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Article

Disproof of the Proposed Structures of Bradyoxetin, a Putative *Bradyrhizobium japonicum* Signaling Molecule, and HMCP, a Putative *Ralstonia solanacearum* Quorum-Sensing Molecule

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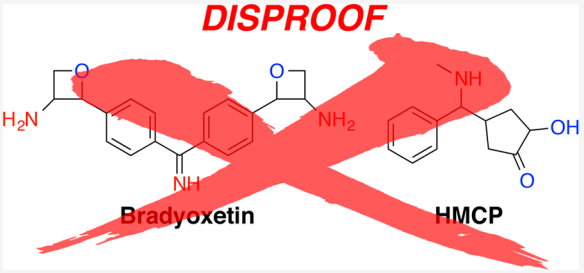
ABSTRACT: First, we revisited the reported NMR data of bradyoxetin, a putative cell density factor of *Bradyrhizobium japonicum*, and found some inconsistencies in the proposed structure. To elucidate the correct structure, we synthesized model oxetane compounds and confirmed that the NMR data of the synthetic compounds did not match those of the reported bradyoxetin. After reinterpreting the reported NMR data, we concluded that bradyoxetin must be chloramphenicol. Next, some derivatives of 2-hydroxy-4-((methylamino)(phenyl)methyl)-cyclopentanone (HMCP), which is a putative quorum-sensing molecule of *Ralstonia solanacearum*, were synthesized. The NMR spectra of the synthesized compounds were completely different from those of the reported natural products. Based on theoretical studies, including the estimation of ^{1}H and ^{13}C NMR chemical shifts using density functional theory calculations, we confirmed the correctness of the structure of the synthesized compound. These results strongly suggest that the proposed structure of HMCP could be incorrect.



Bacteria, fungi, and other microorganisms communicate with each other using small molecules that play an important role in cell-to-cell communication. Among the life regulatory systems, information transfer between individual microorganisms is one of the most important mechanisms for promoting the survival of a species and its interactions with other organisms. This built-up information network relies mainly on the chemical signals between individual cells of the same species. This information network is not limited to the same species, but may be extended to different species or even different kingdoms. One major class of bacterial signaling molecules is known as the autoinducers or quorum-sensing (QS) pheromones.[1] Notably, QS is a cell density-responsive transcriptional system that regulates a variety of life events, such as pathogenicity control, biofilm formation, pigment and antibiotic biosynthesis, and interbacterial competence acquisition. *N*-Acylhomoserine lactones (AHLs, Figure 1), which regulate a wide variety of bacterial activities, are known as one of the major classes of QS signaling molecules, and most Gram-negative bacteria produce AHL(s) to control an internal QS circuit.[2] As represented by AHLs, microbial signaling molecules are often relatively simple in structure. This may be because it is not evolutionarily advantageous to have a complex biosynthetic system for a signaling molecule. Although they are likely to be simple compounds, elucidating the structure of microbial signaling molecules is often difficult because their production levels are extremely low and several physical properties, such as NMR spectra, may not be adequately measured.[3] Consequently, research on the identification of microbial signaling molecules has not been sufficiently advanced, and it is likely that there are still undiscovered types of signaling molecules. However, structurally orphaned (rare and very unusual) compounds, which are interesting from the perspective of natural product chemistry, have been reported. Herein, we discuss the structural disproof of two structurally orphaned microbial signaling molecules, bradyoxetin and 2-hydroxy-4-((methylamino)(phenyl)methyl)-cyclopentanone (HMCP).

Bradyoxetin, a Putative Signaling Molecule of *Bradyrhizobium japonicum*. Rhizobia and legumes form nitrogen-fixing nodules in the roots and live together in a very specific interaction between the bacteria and the plant. The symbiosis involves several steps of exchanging chemical signals, such as flavonoids from the plant and a Nod factor from the bacteria with each other; the various genes are differentially expressed in different ways.[4] Differentiated rhizobia in the

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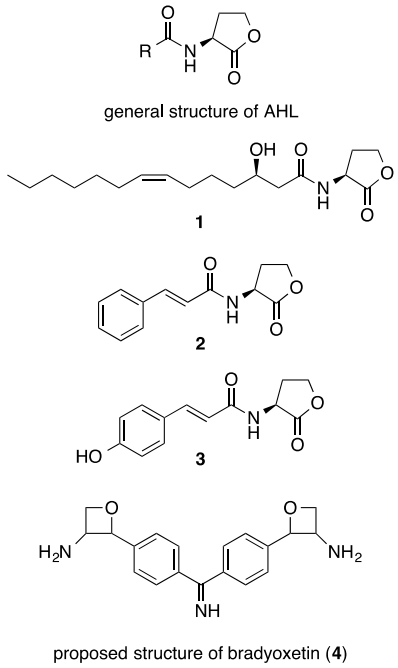


Figure 1. Structures of AHLs and the proposed structure of bradyoxetin.

nodules, referred to as bacteroids, reduce atmospheric nitrogen to ammonium ions that can be available to the plant, and these compounds are exchanged for energy sources from the plant.[5] The AHLs produced by rhizobia are considered the most important QS molecules involved in the symbiotic program of the rhizobia. Both QS mechanisms and molecules have been identified in many rhizobial species.[6] For example, *Rhizobium leguminosarum* produces AHL **1** (Figure 1) as a self-growth inhibition signaling molecule;[6a] furthermore, we have reported that the absolute configuration at the hydroxy-bearing C-3 plays an important role in the biological activity.[7]

