Organic LETTERS

Letter

pubs.acs.org/OrgLett

Curindolizine, an Anti-Inflammatory Agent Assembled via Michael Addition of Pyrrole Alkaloids Inside Fungal Cells

Wen Bo Han,[†] Ai Hua Zhang,[†,‡] Xin Zhao Deng,[†] Xinxiang Lei,[§] and Ren Xiang Tan*[,†]

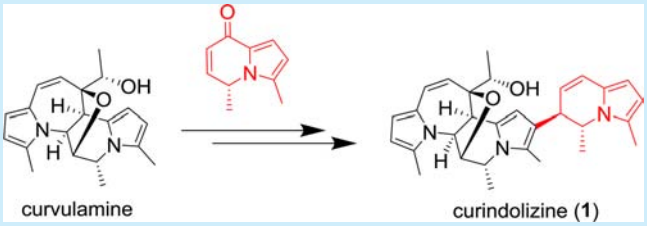
[†]Institute of Functional Biomolecules, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China

[‡]State Key Laboratory of Elemento-organic Chemistry, Nankai University, Tianjin 300071, China

[§]College of Chemistry & Materials Engineering, Wenzhou University, Wenzhou 325035, China

Supporting Information

ABSTRACT: *Curvularia* sp. IFB-Z10, a white croaker-associated fungus, generates a skeletally unprecedented indolizine alkaloid named curindolizine (**1**), which displays an anti-inflammatory action in lipopolyssacharide (LPS)-induced RAW 264.7 macrophages with an IC_{50} value of 5.31 ± 0.21 μM. The enzymatic transformation test demonstrated that the unique curindolizine architecture was most likely produced by the regiospecific in-cell Michael addition reaction between pyrrole alkaloids, curvulamine, and 3,5-dimethylindolizin-8(5*H*)-one.



Inflammation is a crucial pathological process that can be triggered by multiple stimuli such as pathogens, damaged cells, and irritants.[1] During inflammation, macrophages are activated to produce a series of inflammatory mediators including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and nitric oxide (NO).[2] These inflammatory mediators are beneficial to the host defense against challenges, and their actions are restricted to a specific location where necessary. However, the overproduction of these inflammatory mediators definitely leads to chronic inflammation outcomes including rheumatoid arthritis, inflammatory bowel disease, asthma, diabetes, and cancer.[3−5] Therefore, inhibition of these mediators has been documented to be a useful therapeutic strategy, and the anti-inflammatory agents are therefore of particular importance in managing inflammation-derived diseases. Nevertheless, the currently available anti-inflammatory drugs have been shown to possess inevitably severe adverse effects such as atrial fibrillation, gastrointestinal erosions, and renal and hepatic insufficiency.[6,7] Thus, there is an urgent need for new agents useful for the anti-inflammatory therapy. As a diverse microbial community intimately associated with hosts (e.g., plants, insects, and mammals), symbionts are evolved to be a reliable source of biologically potent metabolites, of which some are promisingly anti-inflammatory.[8,9] Accordingly, we have extended our attention to the uniquely structured anti-inflammatory compounds produced by this community of microbes during our characterization of new bioactive secondary metabolites from symbiont cultures.[10,11]

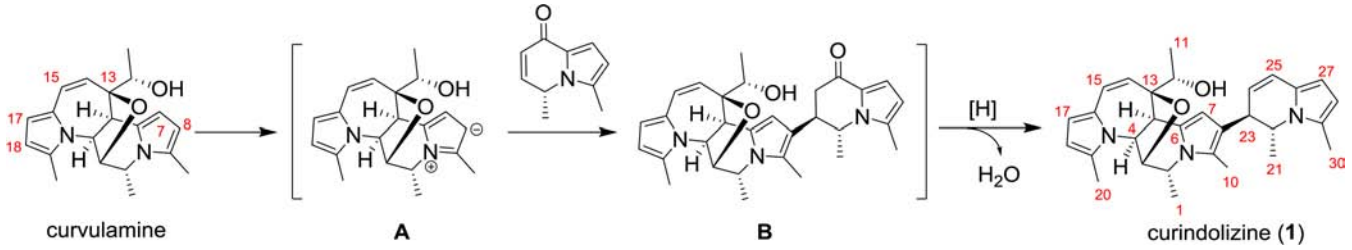
Curvulamine has been discovered as a skeletally undescribed antimicrobial alkaloid from the broth of *Curvularia* sp. IFB-Z10, a white croaker-associated fungus, after cultured in shake flask in the Czapek's medium.[11] To obtain more material of the alkaloid for further investigation, we scaled up the fungal cultivation in a 300 L fermentor in the same medium, but the targeted curvulamine appeared in an unexpectedly low abundance. To identify the "new fate" of curvulamine, the extract derived from the fungal culture was separated by the column chromatography and HPLC purification to give a skeletally unprecedented alkaloid dubbed as curindolizine (**1**). The structure of **1** was demonstrated to derive from curvulamine upon its compounding with 3,5-dimethylindolizin-8(5*H*)-one via an in-cell Michael addition reaction in a regiospecific manner (Scheme 1). Rather than antibacterial, curindolizine (**1**) displayed an anti-inflammatory action in lipopolyssacharide (LPS)-induced RAW 264.7 macrophages by inhibiting the NO production and the expression of TNF-α, IL-1β, and IL-6. Presented below are the structure determination, enzymatic transformation, and bioactivity evaluation conducted for the novel alkaloid.

