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Synthesis of a proposed structure for the diffusible extracellular factor of *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

A proposed structure for the diffusible extracellular factor (DF) of *Xanthomonas campestris* pv. *campestris* (*Xcc*) has been synthesized. Its MS spectrum and biological activity, however, contradict those of the natural product previously reported, suggesting that the structure for DF of *Xcc* must be reexamined.

Xanthomonas campestris is a bacterial species that is responsible for a variety of plant diseases worldwide. *Xanthomonas campestris* pv. *campestris* (*Xcc*) is the causal agent of black rot, which affects crucifers such as Brassica and Arabidopsis. The pathogen produces extracellular polysaccharides (EPS = xanthan gums) and enzymes including proteases, pectinases and endoglucanases which are considered important virulence determinants.[1] In 1997, Barber et al. reported that the pathogenicity of *Xcc* is mediated by a small diffusible signal factor (DSF), which restores production of protease, endoglucanase and polygalacturonate lyase to *rpfF* mutants.[2] The DSF-mediated system was not considered a mechanism for density-dependent regulation, which is a quorum-sensing system. The complete structure of the DSF was reported as (*Z*)-11-methyl-2-dodecenoic acid (**1**) by Wang et al. (Fig. 1).[3] Structure–activity relationship studies suggest that the *cis*-double bond in **1** is the most important feature for DSF activity.[3] Another similar but completely separate diffusible extracellular factor (DF) encoded by the *pigB* gene of *Xcc* was reported by Chun et al. in 1997.[4] They called it a pheromone, and it was reported to regulate both EPS and xanthomonadin pigment production. Furthermore, convincing genetic evidence showed DF to be involved in epiphytic survival and concomitant host infection.[5] The structure of the pheromone was proposed as a butyrolactone derivative (**2**) based on its MS spectrum (Fig. 1). Notably the two DSF-mediated systems independently regulate pigment and endoglucanase production, but both regulate EPS production. In the co-regulating EPS production system, **2** has a stronger effect than **1**. This unique system of *Xcc* is summarized in Figure 1. The structure of **2** is similar to acyl homoserine lactones (AHLs), which are well-known cell density-dependent autoregulators among Gram-negative bacteria. However, there is no published evidence that DF is an autoinducer. Recently, we have reported that the stereochemistry of the acyl side chain in AHL from the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum* is very important for its biological activity.[6] Because pheromone **2** has two stereogenic centers whose absolute configuration remains unknown, we attempted to clarify the stereochemistry-activity relationship of the pheromone. In order to establish the absolute structure of the natural pheromone, we synthesized possible stereoisomers of **2**. This communication describes the synthesis of stereoisomers of **2** and the results of a comparison of the physical and biological properties of the synthetic compounds alongside the natural pheromone.

We anticipated that it might be possible to separate diastereomers of the final product or synthetic intermediates, therefore, the synthesis began with the preparation of a diastereomeric mixture of **2** (Scheme 1) from the known compound **3**.[7] Optically active (*S*)-**3** (>99% ee) was converted into alcohol **4** upon addition of methyl Grignard reagent to the corresponding aldehyde (ca. 1:1 ds). The unseparated diastereomers were used directly in subsequent reactions. Protection of the resultant hydroxyl group of **4** as a PMB ether[8] and deprotection of the TBS group gave alcohol **5**. After conversion of **5** into the corresponding iodide **6**, known **7**[9] was alkylated with **6** using potassium carbonate as the base to give **8** in 85% yield. The ester group of **8** was hydrolyzed with methanolic KOH, followed by careful acidification (pH 4) with

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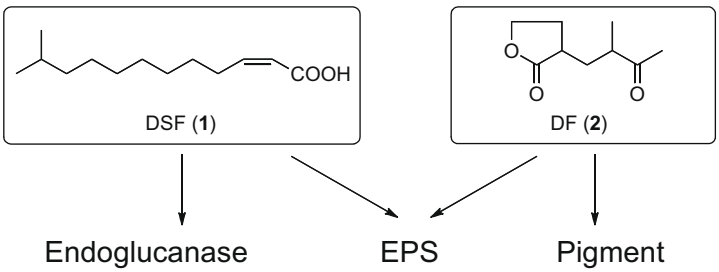
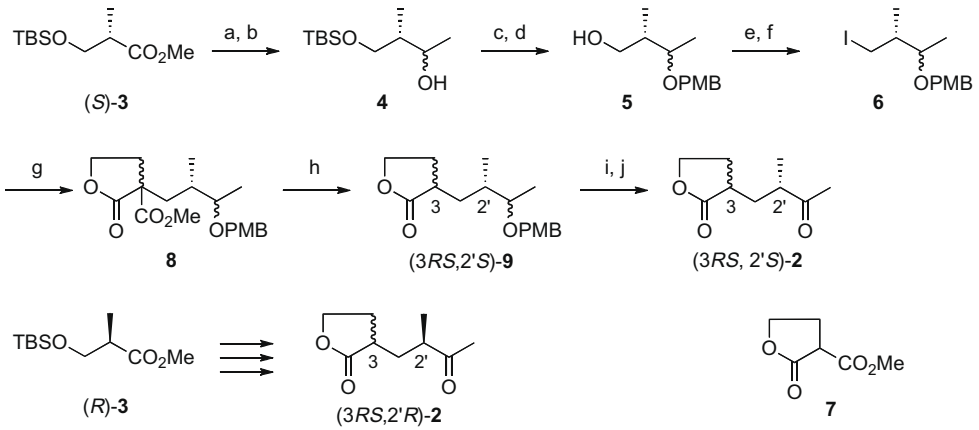