The QS molecules discovered from rhizobia include some structurally unique AHLs, such as cinnamoyl-HSL (**2**)[6g] and *p*-coumaroyl-HSL (**3**).[6i] These unique aromatic classes of AHLs have not been identified in any other bacteria. In 2001, Loh et al. isolated a cell density factor (CDF), bradyoxetin (**4**), which acts as an extracellular signaling molecule and accumulates as a function of culture density from the rhizobia *Bradyrhizobium japonicum*.[8] It has been reported that bradyoxetin induces NolA, which leads to *nod* gene suppression. The bradyoxetin structure was proposed by extensive analyses of its NMR and MS spectra. The proposed structure is unique and contains two symmetrical oxetane rings and a free imine group. However, there is no structurally similar natural product with the features of bradyoxetin. Therefore, we comprehensively examined the description given in Loh's work,[8] and several questions arose. First, we thought that it was necessary to verify whether the *N*-nonsubstituted imine group, which was difficult to isolate because of its high reactivity and susceptibility to hydrolysis, could exist stably as a natural product. Next, some unusual points were found in the NMR data. For instance, in the ^{1}H NMR spectra of known oxetane compounds such as compounds **5** and **6**, the chemical shifts of the methylene protons α to the oxygen atom are reported to be approximately 4−5 ppm,[9] whereas the bradyoxetin signals are reported to be 3.61 and 3.82 ppm, which is similar to general ether derivatives (Figure 2). In addition, the geminal protons

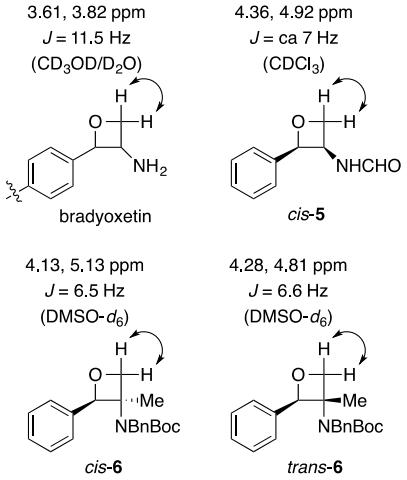
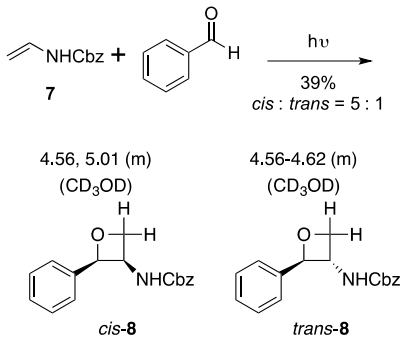


Figure 2. Partial ^{1}H NMR data of known phenyloxetane derivatives.

on the oxetane ring should show a coupling constant of approximately 7 Hz, which could be mistaken for vicinal coupling that would be caused by the strain of the four-membered ring, whereas the coupling constant of those of the bradyoxetin was reported to be 11.5 Hz. Because the NMR spectra of bradyoxetin were recorded in CD_3OD/D_2O, the possibility that these differences are caused by the use of different solvents cannot be excluded. Furthermore, no detailed NMR data of the *trans*-isomer of the phenyloxetane derivative possessing an amino group at C-3, e.g., *trans*-**5**, were available. To resolve these questions and verify the validity of the structural determination of bradyoxetin, we planned to synthesize analogues for NMR analysis.

Scheme 1 shows the synthesis of the phenyloxetane derivatives *cis*-**8** and *trans*-**8**. The known enamine **7** was

Scheme 1. Synthesis of Phenyloxetane (8)



prepared from acryloyl chloride[10] and was subjected to photocycloaddition with benzaldehyde according to Bach's condition[9] to afford *cis*-**8** and *trans*-**8** in a moderate yield. However, the deprotection of the carboxybenzyl (Cbz) group of **8** was unsuccessful, and we obtained undesired ring-opened compounds under various reaction conditions. Although there are several phenyloxetane compounds with nitrogen functionality at C-3 of its oxetane ring, no compound having a free amino group ($-NH_2$) has been reported. This fact might reflect the high reactivity of oxetane, which makes the removal of various substituents difficult. With the phenyloxetane derivatives in hand, 1D and 2D NMR analyses of *cis*-**8** and *trans*-**8** were conducted. Consequently, the NMR data for both *cis*- and *trans*-isomers were similar to those of previously

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J. Nat. Prod. 2021, 84, 495−502

reported compounds. Even considering that the synthetic products were protected by a Cbz group, the signals at the oxetane site of the two products were significantly different from those of the reported bradyoxetin, suggesting that the proposed structure might be incorrect.

Next, we revisited the reported NMR data of bradyoxetin and attempted to speculate on the correct structure. First, the possibility that the nitrogen and oxygen atoms are reversed, i.e., bradyoxetin is an azetidine derivative, was investigated. However, the reported NMR data of similar azetidine compounds are entirely different.[11] Focusing on the reported ^{13}C NMR spectrum of bradyoxetin, it is possible to find the following structural information: bradyoxetin has a *p*-disubstituted-phenyl group, a carbonyl-like carbon, and three sequential carbon atoms possessing oxygen or nitrogen substituents (**9**; Figure 3 and Table 1). Assuming that one

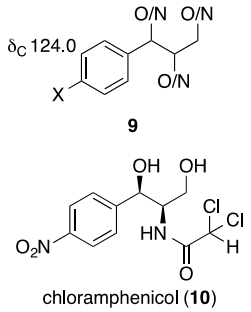
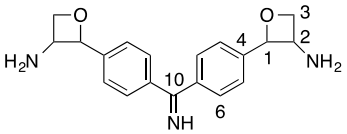
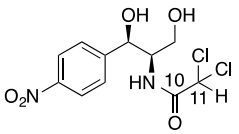


Figure 3. Partial structure **9** based on bradyoxetin NMR data and the structure of chloramphenicol (**10**).