Curindolizine (**1**), afforded as white prisms, had a molecular formula of $C_{30}H_{35}N_3O_2$ evidenced from the Na^+-liganded molecular ion at m/z 492.2608 ($C_{30}H_{35}N_3O_2Na$ requires 492.2614) in its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). The ^1H NMR spectrum of **1** revealed a total of five pyrrolic protons including four equally splitting doublets (J = 3.2 Hz) at δ_H 6.03, 5.88, 5.96, and 5.83, which were ascribable for two 2,5-disubstituted pyrrole moieties.[11] Another singlet at δ_H 5.25 was broadened by its homoallylic coupling with the methyl singlet at δ_H 2.26. This suggested the presence of a 4-

Received: February 26, 2016
Published: April 5, 2016

DOI: 10.1021/acs.orglett.6b00549
Org. Lett. 2016, 18, 1816−1819

Scheme 1. Plausible Biosynthetic Pathway of 1: Constructed from Curvulamine and 3,5-Dimethylindolizin-8(5*H*)-one through Michael Addition Reaction



substituted 2-methylpyrrole motif in the molecule. A pair of doublets at δ_H 5.60 and 6.35 arose likely from a *cis*-disubstituted vinyl group with the coupling constant magnitude (J = 11.6 Hz) suggesting its inclusion in a seven-membered ring as discerned with curvulamine.[11] This observation, along with other ^{1}H NMR signals, highlighted that it was most probably an adduct of curvulamine with 3,5-dimethylindolizin-8(5*H*)-one. The proposal was substantiated by its ^{1}H−^{1}H COSY, HMQC, and HMBC spectra, which allowed the unambiguous assignment of all ^{1}H and ^{13}C NMR data of **1** (Table S1). In particular, the connection of curvulamine with 3,5-dimethylindolizin-8(5*H*)-one was defined by the HMBC correlations of H-7 (δ_H 5.25) with C-23 (δ_C 38.2) and of H-24 (δ_H 5.69) with C-8 (δ_C 127.4). The relative configuration of all chiral centers but C-12 and C-23 was determined by the NOESY spectrum of **1** (Figure 1). To

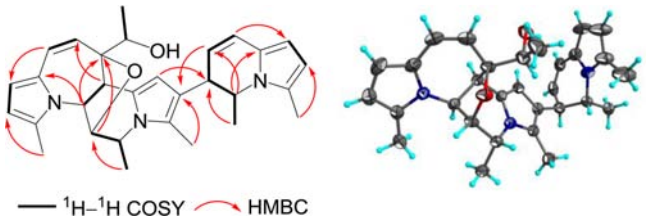


Figure 1. Key HMBC (arrow) and ^{1}H−^{1}H COSY correlations (bolded) of **1** (left) with its structure confirmed by its X-ray crystallographic diffraction (right).

clarify its absolute stereochemistry, curindolizine (**1**) was recrystallized in the dichloromethane/methanol (2:1) mixture to yield prisms. The low-temperature X-ray diffraction (CuKα) of the single crystal revealed that **1** had a (2*R*,3*R*,4*S*,5*R*,12*S*,13*R*,22*R*,23*R*)-configuration (Figure 1).

With the structure in hand, curindolizine (**1**) was tested for its antibacterial action, but it was inactive against the bacterial pathogens used earlier as targets.[11] Subsequently, compound **1** was re-evaluated for anti-inflammatory activity using the Griess method.[12] To our expectation, alkaloid **1** was demonstrated to inhibit the NO production, and the gene expression of TNF-α, IL-6, and IL-1β in LPS-induced RAW 264.7 macrophages (Figure 2).

