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Structural Elucidation of Trace Components Combining GC/MS, GC/IR, DFT-Calculation and Synthesis—Salinilactones, Unprecedented Bicyclic Lactones from *Salinispora* Bacteria

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Abstract

The analysis of volatiles released by marine *Salinispora* bacteria uncovered a new class of natural compounds displaying an unusual bicyclic [3.1.0]-lactone skeleton. Although only sub- μ g quantities of the compounds were available, the combination of analytical methods, computational spectroscopy, and synthesis allowed unambiguous structural identification of the compounds, called salinilactones, without the need for isolation. Orthogonal hyphenated methods, GC/MS and solid-phase GC/IR allowed to propose a small set of structures consistent with the data. A candidate structure was selected by comparison of DFT-calculated IR spectra and the experimental IR-spectrum. Synthesis confirmed the structure and absolute configuration of three bicyclic lactones, salinilactones A–C. The salinilactones are structurally closely related to the A-factor class of compounds, autoregulators from streptomycete bacteria. They exhibited inhibitory activity against *Salinispora* and *Streptomyces* strains.

Keywords

A-factor; autoinducer; bicyclic lactones; GC/IR; structure elucidation

Marine actinomycete bacteria of the genus *Salinispora* are known to produce a wide variety of natural products,^[1–3] for example, the antimalarial salinipostins^[4] or the proteasome inhibitor salinosporamide A.^[5,6] *Salinispora* bacteria also emit an extensive range of volatiles.^[7] In an effort to reveal the function of these volatiles, we analyzed liquid cultures of *Salinispora arenicola* CNQ748 by closed loop stripping analysis (CLSA).^[8] These experiments furnished complex samples of volatiles containing many compounds in just ng amounts. Therefore, isolation and standard NMR procedures for structure elucidation of unknowns cannot be used. GC/EI-MS analyses are well suited for identification of compounds in such samples because of the high chromatographic resolution, sensitivity, and

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Conflict of interest

The authors declare no conflict of interest.

mass spectra revealing skeletal and functional group information. In favorable cases, mass spectrometric analysis and GC retention index data can lead to a structural proposal for an unknown compound, a method frequently used for example, in chemical ecology, geochemistry, or metabolomics. Nonetheless, more complex target compounds are sometimes difficult to identify because the interpretation of mass spectra can become insufficient. At this point, IR offers orthogonal information, for example, on functional groups and double bonds. Solid-phase GC/IR techniques available nowadays allow the use of infrared detectors integrating high sensitivity with high chromatographic resolution comparable to GC/MS.^[9] Finally, structural proposals developed by these methods have to be verified by synthesis, sometimes only successful by a trial-and-error method needing multiple syntheses to finally arrive at the correct structure.

We add here a third type of information to these methods, computational spectroscopy.^[10] Combining solid-phase GC/IR analysis with DFT-simulations and MS data allows to select effectively candidate compounds worth considering for synthetic verification. This combination of methods led to the identification of a hitherto unknown class of volatile bicyclic lactones emitted by several *Salinispora* bacteria with minimal synthetic effort and without the need of isolation of the natural compounds (Figure S1, Supporting Information).

These lactones are structurally related to the A-factor, a bacterial autoregulator that triggers secondary metabolism and cellular differentiation in *Streptomyces griseus*.^[11,12] Several A-factor like autoregulators carrying a monocyclic γ -butyrolactone core structure are already known from different Streptomyces.^[13–16]

The GC/MS analysis of volatile compounds extracted from liquid cultures of *Salinispora arenicola* CNQ748 showed three unknown compounds sharing the same characteristic ions m/z 140 and m/z 122 (Figure 1, A–C). Several other *S. arenicola* strains, CNS205, CNB527, CNH646, CNH996A, and CNQ748, produced the same compounds. Structural analysis was initially performed for compound **B** occurring in the highest concentration. A detailed analysis of the mass spectrum (Figure 2A) revealed the existence of a C₅-acyl side-chain from which the two characteristic fragments arise by McLafferty rearrangement (m/z 140) and subsequent loss of water (m/z 122). Additional peaks resulting from alkyl chain fragmentation at m/z 153 by loss of an ethyl group from M⁺ at m/z 182 and at m/z 125 (α -carbonyl cleavage) supported this assignment (see Figure S3). GC/HR-MS delivered the molecular formula C₁₀H₁₄O₃ (found 182.0972, calc. 182.0943) requiring four double-bond equivalents (DBE). Solid-phase GC/IR confirmed the presence of a ketone and an ester group, recognizable by their C=O double-bond stretching bands at 1696 and 1769 cm⁻¹, respectively (Figure 2B).^[17]

The high energy of the ester stretching vibration indicated ring strain, excluding an open-chain ester. Another important piece of information was the absence of a C=C double-bond stretch vibration (1620–1680 cm⁻¹). In order to reach the required number of DBEs without a C=C double-bond we proposed a [3.1.0]-bicyclic structure. Although a good amount of structural information was obtained, many candidate structures were still in agreement with the data, among them for example, compounds **1–8** (Figure 3). Synthesizing all possible isomers was therefore considered to be ineffective.