Table 1. NMR Data of Chloramphenicol and Reported Data of Bradyoxetin



proposed bradyoxetin (**4**)



chloramphenicol (**10**)

	δ_C		δ_H, mult. (*J*, Hz)	
position[a]	chloramphenicol[b]	reported[c]	chloramphenicol[b]	reported[c]
1	71.5	71.3	5.17, m (2.8)	5.16 (2.9)
2	58.2	58.5	4.17, ddd, (2.8, 6.6, 7.2)	4.14 (2.9, 6.6, −)
3	61.9	62.2	3.64, dd (6.9, 11.4)	3.61 (7.2, 11.5)
			3.83, dd (6.5, 11.4)	3.82 (6.6, 11.5)
4	150.5	151.5		
5, 9	128.2	128.2	7.63, d (8.7)	7.65 (8.8)
6, 8	124.4	124.0	8.20, d (8.7)	8.18 (8.8)
7	148.2	148.5		
10	167.2	166.4		
11	67.1		6.21, s	

[a]According to reported bradyoxetin numbering. [b]Recorded at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) in CD_3OD/D_2O = 1:1. [c]Recorded at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in CD_3OD/D_2O = 1:1.

of the substituents in the benzene ring was the carbon chain, there must be some substituent attached to the other, which Loh et al. assigned to the carbon of the imine. However, we realized that the slightly shielded signal at 124.0 ppm could not be explained unless we assumed that there was a nitro group as X. Following this assumption, it is possible that the 166.4 ppm signal can be a carbonyl carbon and an oxygen or nitrogen in the side chain can be acylated. Although no proton nor carbon signal corresponding to the acyl chain was recorded in the ^{1}H and ^{13}C NMR spectra of bradyoxetin, some of its traces could be found in the reported HSQC and HMBC spectra. For instance, they observed a cross-peak between approximately 6.3 ppm of ^{1}H and 66 ppm of ^{13}C signals in the HSQC spectrum, even though these signals were not recorded in the 1D NMR spectra. This phenomenon leads us to hypothesize that the carbon atom of the acyl chain can be deuterated because it is well known that the reduction of the signal intensity by deuterium quadrupole relaxation or signal broadening due to residual C−D coupling is often observed in the ^{13}C NMR spectrum of a deuterated compound.[12] In the process of estimating the bradyoxetin structure that satisfies (1) a *p*-disubstituted-phenyl moiety, (2) a nitro group and a sequential oxygen/nitrogen trisubstituted propyl chain, and (3) an acyl group with deuterium-exchangeable proton(s), we realize that chloramphenicol (**10**), a common antibiotic, can be a candidate for that compound.

To confirm our hypothesis, we conducted an NMR study using commercial chloramphenicol and found that the ^{1}H and ^{13}C NMR spectra were in good agreement with those of the reported data for bradyoxetin (Table 1). The slight chemical shift discrepancies might be caused by the concentration of the sample or the pH of the solution. This phenomenon is often observed when MeOH, a highly polar solvent, is used as the solvent for NMR.[13] Although signals H-11 (6.21 ppm) and C-11 (67.1 ppm) were observed in the initial measurements, the signal intensities of these peaks were reduced after allowing the sample solution to stand for 2 weeks to promote the H−D exchange at C-11 (Figures S1 and S2). Focusing on the MS data, Loh et al. deduced the molecular formula of bradyoxetin ($C_{19}H_{21}N_3O_2$) based on the observed peak at 324.0 ($[M + H]^+$, theoretical value 324.2). However, the MS peaks assumed from the molecular formula of chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$) are 322.0, 324.0, and 326.0 ($[M]^+$), the major peaks, which are deduced from the isotopes of chlorine atoms. The estimated MS data for chloramphenicol are considered to be consistent with the reported data, although the MS spectrum, including high-resolution MS data for bradyoxetin, is not shown and therefore not comparable. It is unclear why the results of the reported elemental analysis of bradyoxetin do not agree with the calculated chloramphenicol values. From our NMR studies and MS data, we concluded that the compound reported as bradyoxetin must be chloramphenicol. It should be noted that Loh et al. applied chloramphenicol (50 μg/mL) to the culture medium of *B. japonicum*, presumably to inhibit the growth of other microorganisms.

HMCP, a Putative *Ralstonia solanacearum* Quorum-Sensing Molecule. *R. solanacearum* is a Gram-negative *β*-proteobacterium that causes a destructive disease called "bacterial wilt" in more than 200 plant species over a broad geographical range.[14] This pathogen attacks a wide variety of important crops, such as tomato, potato, and tobacco, as well as other weeds, shrubs, and trees by invading the xylem vessels of the plant roots. After the invasion, the bacterium diffuses into the stem, where it grows vigorously and rapidly produces extracellular polysaccharide (EPS), which blocks vascular tissues and prevents water flow, thus blighting the invaded

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plant without xanthochromia. The pathogen also produces plant cell-wall-damaging enzymes, such as endoglucanase and polygalacturonase, as well as ralfranones as secondary metabolites, both of which are required for full bacterial virulence.[15] The production of these virulence and pathogenicity molecules is controlled by an extensive network of interactive signal transduction pathways, wherein PhcA, a LysR-type transcriptional regulator, plays an important role. In this system, the PhcA activity is regulated by the PhcS/PhcR two-component system, and its key molecules have been proven to be 3-hydroxylated-fatty acid methyl esters acting as QS signaling molecules. In 1997, Denny et al. showed that methyl 3-hydroxypalmitate (3-OH PAME, **11**) was a *phc* QS signaling molecule that triggers the expression of an EPS biosynthetic gene in *R. solanacearum* AW1.[16] Although 3-OH PAME was the first QS signaling molecule of an endogenous fatty acid derivative, another fatty acid derivative, 3-hydroxymyristate (3-OH MAME, **12**), was found in other *R. solanacearum* strains, such as OE1-1, as the QS signaling molecule in 2015.[17]

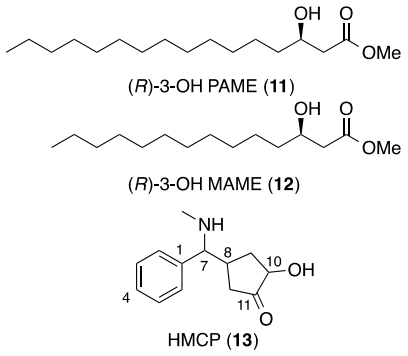

Figure 4. Structures of quorum-sensing molecules of *R. solanacearum* and proposed structure for HMCP.