The half maximal inhibitory concentration (IC_{50}) of **1** was assessed to be 5.31 ± 0.21 μM, which was comparable to that (2.17 ± 0.15 μM) of dexamethasone, a marketed anti-inflammatory drug coassayed as a positive control in the study. Furthermore, the anti-inflammatory activity of **1** was shown to be fairly selective since it exhibited negligible cytotoxicity against RAW264.7 cells (Table 1). This reinforced its significance in the discovery of new anti-inflammatory agents.

The potency and selectivity of **1** in its anti-inflammatory action motivated us to extend our curiosity to the construction of such an unusual curindolizine framework in the fungus. The structural feature of **1** facilitated our postulation that it might have been derived from the covalent coupling between curvulamine[11] and 3,5-dimethylindolizin-8(5*H*)-one (Scheme 1).

As illustrated in Scheme 1, curvulamine might exist in its tautomeric form **A**, which would be able to react with 3,5-dimethylindolizin-8(5*H*)-one to give the intermediate **B** through Michael addition reaction. This intermediate was then transformed into curindolizine (**1**) after the reduction and dehydration. To address the presumption, an LC−MS guided isolation of the singly nitrogenated alkaloid was carried out to identify 3,5-dimethylindolizin-8(5*H*)-one or its precursor from the fungal culture. Fortunately, we obtained a new mononitrogenated compound, named procuramine (**2**), which could be a precursor of 3,5-dimethylindolizin-8(5*H*)-one. Procuramine (**2**) was figured out to have a molecular formula $C_{10}H_{13}NO_2$ according to its Na^+-liganded molecular ion at m/z 202.0842 in its HR−ESI−MS ($C_{10}H_{13}NO_2Na$ requires 202.0839). The structure of **2** was assigned exactly by its 1D (^{1}H and ^{13}C) and 2D NMR spectra (^{1}H−^{1}H COSY, ROESY, HSQC, and HMBC, Table S2). The (2*R*,3*S*)-configuration of **2** was indicated by its low-temperature X-ray diffraction (CuKα) (Figure S1).

Characterization of **2** facilitated our clarification of whether the proposed Michael reaction occurred in or outside fungal cells. Specifically, curvulamine and **2** were coexposed separately to the fungal intra- and extracellular proteins. As expected, curindolizine (**1**) was detected upon the fungal intra- rather than extracellular protein exposed simultaneously to **2** and curvulamine (Figures 3 and S2). This experimentation rationalized the in-cell Michael addition reaction of curvulamine with **2** to form **1** (Figure 3).

As highlighted in Scheme 1, curvulamine could be covalently bonded at all the four pyrrole methine carbons with 3,5-dimethylindolizin-8(5*H*)-one. However, our LC−MS screening of the extract derived from the fungal culture failed to detect any of the other regioisomers of **1**, suggesting that the in-cell Michael addition reaction might be regiospecific. Molecular modeling of curvulamine provided the likely reasons as follows. The left pyrrole moiety of curvulamine is stabilized by its conjugation with the 14,15-double bond. This might have inactivated a possible Michael addition reaction at the pyrrole ring. This rationalized as well why no substitution appeared in the right pyrrole ring of **1**, which conjugates with 24,25-double bond. In the curvulamine molecule, C-8 is spatially more accessible than C-7 that receives the steric hindrance from the fully substituted (or bulky) neighboring quaternary carbon (C-13).