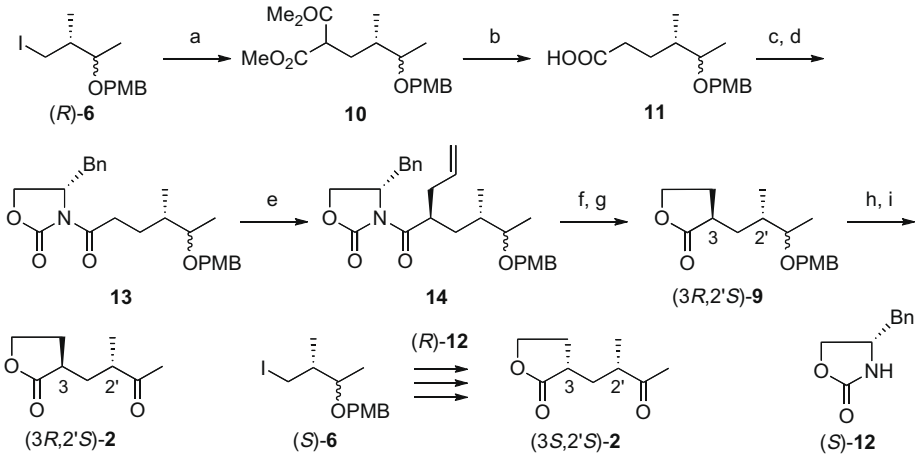
Figure 1. DF and DSF autoregulatory systems in *Xcc*.



Scheme 1. Synthesis of diastereomeric mixtures of **2**. Reagents and conditions: (a) DIBAL, CH_2Cl_2, −78 °C; (b) CH_3MgBr, ether, 0 °C (61% in two steps); (c) PMBOC(=NH)CCl_3, Ph_3CBF_4, ether (90%); (d) TBAF, THF (90%); (e) *p*-TsCl, pyridine, 0 °C; (f) NaI, acetone, reflux (93% in two steps); (g) **7**, K_2CO_3, acetone/DMF = 6:1, reflux (85%); (h) KOH aq, EtOH then AcOH, 23–80 °C (84%); (i) DDQ, CH_2Cl_2/H_2O = 20:1, 0 °C (77%); (j) DMP, CH_2Cl_2, 0 °C (82%).

acetic acid. Decarboxylation by refluxing the mixture gave lactone **9** in 84% yield. The PMB group of **9** was deprotected with DDQ, and the resulting hydroxyl group was oxidized with Dess–Martin periodinane to give the desired (3*RS*,2′*S*)-**2** as a diastereomeric mixture. The overall yield of the synthesis of (3*RS*,2′*S*)-**2** was 21% in 10 steps from **3**. The (3*RS*,2′*R*)-isomer was synthesized in the same manner as described above starting from (*R*)-**3**. Although the products consisted of a mixture of diastereomers, the NMR spectrum was consistent with the proposed structure of synthetic **2**. Unfortunately, diastereoisomers of **2** or any of the synthetic intermediates were difficult to separate by typical chromatographic methods. The ratio of the diastereomers of synthetic **2** was determined to be ca. 2:1 using NMR and GC analyses. However, the relative configuration of each isomer could not be determined. GC analyses of synthetic **2** with a chiral stationary phase showed the C-2′ methyl group was stereochemically pure (>99%).[10]

In an effort to determine the relative configuration of the diastereomers, stereoselective synthesis of **2** was undertaken, as shown in Scheme 2. Dimethyl malonate was alkylated with iodide (*R*)-**6** to give **10** in 94% yield. After the two ester groups were hydrolyzed using excess KOH under reflux, careful acidification (pH 4) with acetic acid and decarboxylation of the resulting di-acid under



