct. To comment more on the covalent modification of conidiogenone C on IRGM1, an additional new paragraph was added in our revised manuscript as following:

Covalent IRGM1 Modification. *In possession of an electrophilic enone moiety which very often serves as reactive warhead that can covalently react with nucleophilic amino acid residues (i.e. cysteine) on target proteins, the potential covalent IRGM1 modifications of conidiogenone C (4) has been further investigated. To decipher the specific covalent binding sites of IRGM1, conidiogenone C (4) was incubated with purified recombinant IRGM1 protein for 1 h at 29 ℃. Then the conidiogenone C-IRGM1 complex was digested with trypsin and the conidiogenone C-labeled IRGM1 fragment was further detected by LC-MS/MS analysis. Much to our delight, one specific peptide 368FLPCVCCCLR377 was identified which contains three conidiogenone C-modified residues C373, C374, C375 (Figures 4J and S6). Later on, the three identified binding sites (C373, C374, C375) of IRGM1 were mutated and then subjected to the pull-down experiments. Whereas site mutagenesis of single cysteine residue (C373A, C374A, C375A) to Ala partially weakened the binding ability of conidiogenone C with IRGM1, the whole mutagenesis of all 3 cysteine residues*

to Ala (3CA) drastically interrupted the interaction (Fig. 4K-L). Therefore, cysteine residues (C373A, C374A, C375A) were identified as nucleophilic amino acid hotspots responsible for the covalent modification of conidiogenone C (4) on IRGM1.

Furthermore, ref 74-77 were also added as the citation to previous studies aiming the exploit the covalent nature of enone in terpenoids in our revised manuscript as following:

74. Jackson, P. A.; Widen, J. C.; Harki, D. A.; Brummond, K. M. Covalent Modifiers: A Chemical Perspective on the Reactivity of α,β-Unsaturated Carbonyls with Thiols via Hetero-Michael Addition Reactions. J. Med. Chem. 60, 839-885 (2017).

75. Berdan, C. A.; Ho, R.; Lehtola, H. S.; To, M.; Hu, X; Huffman, T. R.; Petri, Y.; Altobelli, C. R.; Demeulenaere, S. G.; Olzmann, J. A.; Maimone, T. J.; Nomura, D. K. Parthenolide Covalently Targets and Inhibits Focal Adhesion Kinase in Breast Cancer Cells. Cell Chem Biol. 26,1027-1035 (2019).

76. Davis, D. C.; Hoch, D. G.; Wu, L.; Abegg, D.; Martin, B. S.; Zhang, Z.-Y.; Adibekian, A.; Dai, M. Total Synthesis, Biological Evaluation, and Target Identification of Rare Abies Sesquiterpenoids. J. Am. Chem. Soc. 140, 17465-17473 (2018).

77. Cui, C.; Dwyer, B. G.; Liu, C.; Abegg, D.; Cai, Z.-J.; Hoch, D. G.; Yin, X.; Qiu, N.; Liu, J.-Q.; Adibekian, A.; Dai, M. Total Synthesis and Target Identification of the Curcusone Diterpenes. J. Am. Chem. Soc. 143, 4379-4386 (2021).

We are very grateful to the reviewer for the insightful comments on this part.

Ideally, the responsible nucleophile on IRGM1 would be identified.

The reviewer is correct. To comment more on the covalent modification of conidiogenone C on IRGM1, an additional new paragraph was added in our revised manuscript as following:

Covalent IRGM1 Modification. *In possession of an electrophilic enone moiety which very often serves as reactive warhead that can covalently react with nucleophilic amino acid residues (i.e. cysteine) on target proteins, the potential covalent IRGM1 modifications of conidiogenone C (4) has been further investigated. To decipher the specific covalent binding sites of IRGM1, conidiogenone C (4) was incubated with purified recombinant IRGM1 protein for 1 h at 29 ℃. Then the conidiogenone C-IRGM1 complex was digested with trypsin and the conidiogenone C-labeled IRGM1 fragment was further detected by LC-MS/MS analysis. Much to our delight, one specific peptide 368FLPCVCCCLR377 was identified which contains three conidiogenone C-modified residues C373, C374, C375 (Figures 4J and S6). Later on, the three identified binding sites (C373, C374, C375) of IRGM1 were mutated and then subjected to the pull-down experiments. Whereas site mutagenesis of single cysteine residue (C373A, C374A, C375A) to Ala partially weakened the binding ability of conidiogenone C with IRGM1, the whole mutagenesis of all 3 cysteine residues to Ala (3CA) drastically interrupted the interaction (Fig. 4K-L). Therefore, cysteine residues (C373A,*

C374A, C375A) were identified as nucleophilic amino acid hotspots responsible for the covalent modification of conidiogenone C (4) on IRGM1.