R. solanacearum also employs some AHLs as QS signaling molecules.[18] Although only two classes, fatty acid derivatives and AHLs, had been identified as QS signaling molecules in *R. solanacearum*, Umesha et al. found a new class in 2016.[19] Spectroscopic analyses, such as ^{1}H NMR and MS, revealed the structure of the compound as HMCP (**13**). They claimed that the structure of the compound was confirmed by chemical synthesis. This is a surprising discovery in the history of QS science because the reported HMCP structure does not share any common structural features with that of the known QS molecules of any microorganisms. The reported HMCP structure has unique arrangements as a secondary amino group at the benzylic position and α-hydroxy ketone in its five-membered ring system. It is well known that some QS signaling molecules bearing an amino group derived from an amino acid (e.g., ComX pheromone[20]) or anthranilic acid (e.g., PQS[21]) or bearing five-membered ring systems, such as lactone (e.g., AHL) or thiazole (e.g., IQS[22]), exist. However, the reported HMCP structure is prominent from the perspective of its structure. Furthermore, although HMCP has three stereogenic centers, leading to eight possible stereoisomers, no information about its relative or absolute configuration has been reported. Owing to its high biological and chemical importance, we were interested in synthesizing HMCP.

Before starting our synthetic study of HMCP, we carefully checked the synthetic procedure reported by Umesha et al. Figure 5 summarizes the synthesis of HMCP as described by Umesha et al.[19] Benzylmethylamine (**14**) was treated with *n*-BuLi at low temperatures, and the obtained intermediate was then reacted with 2-hydroxy-4-methylcyclopentanone (**15**) to obtain the desired HMCP as a colorless solid. Although the synthetic procedure was not reported in detail, we noticed that there were some suspicious features with this method. First, the corresponding lithium amide should be formed first when benzylmethylamine is treated with *n*-BuLi. It would be very difficult to form a benzyl anion even if an excess amount of *n*-BuLi is employed. Second, assuming that the benzyl anion can be formed, it is impossible for the employed electrophile (**15**) to react with the anion at its methyl group. Furthermore, if the intended carbon−carbon bond formation occurs, the number of carbon atoms in the product would not match those of the proposed HMCP structure.

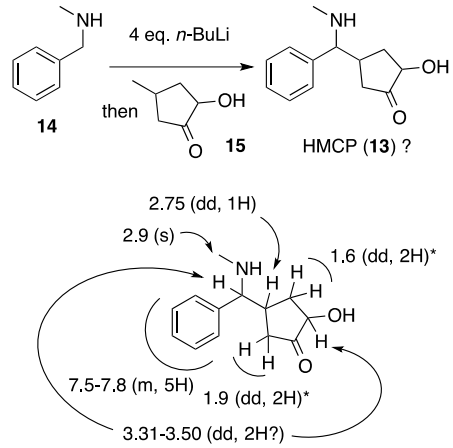

Figure 5. Synthetic procedure and ^{1}H NMR data of HMCP reported by Umesha. *It was not specified which of these two signals each corresponded to.

Next, we carefully checked the reported ^{1}H NMR data of HMCP. The reported assignments of each resonance are summarized in Figure 5. Again, we found many unusual descriptions in their claims. Therefore, we performed a simulation on the ^{1}H NMR spectrum of the reported HMCP structure using commercial software (Table S1). We found many differences between the reported and predicted assignments. Unfortunately, because the ^{13}C NMR spectrum of the natural product has not been reported, it is impossible to verify the data. Although the prediction of the chemical shifts using computational methods is not always accurate, the obtained shifts appeared to be highly reasonable. On the basis of these results, we speculated that the reported structure of the natural product might be incorrect. Thus, we decided to synthesize the sample with the proposed HMCP structure to conduct a direct comparison with their NMR spectra.

Scheme 2 summarizes the synthesis of the HMCP derivatives. First, the commercially available cyclopent-3-ene carboxylic acid was converted into ketone **17** via the known amide **16**.[23] The reductive amination of the resulting keto group of **17** with methylamine yielded the secondary amine **18**. Prior to the introduction of some oxygen functionalities into the cyclopentenyl moiety of **18**, it was necessary to protect the amino group. We first selected an acetyl group (**19**) because the Ac group could be tolerant toward various reaction conditions. In the NMR spectra of **19**, the rotational isomers

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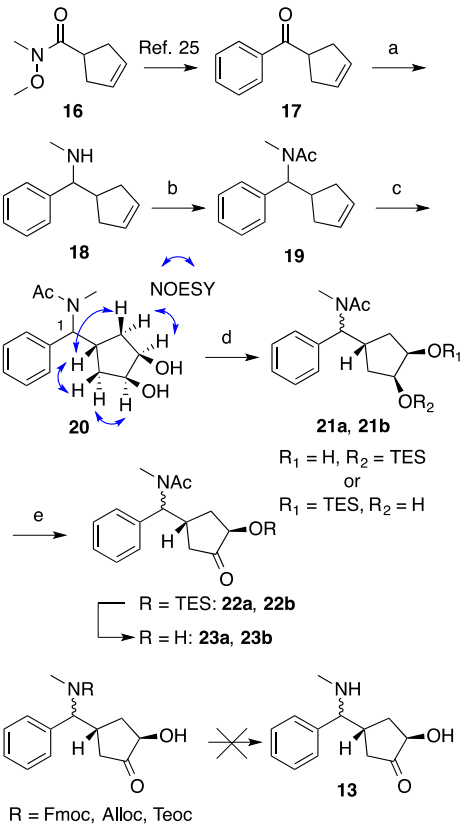
J. Nat. Prod. 2021, 84, 495−502

Scheme 2. Synthesis of *N*-Acetyl HMCP[a]



[a]Reagents and conditions: (a) $MeNH_2$, Ti(O*i*-Pr)$_4$, EtOH, $NaBH_4$, 0 °C (96%); (b) AcCl, Et_3N, THF, 0 °C (90%); (c) OsO_4, NMO, *t*-BuOH, acetone, H_2O (91%); (d) NaH, TESCl, THF, 0 °C (28% for **21a**, 39% for **21b**); (e) Dess-Martin periodinane, CH_2Cl_2, 0 °C (89% for **22a**, 89% for **22b**); (f) HF-pyridine, MeCN, 0 °C (40% for **23a**, 89% for **23b**).