Scheme 2. Synthesis of (3*R*,2′*S*)- and (3*S*,2′*S*)-**2**. Reagents and conditions: (a) dimethyl malonate, K_2CO_3, acetone/DMF = 6:1, reflux (94%); (b) (i) 1 M KOH in MeOH, 75 °C then acidification with AcOH (ii) toluene, reflux (88%); (c) PivCl, Et_3N, toluene, 0 °C; (d) LiHMDS, (*S*)-**12**, THF (74% in two steps); (e) NaHMDS, allyl iodide, THF, −78 °C (78%); (f) OsO_4, 2,6-lutidine, $NaIO_4$, *t*-BuOH/H_2O = 3:1; (g) $NaBH_4$, MeOH (59% in two steps); (h) DDQ, CH_2Cl_2/H_2O = 20:1, 0 °C (83%); (i) DMP, CH_2Cl_2, 0 °C (quant).

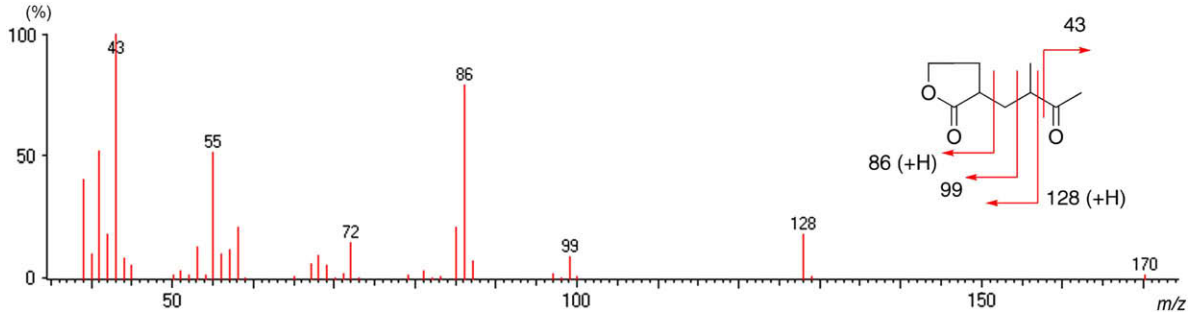


Figure 2. MS spectrum of synthetic **2**.

reflux in toluene gave acid **11** in two steps (88% yield). The PMB group of **11** was relatively labile therefore, the decarboxylation process was carried out under controlled conditions. Acid **11** was condensed with Evans oxazolidinone (*S*)-**12**,[11] and the resulting **13** was diastereoselectively alkylated using allyl iodide to give **14**. Oxidative cleavage of the terminal olefin of **14** with OsO_4 and $NaIO_4$, and subsequent reduction of the resulting aldehyde with $NaBH_4$ gave (3*R*,2′*S*)-**9** with spontaneous lactone formation. Treatment with DDQ followed by Dess–Martin oxidation of the resulting alcohol afforded the desired (3*R*,2′*S*)-**2**.[12] (3*S*,2′*S*)-**2** was synthesized in the same manner from (*S*)-**6** using (*R*)-**12** as the chiral auxiliary.[12] GC analyses of the synthetic samples using a chiral stationary phase showed that the diastereoselectivity of the Evans alkylation of (4*S*)- and (4*R*)-**13** with allyl iodide was 96:4 and >99:1, respectively.[10] The difference in ^1H and ^{13}C NMR spectra of the two isomers distinguished the major isomer in the diastereomeric mixtures, (3*RS*,2′*S*)- and (3*RS*,2′*R*)-**2**, as the *syn*-isomer (Scheme 1). The lactone ring of **9** was formed under thermal conditions, therefore, the major *syn*-isomer is expected to be the thermodynamically favored isomer, consistent with higher diastereoselectivity in Evans alkylation giving the *syn*-isomer, (2′*R*,4′*S*)-**14**.

The ^1H and ^{13}C NMR spectra of natural DF are unavailable due to the scarcity of the natural product; only the MS spectrum of DF has been reported.[4] To compare the MS spectrum of synthetic **2** with that of the natural product, the MS spectra of the four stereoisomers of **2** were recorded using GC–MS under similar conditions as reported for the natural compound (Fig. 2). The MS spectra of the four stereoisomers were identical, but none were in agreement with that reported for the natural compound.[4] A comparison of MS data for synthetic **2** with that of the reported natural DF is shown in Table 1. Mass spectra of **2** acquired under various conditions, including both LC–MS and GC–MS in positive and negative modes, also disagreed with the data reported for the natural compound.