We are very grateful to the reviewer for the insightful comments on this part.

In summary this is an impressive piece of work that contributes an alternative synthetic strategy to conidiogenones and more importantly, sheds light on its biological activity. It is regrettable that the most promising activity from conidiogenones (nM cytotoxicity again HL60) could not be replicated. The authors give very little prospective value to IRGM1-inhibition. What is the importance of an IRGM1-selective inhibitor?

Prior to our studies, IRGM1 has been identified as a master suppressor of type I interferonopathy (ref 70-73), on the other word, IRGM1 is a guardian of mitochondrial DAMPs–mediated autoinflammation. However, the potential therapeutical value of IRGM1 for the treatment of a series of autoimmune diseases (IBD, Psoriasis, SLE, etc.) has been hampered by the absence of small-molecule modulators. Through our studies, conidiogenone C has been identified as the very first small-molecule activator of IRGM1, and we expect the identification of conidiogenone C as the very first small-molecule activator of IRGM1 could potentially shed light on the further investigation of IRGM1 biology and pharmacology for the treatment of a series of autoimmune diseases (IBD, Psoriasis, SLE, etc.).

To further clarify the significance of IRGM1 activator, we also revised the final part of our manuscript as:

Our present total synthesis has opened the gate for SAR studies and further preparation of conidiogenone C (4) analogues. Meanwhile, since IRGM1 is a guardian of mitochondrial DAMPs–mediated autoinflammation, the identification of conidiogenone C as the very first small-molecule activator of IRGM1 could potentially shed light on the further investigation of IRGM1 biology and potential therapeutical application for the treatment of a series of autoimmune diseases (IBD, Psoriasis, SLE, etc.).

We are very grateful to the reviewer for the insightful comments on this part.

Given that IRGM1 is highly implicated in T-cell replication, could this explain the HL60 activity reported? It is not uncommon for immortalized cell lines to drift genetically.

As shown in Fig. S3, we evaluated a series of both human and mouse leukemia cell lines where IRGM1 are expressed (Raji, HL-60, Jurkat, K562, A20, etc.), and no inhibition of IFN-stimulated genes (ISGs) (Ifnb, Mx1) expression by conidiogenone C (4) was observed.

For the time being, it is unclear and hard to draw any conclusion that HL60 activity is relevant to IRGM1 or not. BUT it is clear that the three Cyclopiane diterpenes tested herein showed no anti-tumor activities against HL60 cells at 40 μM concentration.

We are very grateful to the reviewer for the insightful comments on this part.

Are the cell lines tested in this report and in ref 5 identical?

Yes, we performed initial antitumor activities evaluation experiments following exactly ref 5, and none of these three cyclopiane diterpenes probed to be cytotoxic at 40 μM concentration.

I would strongly encourage the authors to test conidiogenone C against a panel of cells that express IRGM1 (most leukemia cell lines). This part of the work is not fully matured.

To investigate the correlation between IRGM1 expression within different cell lines and anti-inflammatory activity of the cyclopiane class. We conducted a series of additional experiments.