(approximately 3:1 in DMSO-d_6) were observed, and the ratio of the isomers was changed to approximately 2:1 at 90 °C. The double bond of **19** was oxidized with OsO_4 to afford the corresponding diol **20**. The facial selectivity of this oxidation was estimated to be >97:3 by the ^{1}H NMR spectrum, and the isomers were inseparable. The relative configuration of the five-membered ring of the major isomer of **20** was deduced by a NOESY experiment. It should be noted that compound **20** is not a diastereomeric mixture relative to the cyclopentane ring. The monoprotection of the hydroxy group of **20** as triethylsilyl (TES) ether yielded **21a** and **21b**. Although the four stereoisomers could be produced in this step (see Figure S3), only two compounds were separated. There were two possibilities: (1) two of the four isomers were selectively produced, and (2) the separated **21a** and **21b** were both diastereomeric mixtures that were indistinguishable by NMR analysis. In any case, it was impossible to determine the relative configuration of each compound. Finally, the Dess−Martin oxidation of the remaining secondary alcohol of **21a** and **21b** was followed by the removal of the TES group with HF-pyridine to produce *N*-acetyl HMCP **23a** and **23b**, respectively. Even if the relative configurations of compounds **21a** and **21b** were unknown, this subsequent two-step conversion reaction narrows the number of possible structures of *N*-acetyl HMCP to two (Figure S3). The ^{1}H and ^{13}C NMR spectra provided good support for the structures of the synthesized **23a** and **23b**, although it was not possible to determine which stereoisomers they were. Because the acetyl groups of **23a** and **23b** could not be removed under standard conditions, we could not obtain HMCP. Next, we employed Fmoc, Alloc, and Teoc groups as the protecting groups of the amino group of **18** because the protecting groups could be removed under various reaction conditions. We synthesized the corresponding *N*-protected HMCPs using the same procedures as described above. Unfortunately, however, all attempts to remove the protecting groups resulted in the decomposition of the product under all examined reaction conditions. These results indicate that the proposed structure of HMCP (**13**) would be highly unstable toward basic, acidic, or even neutral transition metal-catalyzed reaction conditions. We assumed that the free amino group of HMCP may be condensed with the acyloin moiety via Maillard-like reactions to obtain a complex mixture.

Although we could not prepare the pure sample of HMCP, some *N*-protected HMCPs were obtained. Essentially, there was no difference among the NMR spectra, except for the signals originating from the substituted groups on the nitrogen atom. This finding indicates that it is possible to compare the NMR spectra of *N*-protected HMCPs with those of the reported HMCP directly. To simplify the discussion, the simplest compounds, *N*-acetyl HMCP (**23a** and **23b**), were selected. The observed NMR spectra were completely different from those of the reported HMCP data and were in good agreement with the expected values (Tables S2 and S3), except for those of an additional acetyl group. However, it was still possible that the differences might be attributed to the difference in the configuration of the hydroxy-bearing C-10 because we prepared two out of four stereoisomers. To exclude this possibility, we conducted a more reliable estimation of the NMR spectra of the stereoisomers of *N*-acetyl HMCP by means of density functional theory (DFT) calculations.[24] The observed NMR data of our synthesized **23a** and **23b** and theoretical NMR data of four stereoisomers are shown in Tables S2 and S3. The observed data are in good agreement with the estimated data, supporting the correctness of the structure of our synthetic compounds. Because there were significant but very small differences between the estimated data of the two stereoisomers, we concluded that the disagreement between the ^{1}H NMR spectrum of our sample and that of the reported HMCP should not be caused by the difference in their configuration. There are two significant differences between the reported data of HMCP and our synthetic compounds: (1) HMCP appears to have multiple substituents on the benzene ring, whereas our compounds give typical monosubstituted-benzene signals, and (2) the chemical shifts and coupling patterns of the protons on the cyclopentane ring do not match at all. Thus, we concluded that the reported structure of the natural product is incorrect. Although we conducted several bioassays using our synthesized *N*-acetyl HMCP, no activity was observed against *R. solanacearum*.

We focused on two structurally unique microbial signaling molecules, bradyoxetin and HMCP, and disproved their assigned structures. First, we synthesized the model phenyl-oxetane derivatives and found that the ^{1}H NMR spectra of the compounds were completely different from those in the data of the reported natural bradyoxetin. Thus, we revisited the reported NMR data of bradyoxetin. On the basis of NMR and MS studies, we concluded that the compound reported as bradyoxetin must be chloramphenicol. It was reported that

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J. Nat. Prod. 2021, 84, 495−502

MerR family proteins, such as NolA, activate their target genes in the presence of some toxic compounds; thus, chloramphenicol as an antibiotic may cause Loh's observations. They reported that the production of bradyoxetin was reduced under Fe^{3+} replete culture conditions. This might be rationalized by the chelation ability of chloramphenicol caused by two free hydroxy groups and an amide nitrogen to Fe^{3+}, which prevented its extraction into the EtOAc phase. Even if the observation of chloramphenicol was the result of an experimental application from a reagent bottle, its signaling molecule-like activity remains intriguing. Chloramphenicol was isolated from *Streptomyces* sp. in 1948 by the Parke-Davis group. Because there is no report of chloramphenicol being identified from other microorganisms, Loh's findings can facilitate more important insights in microbiology if the isolated chloramphenicol is a product of *B. japonicum*. Second, we synthesized some HMCP derivatives, such as *N*-acetyl HMCP, and found that the ^{1}H NMR spectra of the compounds were completely different from those reported for natural HMCP. We carefully assessed the structural adequacy of our synthetic compounds through computational estimations of the NMR chemical shifts of the *N*-acetyl HMCP stereoisomers. The significant difference in the NMR spectra between the reported HMCP and our synthetic sample, as well as theoretical data, strongly suggests that the assigned structure of the natural product is incorrect, and the reported HMCP synthesis procedure must be reconsidered. There is an urgent need to reisolate the natural product from *R. solanacearum*, and the correct structure of the active compound should be determined using extensive NMR studies that include ^{13}C NMR and 2D experiments.