Consequently, we decided to apply dispersion-corrected DFT^[18] (density functional theory) calculations at the B3LYP level of theory^[19] to simulate the IR spectra of the relevant lactone isomers in question. The lowest energy conformer of each isomer was determined using a hybrid Monte Carlo/Low Frequency Mode algorithm^[20] applying the MMFF^[21] force field. In a second step, each of the global force field minima was subject to a DFT-based optimization and frequency calculation applying the B3LYP-D/6–311G(p,d) level of theory (Figure 3). Though we are comparing solid-state IR data with gas-phase computations,^[22] the agreement between theory and experiment was sufficient for our purpose. The visual comparison of the fingerprint areas of the spectra revealed **8** as the most likely structure for **B**. A detailed discussion of the spectra is given in Figures S10–S17 in the Supporting Information. To quantify our results a computer-assisted comparison based on a first derivative correlation algorithm available through the GRAMS software package was performed, also revealing **8** as the most similar structure (see Figure S9 and Table S5).

A more sophisticated calculation with additional optimization steps and Boltzmann averaging of several conformers further emphasized the degree of similarity (Figure S18). The close resemblance between this simulation and the spectrum of the natural product justified the synthesis of compound **8**.

The racemic synthesis started from commercially available ester **9** (Scheme 1). Saponification and subsequent esterification with allyl alcohol gave the enyne ester **16** that was converted into the target compound **8** under oxidizing conditions and Pd catalysis according to Sanford et al.^[23] Structural proposals for compounds **A** and **C**, **18** and **19**, were made with respect to the gas chromatographic retention index of each compound. The synthesis of **18** and **19** started from the terminal alkynes **10** and **11**, which were transformed into acids **12** and **14** with CO₂ and further developed as outlined in Scheme 1. Mass and IR spectra as well as gas chromatographic retention indices of compounds **8**, **18**, and **19** matched those of the natural products **A–C**, thus confirming their identity (see Figure S2).

To determine the absolute configuration of **B**, an asymmetric synthesis was performed. Valeroyl chloride (**20**) was converted into the β -keto ester **21** utilizing Meldrum's acid and allyl alcohol.^[24] The carbene precursor **22** was synthesized by diazo transfer under basic conditions.^[25] The key step was the asymmetric copper-catalyzed intramolecular cyclopropanation described by Nakada and co-workers (Scheme 2).^[26]

The mediocre enantioselectivity of 52% *ee* under standard conditions could be slightly enhanced to 58% *ee* by lowering the reaction temperature, yet the yield dropped significantly. The absolute configuration of the major enantiomer suggested by the proposed transition state from Nakada et al. was proven by electronic circular dichroism measurements, DFT simulations, and literature comparison of the specific rotation of structurally related compounds (see Figures S19 and S20). Chiral GC/MS investigations disclosed the absolute configuration of the natural compound **B** using the synthesized scalemic mixture (see Figure S21). The natural compound **B** occurred in a 97:3 (1*R*,5*S*)/(1*S*,5*R*)-mixture. Both the natural compounds **A** and **C** were also submitted to chiral GC analyses and compared to the corresponding racemic mixtures as references. In each case

the natural product was identical with the later eluting enantiomer (see Figure S12), indicating the same absolute configuration as found in **B**.

The biosynthesis of the A-factor (**30**), related to the salinilactones, has been investigated in detail (Scheme 3).^[27] Horinouchi et al. showed that the enzyme A-factor synthase AfsA synthesizes ester **25** by coupling an acyl carrier protein (ACP) bound β -ketoacyl unit (**24**) and dihydroxyacetone phosphate (**23**).^[28] The phosphorylated ester **25** is then converted into lactone **26**, followed by reduction to lactone **27** that only needs a phosphatase to release **30**. We hypothesized that the salinilactones might be biosynthesized by the same pathway.

We recently reported the creation of a mutant of the AfsA-like gene called *spt9* in the related isolate *S. arenicola* CNS205 associated with salinipostin biosynthesis.^[29] In contrast to the wildtype, no salinilactones were detected in the mutant. Therefore, the biosynthetic routes of γ -butyrolactone signaling compounds and salinilactones seemed to be closely connected. Especially phosphate **27** seems to be a suitable precursor for salinilactone biosynthesis, offering a leaving group for the construction of the three-membered ring. We propose that compound **27** is enzymatically deprotonated to form enolate **28**. This enolate can then form the cyclopropane unit of the salinilactones by C-alkylation via nucleophilic displacement of the phosphate group. We suspect that the phosphate **27** also gives rise to the recently discovered salinipostins (**29**), potent anti-malaria compounds also isolated from *Salinispora*,^[4] as the *spt9* knockout mutant also abolishes its biosynthesis.^[29] The oxygen of enolate **28** likely attacks alternatively the phosphate group, or a derivative thereof, leading finally to **29**.