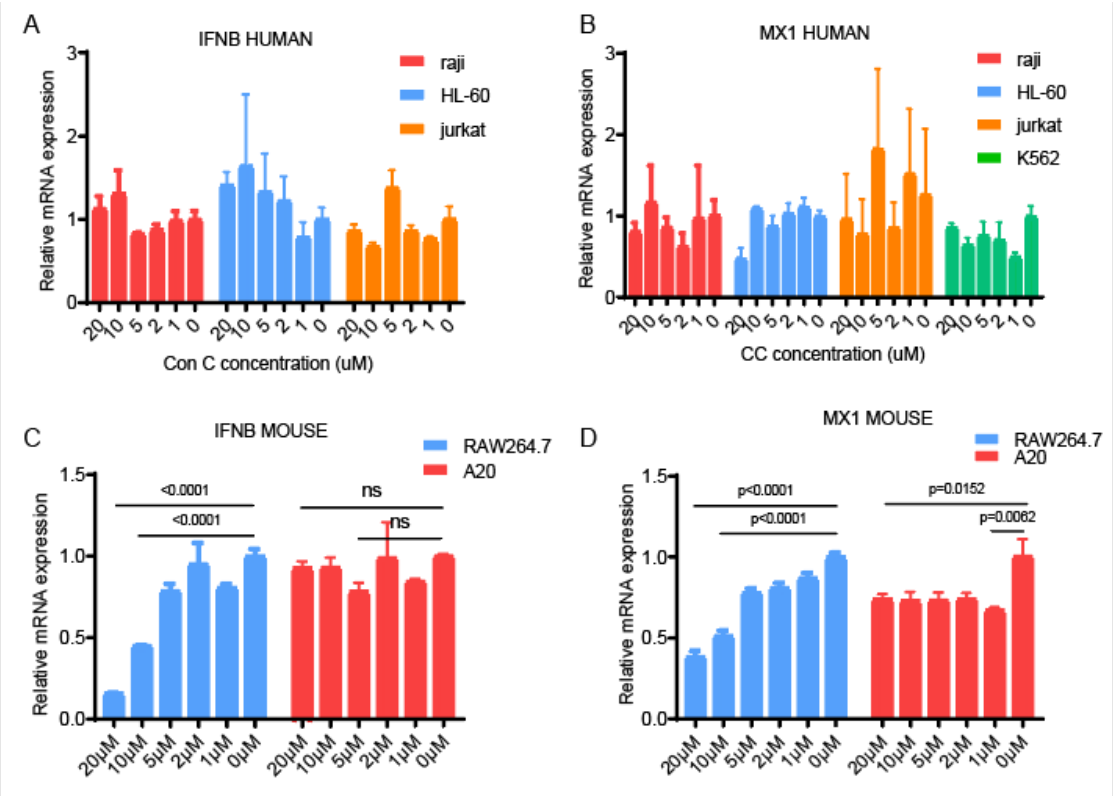

Fig. S3 IFN-stimulated genes (ISGs) (Ifnb, Mx1) expression in in different cell lines

As shown in Fig. S3A and S3B, a series of human cancer (mostly leukemia) cell lines where IRGM1 are all expressed (Raji, HL-60, Jurkat, K562, etc.) has been evaluated, and no inhibition of IFN-stimulated genes (ISGs) (Ifnb, Mx1) expression by conidiogenone C (4) was observed. We

further compared with mouse macrophages RAW26 and mouse leukemia cell line A20 (Fig. S3C and S3D). Whereas a concentration-dependent inhibition of IFN-stimulated genes (ISGs) (Ifnb, Mx1) expression by conidiogenone C (4) was observed in the former case, again no inhibition was observed in the latter. Taken together, we believe the anti-inflammatory activity of the Cyclopiane class was very specific and selective in inflammatory macrophages.

Accordingly, the last sentence in the ***Biological Evaluation and Probe Synthesis*** *section has also been revised as following:*

Of note, a series of both human and mouse cancer (mostly leukemia) cell lines (Raji, HL-60, Jurkat, K562, A20, etc.) has also been evaluated, and no inhibition of IFN-stimulated genes (ISGs) (Ifnb, Mx1) expression by conidiogenone C (4) was observed (Fig. S3). Taken together, since the anti-inflammatory activity was very specific and selective in inflammatory macrophages, thereby it would be worthwhile to further decipher the cellular mechanism of action (MOA) of immunosuppressant conidiogenone C (4), given that IFN-I plays a central role in the pathogenesis of autoimmune diseases.

We also added Figure. S3 in our revised Supporting Information.

We are very grateful to the reviewer for the insightful comments on this part.

Technical points:

The diastereoselectivity of C16 (46 -> 47) needs to be explained.

Thanks for pointing this out. The alkylation is both kinetic and thermodynamically favored, and only the β face was accessible as the α face was blocked by the B ring. Also there are several relevant alkylation examples reported in the literatures (Figure 3).