Determining the structure of natural organic compounds is often a challenging task, and errors occasionally occur.[25] The causes might be a simple error in the interpretation of the spectra, insufficient data due to the limited amount of compound obtained, and, rarely, scientific fraud. The elucidation of the structure of a microbial signaling molecule is particularly difficult because its production is extremely low and its physical properties, such as an NMR spectrum, may not be sufficiently measured.[26] If the structure of a compound deduced by spectroscopic analyses is so unique that it has never been found before, it would be a very exciting and important discovery, but if the structure is rare and very unusual, there is no doubt that it is necessary to collect all sufficient data and reconsider.

EXPERIMENTAL SECTION

General Experimental Procedures. The IR spectra were measured using a Jasco IR-4100 spectrometer. The ^{1}H NMR spectra were recorded on a Jeol JNM ECX400 (400 MHz) spectrometer using $CDCl_3$ at δ 7.26, CD_3OD at δ 3.30, or TMS at δ 0.00 as the internal standards. The ^{13}C NMR spectra were recorded on a Jeol JNM ECX400 (100 MHz) spectrometer using $CDCl_3$ at δ 77.0 and CD_3OD at δ 49.0 as the internal standards. High-resolution MS data were recorded on an Agilent 6530 Accurate-Mass Q-TOF (ESI) and Jeol JMS-T100LP (DART) spectrometers. Column chromatography was performed with Wakogel-C200 silica gel. Conformational analyses, structural optimizations, and chemical shift calculations were performed using Spartan'14 (version 1.1.8, Wavefunction, Inc.). All of the air- or moisture-sensitive reactions were conducted under an argon atmosphere. The dried solvents used for the reactions (THF and CH_2Cl_2) were purchased from FUJIFILM Wako Pure Chemical Corporation.

Benzyl 2-Phenyloxetan-3-ylcarbamate (*cis*-8 and *trans*-8). In a quartz tube, benzaldehyde (152 μL, 1.50 mmol) and **7** (532 mg, 3.00 mmol) were dissolved in MeCN (10 mL). This mixture was irradiated for 19 h (λ = 365 nm; light source: Sen Lights Co., HL100CH-4). After concentration under reduced pressure, the residue was chromatographed on SiO_2 (hexane/EtOAc = 20:1) to give a mixture of two isomers (5:1), *cis*-**8** and *trans*-**8** (167 mg, 39%). Analytical samples were obtained by further chromatography (CH_2Cl_2/acetone = 100:1).

*Properties of cis-**8**:* colorless oil: IR (film) ν_{max} 3291, 1714 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.37−7.23 (8H, m), 7.08 (2H, m), 5.91 (1H, m), 5.03−5.00 (2H, m), 4.95 (1H, d, J = 12.8 Hz), 4.81 (1H, d, J = 12.8 Hz), 4.56 (1H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.2, 136.9, 136.0, 128.7, 128.5, 128.2, 128.0, 125.5, 86.5, 76.9, 66.8, 48.7; HRDARTMS m/z 284.1280 [M + H]$^+$ (calcd for $C_{17}H_{18}O_3$, 284.1281).

*Properties of trans-**8**:* colorless oil: IR (film) ν_{max} 3314, 1683 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.44−7.25 (10H, m), 5.54 (1H, d, J = 5.5 Hz), 5.06 (1H, d. J = 12.3 Hz), 5.04 (1H, d. J = 12.3 Hz), 4.75 (1H, m), 4.62−4.55 (2H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.1, 139.8, 128.6, 128.4, 128.3, 128.2, 125.5, 90.3, 74.4, 67.1, 53.5; HRDARTMS m/z 284.1285 [M + H]$^+$ (calcd for $C_{17}H_{18}O_3$, 284.1281).

Cyclopent-3-en-1-yl(phenyl)methanone (17). To a solution of **16** (5.00 g, 32.2 mmol) in THF (70 mL) was added dropwise phenyllithium (1.6 M in *n*-dibutyl ether, 22.4 mL, 35.4 mmol) at −78 °C under Ar. After stirring for 1 h, the mixture was warmed to room temperature (rt) and stirred for 1 h. The reaction mixture was quenched with saturated aqueous NH_4Cl and extracted with EtOAc. The organic layer was washed with saturated aqueous $NaHCO_3$, H_2O, and brine and dried with Na_2SO_4. After concentrating under reduced pressure, the residue was chromatographed on SiO_2 (hexane/EtOAc = 10:1) to give **17** (4.97 g, 90%) as a colorless oil: IR (film) ν_{max} 1756, 1697 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.98 (2H, m), 7.56 (1H, m), 7.47 (2H, m), 5.69 (2H, s), 4.09 (1H, m), 2.76 (4H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.4, 136.4, 132.8, 128.9, 128.6, 44.1, 36.2; HRESIMS m/z 173.0947 [M + H]$^+$ (calcd for $C_{12}H_{13}O$, 173.0961).