DOI: 10.1021/acs.orglett.6b00549
Org. Lett. 2016, 18, 1816−1819

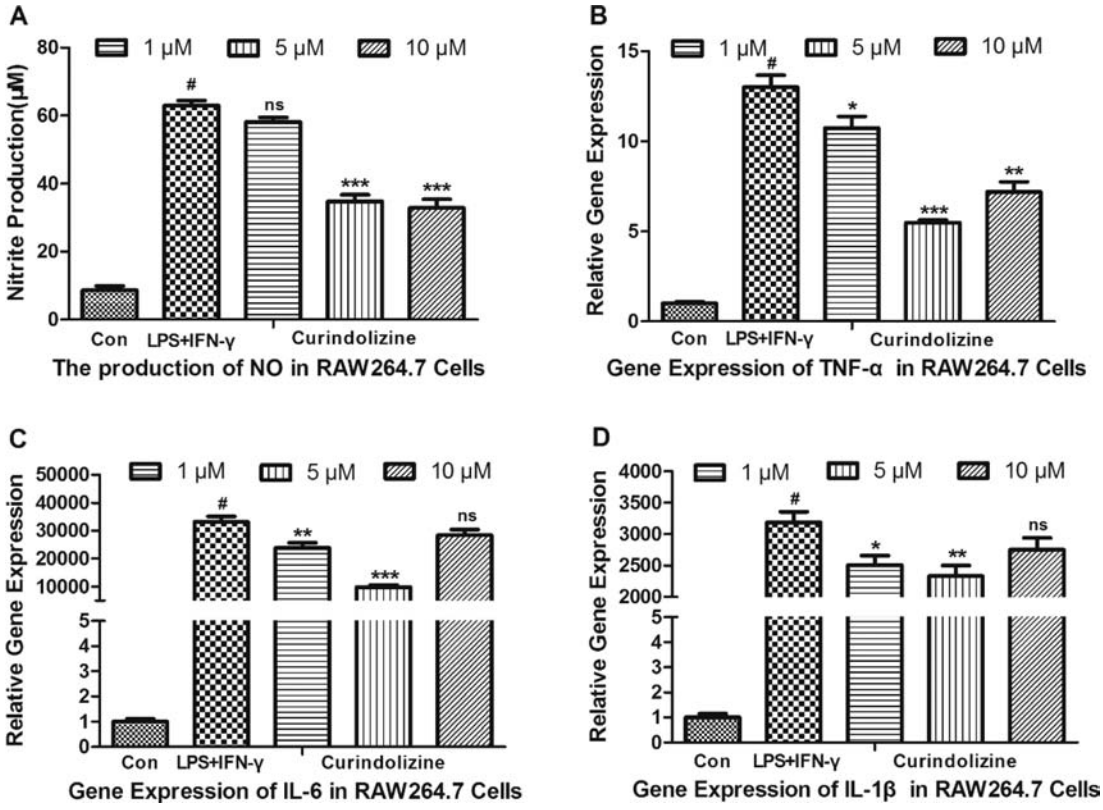


Figure 2. Inhibition of curindolizine (**1**) on the NO production and the gene expression of TNF-α, IL-6, and IL-1β in LPS and IFN-γ stimulated RAW 264.7 cells. Macrophage cells were normally cultured (Con group) or stimulated with LPS and IFN-γ (with or without curindolizine treatment, curindolizine group, and LPS + IFN-γ group). (A) The inhibition of **1** on the production of NO in cell culture supernatants from LPS and IFN-γ stimulated RAW 264.7 cells. (B) The inhibition of **1** on the gene expression of TNF-α in LPS and IFN-γ stimulated RAW264.7 cells. (C) The inhibition of **1** on the gene expression of IL-6 in LPS and IFN-γ stimulated RAW264.7 cells. (D) The inhibition of **1** on the gene expression of IL-1β in LPS and IFN-γ stimulated RAW264.7 cells. Data were derived from three independent experiments and summarized as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the LPS + IFN-γ group. $^{\#}P < 0.001$ for LPS + IFN-γ group compared to the vehicle control group. ns, $P > 0.05$ compared to the LPS + IFN-γ group.

Table 1. Anti-Inflammatory Activity of 1 (IC_{50} in μM)

compd	IC_{50} (μM)	survival rate (%)[a]
curindolizine (**1**)	5.31 ± 0.21[b]	97.60 ± 3.42
dexamethasone[c]	2.17 ± 0.15	96.33 ± 2.11

[a]Survival rate (%) of RAW264.7 at 10 μM expressed as the mean of three independent experiments. [b]Mean ± SEM. [c]Positive control.

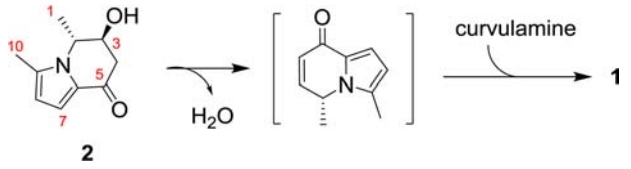


Figure 3. Generation of curindolizine (**1**) upon the fungal intracellular protein exposure to curvulamine and procuramine (**2**).