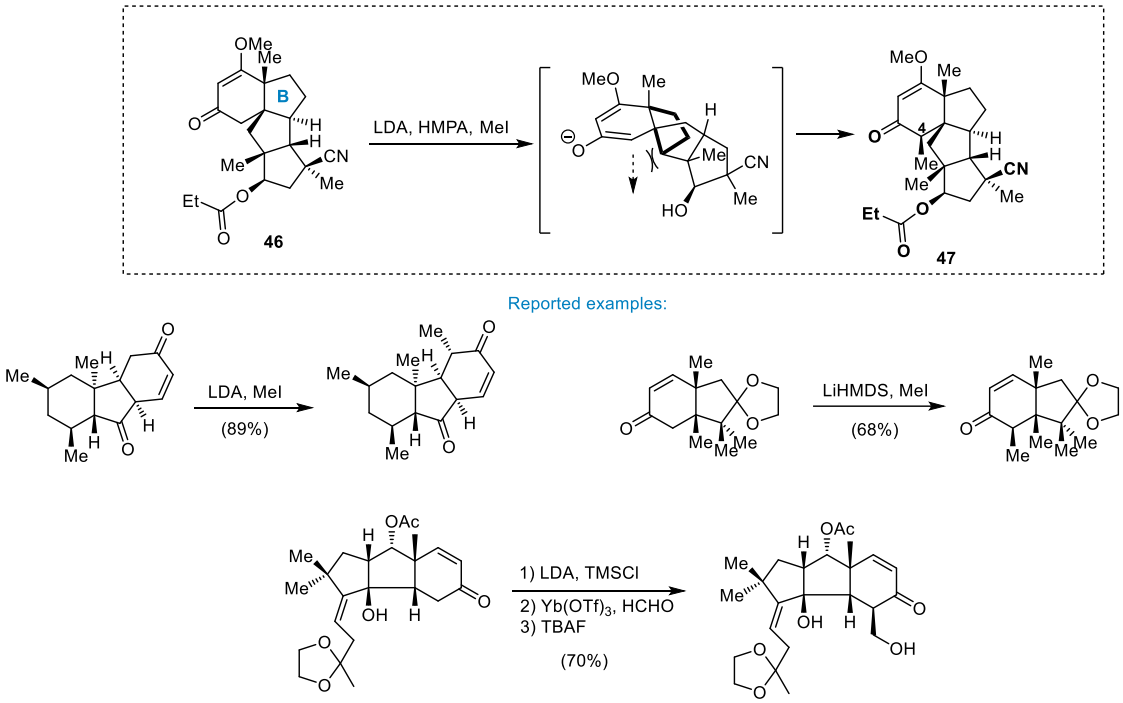

Fig. 3 Stereoselective alkylation

Accordingly, in the main text of our revised manuscript, we added additional rationale as following:

From the enone intermediate, after deprotonation by LDA, the α face of in-situ formed enolate was blocked by the adjacent B ring, thereby the C4 methyl group was correctly installed through this stereoselective alkylation.

Meanwhile, we also added two relevant references (ref 58, 59) as following:

58 Tanaka, R.; Ohishi, K.; Takanashi, N.; Nagano, T.; Suizu, H.; Suzuki, T.; Kobayashi, S. Synthetic Study of Pyrrocidines: First Entry to the Decahydrofluorene Core of Pyrrocidines. Org. Lett. 14, 4886-4889 (2012).

59 Srikrishna, A.; Mahesh, K. Enantiospecific First Total Synthesis of ent-Allothapsenol. Synlett 23, 1021-1024 (2012).

We sincerely appreciate the reviewer for pointing out the diastereoselectivity issue of the alkylation, which dramatically improved our manuscript.

Gels in Fig 4 should have molecular weight markers shown.

All the molecular weight markers have been added into the Gels throughout the revised manuscript and the revised Supporting Information.

We are very grateful to the reviewer for the insightful comments on this part.

The gel in Fig 4I is difficult to interpret. Why is the input protein impure? Why is there such a change in MW with the drug?

For the moment, The IRGM1 antibody could only be purchased from CST. As shown in Figure 4A, from CST website, we can also see the impurity above IRGM1 band. Thus, the impurity above in the input protein is mostly likely be the trace impurity derived from the CST IRGM1 antibody.

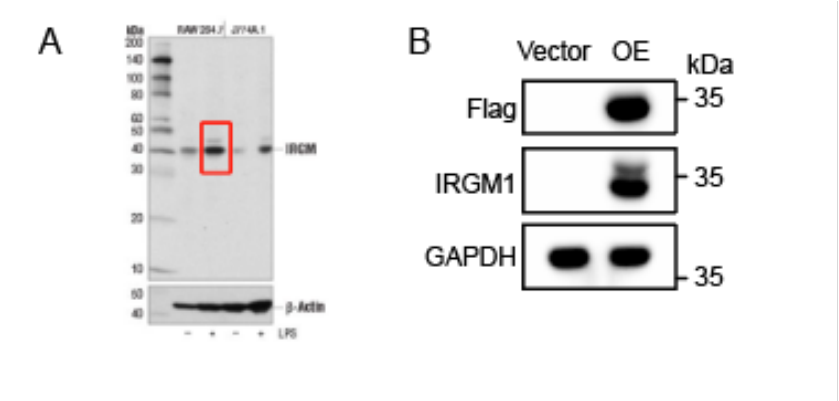

Fig. 4 IRGM1 protein. (A) picture from CST website. (B) WB using Flag antibody

To further clarify the abovementioned hypothesis, we performed WB in parallel using both Flag antibody and IRGM antibody. Indeed, as seen in Fg. 4B, we can again see the impurity above when using IRGM antibody, but a clear band when switching to Flag antibody. Therefore, the impurity above in the input protein should be the trace impurity derived from the CST IRGM1 antibody.