***N*-(Cyclopent-3-en-1-yl(phenyl)methyl)-*N*-methylacetamide (19).** To a solution of **17** (5.83 g, 33.9 mmol) in methylamine (2.0 M in EtOH, 67.7 mL, 135 mmol) was added dropwise $Ti(O^iPr)_4$ (15.1 mL, 50.8 mmol) under Ar, and the solution was stirred for 5 h. After cooling to 0 °C, $NaBH_4$ (1.92 g, 50.8 mmol) was added, and the mixture was stirred for 2 h. The reaction mixture was quenched with H_2O. The resulting inorganic precipitate was filtered off and was washed with diethyl ether. The combined organic layer was washed with 2 M HCl(aq) and 10% NaOH(aq) and dried with K_2CO_3. Concentration under reduced pressure gave crude **18** (6.12 g, 96%) as a brown oil. This compound was then used in the next step without further purification. To a solution of crude **18** (1.28 g, 4.39 mmol) in THF (6.4 mL) was added triethylamine (2.86 mL, 2.05 mmol) at 0 °C under Ar. After stirring for 30 min, acetyl chloride (732 μL, 10.3 mmol) was added. After stirring for 3 h, the reaction mixture was quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with H_2O and brine and dried with Na_2SO_4. After concentration under reduced pressure, the residue was chromatographed on SiO_2 (hexane/EtOAc = 7:3) to give **19** (1.42 g, 90%) as a mixture of rotational isomers (3:1): colorless oil; IR (film) ν_{max} 1630 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.46−7.24 (5H, m), 5.77−5.62 (2H, m), 5.53 (0.75H, d, J = 11.9 Hz), 4.69 (0.25H, d, J = 11.4 Hz), 3.26−2.98 (1H, m), 2.68 (2.25H, s), 2.57 (0.75H, s), 2.46−2.26 (2H, m), 2.20 (0.75H, s), 2.15−2.01 (1H, m), 1.99 (2.25H, s), 1.90−1.72 (1H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.8, 170.4, 139.6, 138.9, 129.7, 129.6, 129.5, 129.3, 128.64, 128.59, 128.4, 127.8, 127.4, 65.1, 59.4, 37.54, 37.46, 36.4, 35.9, 35.6, 30.0, 27.3, 22.31, 22.26; HRESIMS m/z 230.1535 [M + H]$^+$ (calcd for $C_{15}H_{20}NO$, 230.1539).

***N*-((3,4-Dihydroxycyclopentyl)(phenyl)methyl)-*N*-methylacetamide (20).** To a solution of **19** (1.00 g, 1.05 μmol) in *t*-BuOH/acetone/H_2O (5:1:1, 14 mL) were added *N*-methylmorpholine-*N*-

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J. Nat. Prod. 2021, 84, 495−502

oxide (1.34 g, 11.4 μmol) and OsO_4 (1% in *t*-BuOH, 1 mL), and the solution was stirred for 12 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated under reduced pressure. The residue was diluted with EtOAc, and the resulting solution was dried with Na_2SO_4. After concentration under reduced pressure, the residue was chromatographed on SiO_2 (hexane/EtOAc = 1:1, EtOAc and MeOH) to give **20** (1.05 g, 91%) as a mixture of rotational isomers (9:1). Properties of the major isomer: colorless oil; IR (film) ν_{max} 3344, 1613 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.28−7.15 (5H, m), 5.55 (1H, d, J = 12.4 Hz), 4.09 (2H, m), 3.42 (2H, br), 3.02 (1H, m), 2.57 (3H, s), 1.99 (3H, s), 1.78 (2H, m), 1.58 (1H, m), 1.36 (1H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.2, 138.6, 128.5, 128.4, 127.5, 73.7, 73.4, 60.0, 35.9, 34.5, 33.7, 30.1, 22.1; HRESIMS m/z 264.1596 $[M + H]^+$ (calcd for $C_{15}H_{22}NO_3$, 264.1594).

***N*-((3-Hydroxy-4-((triethylsilyl)oxy)cyclopentyl)(phenyl)-methyl)-*N*-methylacetamide (21a and 21b).** To a solution of **20** (405 mg, 1.54 mmol) in THF (12 mL) was added sodium hydride (60%, 111 mg, 2.78 mmol) at 0 °C under Ar. After stirring for 30 min, chlorotriethylsilane (271 μL, 1.54 mmol) was added, and the mixture was stirred for 5 h. The reaction mixture was quenched with saturated aqueous NH_4Cl, and the aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with H_2O and brine and dried with Na_2SO_4. After concentration under reduced pressure, the residue was chromatographed on SiO_2 (hexane/EtOAc = 10:1 to 1:1) to give **21a** (167 mg, 28%) and **21b** (158 mg, 39%).

*Less polar isomer **21a**:* colorless oil; IR (film) ν_{max} 3460, 1644 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.37−7.25 (5H, m), 5.65 (1H, d, J = 11.9 Hz), 4.24 (1H, m), 4.00 (1H, m), 3.08 (1H, m), 2.75 (1H, br), 2.67 (3H, s), 2.08 (3H, s), 1.86 (1H, m), 1.71 (1H, dd, J = 6.9, 7.8 Hz), 1.63 (1H, m), 1.30 (1H, m), 0.97 (9H, m), 0.64 (6H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.8, 138.8, 128.6, 128.4, 127.5, 74.1, 73.9, 59.8, 36.5, 35.2, 34.1, 30.0, 22.3, 6.7, 4.7; HRDARTMS m/z 378.2466 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_3Si$, 378.2459).

*More polar isomer **21b**:* colorless oil; IR (film) ν_{max} 3460, 1633 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.35−7.24 (5H, m), 5.68 (1H, d, J = 11.9 Hz), 4.20 (1H, m), 4.00 (1H, m), 3.10 (1H, m), 2.77 (1H, br), 2.63 (3H, s), 2.07 (3H, s), 1.82 (2H, m), 1.50 (2H, m), 0.92 (9H, t, J = 7.8 Hz), 0.56 (6H, q, J = 7.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.9, 139.2, 128.5, 128.4, 127.5, 74.4, 73.7, 59.8, 36.3, 34.8, 34.1, 30.1, 22.2, 6.6, 4.6; HRDARTMS m/z 378.2442 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_3Si$, 378.2459).