Next, synthetic (3*RS*,2′*S*)-**2** and (3*RS*,2′*R*)-**2** were subjected to a biological assay according to the reported procedure.[4] The compounds were tested at seven different concentrations, varying from 0.64 to 10,000 mg/ml. No effects on pigment or EPS production of the test strain were observed with either synthetic compound, regardless of the concentration used. These results strongly suggest that natural DF does not correspond to the structure of **2**.

In summary, we report the synthesis of a diastereomeric mixture and the stereochemically pure form of a proposed compound for the diffusible extracellular factor of *Xcc*. However, the results of the MS spectra and biological activities of the natural product and the synthetic compounds were inconsistent. This finding clearly shows that the structure proposed for DF of *Xcc* must be revised. In this study, an intense peak at 86 was observed in the MS spectra of both the natural product and synthetic **2** (Table 1), indicating that the natural DF possesses at least a butyrolactone structure

Table 1
Selected MS data for synthetic **2** and reported natural DF

Fragment ion (relative intensity, %)	
Natural DF	Synthetic **2**
170 (M^+, 100)	170 (M^+, 2.7)
155 (3.7)	—[a]
140 (12.3)	—[a]
128 (5.6)	128 (17.8)
112 (5.4)	—[a]
—[a]	99 (8.1)
86 (85.1)	86 (80.0)
—[a]	72 (14.9)
69[b]	—[a]
—[a,c]	55 (52.4)
—[a,c]	43 (100)

[a] Not observed.
[b] Relative intensity was not reported.
[c] MS data below *m*/*z* 60 were not reported.

as originally proposed by Chun et al. Previous work indicates that DF is probably not an AHL, and probably is a butyrolactone. AHLs from several other bacterial genera were unable to restore *pigB* mutant strains for pigment and EPS production,[4,13] and several *Streptomyces* strains which produce butyrolactones (but not AHLs) were able to restore the *pigB* mutant strain for both these traits.[14] Re-investigation of the structure of DF of *Xcc* is currently underway.

Acknowledgment

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10. Conditions of GC analysis; column: CP-Chirasil-Dex CB (0.25 mm × 25 m), carrier gas He (50 kPa), 10–180 °C (3 °C/min), t_R(3*S*,2′*R*) = 23.7, t_R(3*R*,2′*S*) = 24.0, t_R(3*R*,2′*R*) = 25.3 and t_R(3*S*,2′*S*) = 25.6.
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12. *Properties of synthetic* **2**. (a) (3*R*,2′*S*)-isomer: colorless oil; $[\alpha]_D^{22}$ −10.4 (*c* 0.14, $CHCl_3$). IR ν_{max} (film) cm^{-1}: 1711 (s), 1767 (s). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.15 (d, *J* = 7.3 Hz, 3H, 2′-CH_3), 1.75 (ddd, J = 5.4, 7.3, 12.7 Hz, 1H, 1′-H),

1.85−1.97 (m, 2H, 1′-H, 4-H), 2.20 (s, 3H, 4′-CH_3), 2.39 (m, 1H, 4-H), 2.49 (m, 1H, 3-H), 2.95 (m, 1H, 2′-H), 4.17 (ddd, J = 6.5, 9.4, 16.0 Hz, 1H, 5-H), 4.33 (ddd, J = 3.1, 8.3, 16.0 Hz, 5-C*H*H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.2, 28.6, 29.6, 33.2, 37.2, 44.5, 66.5 (C-5), 179.2 (C-3′), 212.0 (C-2). HREIMS m/z [M]$^+$: calcd for $C_9H_{14}O_3$, 170.0943; found, 170.0937. (b) (3*S*,2′*S*)-isomer: colorless oil; $[\alpha]_D^{22}$ −23.5 (c 0.12, $CHCl_3$). IR ν_{max} (film) cm^{-1}: 1712 (s), 1767 (s). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.19 (d, J = 7.0 Hz, 3H, 2′-CH_3), 1.49 (ddd, J = 7.0, 8.2, 14.3 Hz, 1H, 1′-H), 1.90 (m, 1H, 4-H), 2.18 (s, 3H, 4′-CH_3), 2.24 (ddd, J = 7.0, 7.9, 14.3 Hz, 1H, 1′-H), 2.39 (m, 1H, 4-H), 2.52 (m, 1H, 3-H), 2.79 (sxt, J = 7.0 Hz, 2′-H), 4.17 (ddd, J = 6.5, 9.4, 16.0 Hz, 1H, 5-H), 4.39 (ddd, J = 2.8, 8.7, 16.0 Hz, 1H, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 16.7, 27.7, 29.4, 33.1, 37.0, 44.7, 66.4 (C-5), 178.9 (C-3′), 211.6 (C-2). HREIMS m/z [M]$^+$: calcd for $C_9H_{14}O_3$, 170.0943; found, 170.0947.

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