In terms of a slight change in MW with the drug shown in Fig 4I, we fully agree with the reviewer that the MW change after pull down with the Conidiogenone C probe should not be dramatic, we suspect this might be due to technical reason-because of our western blot experiment.

We are very grateful to the reviewer for the insightful comments on this part.

A western should be performed on the gels shown in Fig 4D to demonstrate that the most prominent band is indeed IRGM1.

The reviewer is correct in terms of the identification of the most prominent band to be IRGM1. In our case, as shown in Fig. S5, we performed in-gel fluorescence profiling using conidiogenone C probe, followed by western blot together to confirm the outstanding band in the gel is IRGM1.

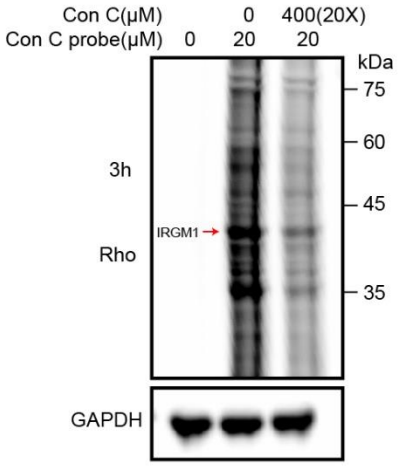

Fig. S5 The IRGM1 in the gel.

We added Fig. S5 in our revised Supporting Information. We are very grateful to the reviewer for the insightful comments on this part.

Reviewer #3:

This manuscript from Hou, Li, Zhou, and co-workers describes the chemical synthesis of several members of the conidiogenone family of natural products as well as an initial biochemical identification identifying the molecular target of conidiogenone C which is responsible for its anti-inflammatory content. Collectively, these targets have been of significant synthetic interest from many groups throughout the world, with several syntheses accomplished to date via routes which are well-described and depicted within the opening paragraphs and figures of the manuscript. The present approach, while similar in length to several of these approaches, is entirely distinct, leveraging the lone all-carbon quaternary center within the Wieland-Miescher ketone to then forge all the remaining chiral centers (and three additional all-carbon quaternary centers) with relative stereocontrol. Of particular value is how the route contemplates the synthesis of the lowest ring of the targets (as drawn), one that allows far more facile access to differentially oxidized materials than previous approaches. Overall, this referee is in strong support of publication of this manuscript in Nature: Communications. In truth, the substance of this paper, including its biological findings, would absolutely rate in the very top tier for this referee for the high standards within this journal (with arguably enough significant content to justify 1.5 papers if not 2). This referee has no substantive comments for improvement for these authors, and definitely believes the paper should not be truncated in any way as it is a complete and compelling story for a hot collection of targets in the natural product synthesis space. This work should be well received by the synthesis community at large.

Response: We sincerely acknowledge the referee for this encouraging comment.

Collectively, we believe that we have addressed all the key issues from the comments of three expert reviewers and we sincerely thank both the editor and the reviewers for greatly improving the quality of this work through additional experiments, analysis and revisions.

Point-by-point response letter (NCOMMS-23-57966A)

Below are provided a point-by-point reply to all the comments raised by three expert reviewers. Note that all revisions and changes have been highlighted in yellow in the revised versions of manuscript.

Reviewer comments:

Reviewer #2:

The revised manuscript has addressed all the points that had been raised in the first evaluation. My only suggestion would be to include DOI10.1038/ncomms12470 as an additional reference or instead of ref 77 as the work more closely parallels the work flow of the present publication.

Given the that all the points have been addressed, I reiterate my enthusiasm for this impressive piece of work that contributes an alternative synthetic strategy to conidiogenones and more importantly, sheds important light on its biological activity and covalent interactome. This advance is worthy of publication in Nat. Commun. and will interest a broad readership and the interface of synthetic chemistry and chemical biology.

Response:

We sincerely acknowledge the referee for this encouraging comment. The corresponding reference has been cited as ref.75 in the revised Manuscript.

Collectively, we sincerely thank both the editor and the reviewers for greatly improving the quality of this work through additional experiments, analysis and revisions.