***N*-((3-Hydroxy-4-oxocyclopentyl)(phenyl)methyl)-*N*-methylacetamide (22a and 22b).** To a solution of **21a** (173 mg, 458 μmol) in CH_2Cl_2 (11 mL) was added Dess-Martin periodinane (607 mg, 1.43 mmol) at 0 °C. After stirring for 2 h, saturated aqueous $NaHCO_3$ and saturated aqueous $Na_2S_2O_3$ were added, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with saturated aqueous $NaHCO_3$, H_2O, and brine and dried with Na_2SO_4. After concentration under reduced pressure, the residue was chromatographed on SiO_2 (hexane/EtOAc = 3:1) to give **22a** (153 mg, 89%). **22b** (161 mg, 89%) was synthesized from **21b** (182 mg) in the same manner.

*Properties of **22a**:* colorless oil; IR (film) ν_{max} 1755, 1645 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.38−7.28 (5H, m), 5.84 (1H, d, J = 12.3 Hz), 4.26 (1H, dd, J = 6.9, 6.9 Hz), 3.09 (1H, m), 2.69 (3H, s), 2.47 (1H, dd, J = 8.7, 19.2 Hz), 2.13 (3H, s), 2.01−1.82 (3H, m), 0.97 (9H, t, J = 7.8 Hz), 0.65 (6H, q, J = 7.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 214.5, 170.9, 137.8, 128.7, 128.6, 127.9, 73.5, 58.5, 40.1, 35.2, 30.7, 30.1, 22.0, 6.6, 4.7; HRDARTMS m/z 376.2302 $[M + H]^+$ (calcd for $C_{21}H_{34}NO_3Si$, 376.2303).

*Properties of **22b**:* colorless oil; IR (film) ν_{max} 1755, 1644 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.41−7.28 (5H, m), 5.83 (1H, d, J = 11.9 Hz), 4.09 (1H, dd, J = 5.5, 5.5 Hz), 3.18 (1H, m), 2.70 (3H, s), 2.39 (1H, dd, J = 8.2, 19.2 Hz), 2.17 (1H, dd, J = 8.2, 19.2 Hz), 2.08 (3H, s), 1.95 (1H, m), 1.74 (1H, m), 0.93 (9H, t, J = 7.8 Hz), 0.60 (6H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 214.2, 170.9, 137.7, 128.7, 128.5, 127.9, 73.4, 59.3, 39.0, 36.5, 31.7, 30.1, 22.2, 6.6, 4.7; HRDARTMS m/z 376.2301 $[M + H]^+$ (calcd for $C_{21}H_{34}NO_3Si$, 376.2303).

***N*-Acetyl HMCP (23a and 23b).** To a solution of **22a** (20 mg, 53.3 μmol) in CH_3CN (2 mL) was added HF-pyridine complex (150 μL, 207 μmol) at 0 °C. After stirring for 2 h, the mixture was concentrated under reduced pressure. The residue was extracted with diethyl ether. The organic layer was washed with saturated aqueous $NaHCO_3$, H_2O, and brine and dried with Na_2SO_4. After concentration under reduced pressure, the residue was chromatographed on SiO_2 (hexane/EtOAc = 1:1, $CHCl_3$, $CHCl_3$/MeOH = 8:1) to give **23a** (5.6 mg, 40%) as a yellow oil. **23b** (161 mg, 89%) was synthesized from **22b** (182 mg) in the same manner.

*Properties of **23a**:* colorless oil; IR (film) ν_{max} 3399, 1750, 1638 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.37−7.28 (5H, m), 5.88 (1H, d, J = 12.4 Hz), 4.41 (1H, dd, J = 8.3, 8.3 Hz), 3.07 (1H, m), 2.70 (3H, s), 2.49 (1H, dd, J = 8.3, 19.2 Hz), 2.19 (1H, m), 2.13 (3H, s), 2.06 (1H, m), 1.91 (1H, ddd, J = 8.3, 8.3, 12.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.3, 171.2, 137.6, 128.8, 128.5, 128.0, 73.1, 58.2, 39.7, 32.6, 30.1, 30.0, 22.1; HRESIMS m/z 262.1453 $[M + H]^+$ (calcd for $C_{15}H_{20}NO_3$, 262.1438).

*Properties of **23b**:* colorless oil; IR (film) ν_{max} 3357, 1750, 1624 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.38−7.27 (5H, m), 5.88 (1H, d, J = 11.9 Hz), 4.26 (1H, dd, J = 8.2, 8.2 Hz), 3.12 (1H, m), 2.68 (3H, s), 2.40 (1H, m), 2.31 (1H, m), 2.09 (3H, s), 1.98 (1H, m), 1.97 (1H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.1, 171.2, 137.3, 128.8, 128.6, 128.0, 72.8, 58.9, 38.3, 33.7, 30.7, 30.1, 22.2; HRDARTMS m/z 262.1453 $[M + H]^+$ (calcd for $C_{15}H_{20}NO_3$, 262.1438).

Calculations. The conformational analyses of each stereoisomer of HMCP and *N*-acetyl HMCP using the MMFF94 force field gave 7 to 11 conformers within 20 kJ/mol from the global minimum. Geometry optimization of each conformer and ^1H and ^{13}C NMR chemical shift calculations were both performed by DFT calculations using the B3LYP/6-31G* level of theory. The calculated chemical shifts of each compound were corrected based on the Boltzmann distribution of the conformers.

Biofilm Formation Assay. To measure biofilm formation of *R. solanacearum*, we used a standard PVC assay. Strain OE1-1 cells from overnight bacterial cultures in CPG were diluted to an OD_{600} of 0.1 in new medium. A sample (5 μL) of the cell suspension was seeded into each well of a PVC 96-well microtiter plate (Thermo Fisher Scientific) containing CPG media (95 μL) and compounds. Plates were sealed with Breathe-Easy membrane (Sigma-Aldrich) and incubated statically at 30 °C. After 24−30 h, each well was stained with 25 μL of 1% crystal violet for 25 min at rt. PVC plates were washed twice with Milli-Q water (200 μL), and the remaining liquid was aspirated from the bottom of the plate. Adhering crystal violet was dissolved in 95% EtOH (200 μL), transferred to a new polystyrene plate, and measured as absorbance at 595 nm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c01369.

Supplemental figures and tables, copies of ^1H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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