In conclusion, as an unexpected "byproduct" of reisolating curvulamine (an antibacterial alkaloid from the title fungus),[11] curindolizine (**1**) was discovered as an anti-inflammatory agent with an undescribed skeleton. The enzymatic transformation experiments have addressed that the curindolizine framework results from an in-cell Michael addition reaction of curvulamine with 3,5-dimethylindolizin-8(5*H*)-one derived from procuramine (**2**), a new mononitrogenated pyrrole alkaloid characterized in the work. Though beyond the scope of the present investigation, the enzymes involved in the fungal transformation include most likely aldolase, oxidoreductase, and dehydratase, which are all common in the fungal kingdom.[13−15] Furthermore, the in-cell generation of curindolizine (**1**) has consumed a remarkable amount of curvulamine to lower substantially the abundance of the alkaloid in the refermentation of the fungus. Collectively, the findings are of value in searching for new anti-inflammatory agents, and the unprecedented curindolizine (**1**) framework opens additionally a fresh topic for the chemists and pharmacologists to develop novel bioactive alkaloids that may be of significance in the drug discovery efforts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00549.

General methods and details of isolation of metabolite, and 1D and 2D NMR spectra (PDF)
Compound **1** (CIF)
Compound **2** (CIF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rxtan@nju.edu.cn. Fax: +86-25-8330 2728.

DOI: 10.1021/acs.orglett.6b00549
Org. Lett. 2016, 18, 1816−1819

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was cofinanced by the NSFC grants (81530089, 81421091, and 21132004).

■ REFERENCES

(1) Yeom, M.; Park, J.; Lim, C.; Sur, B.; Lee, B.; Han, J. J.; Choi, H. D.; Lee, H.; Hahm, D. H. *Nutr. Res. (N. Y., NY, U. S.)* **2015**, *35*, 241−250.
(2) Aderem, A.; Ulevitch, R. J. *Nature* **2000**, *406*, 782−787.
(3) Lawrence, T.; Willoughby, D. A.; Gilroy, D. W. *Nat. Rev. Immunol.* **2002**, *2*, 787−795.
(4) Dong, J.; Shao, W.; Yan, P.; Cai, X.; Fang, L.; Zhao, X.; Lin, W.; Cai, Y. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 198−202.
(5) Elinav, E.; Nowarski, R.; Thaiss, C. A.; Hu, B.; Jin, C.; Flavell, R. A. *Nat. Rev. Cancer* **2013**, *13*, 759−771.
(6) Hennekens, C. H.; Borzak, S.; Bjorkman, D. J. *Expert Rev. Cardiovasc. Ther.* **2014**, *12*, 291−293.
(7) Süleyman, H.; Demircan, B.; Karagöz, Y. *Pharmacol. Rep.* **2007**, *59*, 247−258.
(8) Xu, Y. M.; Espinosa-Artiles, P.; Liu, M. X.; Arnold, A. E.; Gunatilaka, A. A. *J. Nat. Prod.* **2013**, *76*, 2330−2336.
(9) Wei, W. C.; Sung, P. J.; Duh, C. Y.; Chen, B. W.; Sheu, J. H.; Yang, N. S. *Mar. Drugs* **2013**, *11*, 4083−4126.
(10) Zhang, X.; Wei, W.; Tan, R. X. *Sci. China: Chem.* **2015**, *58*, 1097−1109.
(11) Han, W. B.; Lu, Y. H.; Zhang, A. H.; Zhang, G. F.; Mei, Y. N.; Jiang, N.; Lei, X.; Song, Y. C.; Ng, S. W.; Tan, R. X. *Org. Lett.* **2014**, *16*, 5366−5369.
(12) Costa, J. F.; Barbosa-Filho, J. M.; Maia, G. L.; Guimarães, E. T.; Meira, C. S.; Ribeiro-dos-Santos, R.; de Carvalho, L. C.; Soares, M. B. *Int. Immunopharmacol.* **2014**, *23*, 469−474.
(13) Lin, L. P.; Yuan, P.; Jiang, N.; Mei, Y. N.; Zhang, W. J.; Wu, H. M.; Zhang, A. H.; Cao, J. M.; Xiong, Z. X.; Lu, Y.; Tan, R. X. *Org. Lett.* **2015**, *17*, 2610−2613.
(14) Brakhage, A. A. *Nat. Rev. Microbiol.* **2013**, *11*, 21−32.
(15) Yan, W.; Ge, H. M.; Wang, G.; Jiang, N.; Mei, Y. N.; Jiang, R.; Li, S. J.; Chen, C. J.; Jiao, R. H.; Xu, Q.; Ng, S. W.; Tan, R. X. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 18138−18143.

DOI: 10.1021/acs.orglett.6b00549
Org. Lett. 2016, 18, 1816−1819