γ -Butyrolactones like A-factor are generally produced in small amounts in *Streptomyces*. To determine the amount of salinilactones produced by *Salinispora*, we opted for an isotope dilution method. Therefore, the deuterated analogue *d*₂-**8** was synthesized and spiked to bacterial cultures. The following headspace analysis of liquid cultures, performed at different time points during the culture growth allowed determination of the amount of **8** produced and its time course.

Reduction of acryloyl chloride gave crude 1,1-*d*₂-allyl alcohol^[30] used for the synthesis of ester *d*₂-**16** that was converted to the deuterated bicyclic lactone *d*₂-**8** as described (see Scheme S1). *S. arenicola* CNS205, the strain with the highest production rate of salinilactones A–C was used for the spiking experiments (Figure 4). A well grown liquid culture of strain CNS205 yielded about 500 μL^{-1} of salinilactone B. Quantification of salinilactones A and C was possible by referencing characteristic ions to the deuterated internal standard (Figure 4). The amounts of salinilactones produced by *S. arenicola* CNS205 are one order of magnitude higher than that of A-factor in *Streptomyces griseus* that produces 25–30 $\mu\text{g L}^{-1}$.^[31]

Preliminary experiments were performed to test the biological effects of these compounds. Agar diffusion assays of the salinilactones A–C showed inhibition of growth of the test strain *S. arenicola* CNH996A that also can produce these metabolites. Similarly, growth of *Streptomyces violaceoruber* A3(2), producer of the γ -butyrolactone SCB1 closely related to the A-factor, was inhibited.^[13] No inhibition was observed with loadings below 50 mg (see Supporting Information). The analysis of *S. violaceoruber* A3(2) showed no production of

salinilactones. The self-inhibitory activity of the salinilactones may hint to a signaling function, but further experiments are needed to clarify the function of these compounds in the biology of *Salinispora*.

The salinilactones have an interesting structural feature, the reactive cyclopropane ring. An analysis of the relaxed DFT force constants revealed kinetically labile C–C bonds in the three-membered ring of **8** indicated by a deviation from the Badger rule by more than 20%, not far from the value for fluxional molecules.^[32] The β -keto functionality on one side and the alkyl group on the other side are structural features of push-pull cyclopropanes, which show enhanced reactivity against nucleophiles and/or electrophiles.^[33] Several synthetic strategies using this concept have been developed. Therefore, multiple activities of salinilactones in biological systems can be assumed. The bicyclic fused cyclopropabutyrolactone motif has not been reported before as a natural product.

In summary, we were able to combine solid-phase GC/IR and DFT-based simulation of infrared spectra to complement GC/MS analysis, thus enabling identification of natural compounds present in trace amounts without NMR spectroscopy. This approach led to the discovery of a new class of A-factor related γ -butyrolactones produced by *Salinispora* bacteria. Research on naturally occurring derivatives and the biological function of these unique fused bicyclic lactones is currently ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Total-ion chromatogram of a headspace extract of a liquid culture of *Salinispora arenicola* CNQ748 obtained by CLSA. Compounds A–C are indicated.

Figure 2.

A) Mass spectrum and B) infrared spectrum of natural compound **B**.

Figure 3.

Experimental infrared spectrum (bottom) and simulated IR spectra of the depicted structures based on lowest energy conformers. Conformational analysis with MMFF and optimization/frequency calculation using B3LYP-D/6-311G(p,d), scaling factor 0.975.

Figure 4. Production of salinilactones A–C in a liquid culture of *S. arenicola* CNS205 within a six-day period. square: salinilactone A; circle: salinilactone B; triangle: salinilactone C.

Scheme 1.
 Racemic synthesis of bicyclic lactones **8**, **18** and **19**.

Scheme 2.

Asymmetric synthesis of **8** via intramolecular cyclopropanation.^[25] ligand*: 2,2-bis((4*S*)-(—)-4-isopropylloxazoline)propane, *p*-ABSA: 4-acetamidobenzene sulfonyl azide.

Scheme3.

Proposed biosynthesis of salinilactones based on A-factor biosynthesis in *Streptomyces griseus*.^[4,27] The key intermediate, enolate **28**, gives salinilactones via C-alkylation, while O-phosphorylation leads to salinipostins (**29**).