

Structure elucidation and chemical synthesis of stigmolone, a novel type of prokaryotic pheromone

(myxobacteria/*Stigmatella aurantiaca*/microbial development/NMR)

WILLIAM E. HULL[†], ALBRECHT BERKESSEL[‡], AND WULF PLAGA^{§¶}

[†]Central Spectroscopy Department, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany; [‡]Institut für Organische Chemie der Universität zu Köln, Greinstrasse 4, D-50939 Cologne, Germany; and [§]Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany

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ABSTRACT Approximately 2 μ mol of a novel prokaryotic pheromone, involved in starvation-induced aggregation and formation of fruiting bodies by the myxobacterium *Stigmatella aurantiaca*, were isolated by a large-scale elution procedure. The pheromone was purified by HPLC, and high-resolution MS, IR, ¹H-NMR, and ¹³C-NMR were used to identify the active substance as the hydroxy ketone 2,5,8-trimethyl-8-hydroxy-nonan-4-one, which has been named stigmolone. The analysis was complicated by a solvent-dependent equilibrium between stigmolone and the cyclic enol-ether 3,4-dihydro-2,2,5-trimethyl-6-(2-methylpropyl)-2H-pyran formed by intramolecular nucleophilic attack of the 8-OH group at the ketone C4 followed by loss of H₂O. Both compounds were synthesized chemically, and their structures were confirmed by NMR analysis. Natural and synthetic stigmolone have the same biological activity at ca. 1 nM concentration.

Pheromones are defined as substances that are used for communication between individuals of the same species (1). The structure elucidation of acrasin (2) and bombykol (3) are well-known landmarks in early pheromone research. Since then the exchange of chemical signals between individuals of a given species has been observed for many unicellular and multicellular organisms, e.g., for bacteria (4, 5), plants (6–8), and animals (9). It seems that only the archaeobacteria have not yet been examined in this respect. It should be noted that some pheromones, such as (*Z*)-7-dodecen-1-yl acetate, are used by more than one species (9, 10). Because of their potential in pest control, the pheromones of insects have been studied most extensively and represent the largest known class. For bacteria, pheromones of the lactone type (4, 11) make up an important group, and further classes involve specific (modified) peptides (4).

Myxobacteria are on the borderline between unicellular and multicellular organisms. They live as single cells associated in swarms during the vegetative part of their life cycle to manage cooperative feeding (12, 13). In the developmental part of the life cycle the cells of a swarm form multicellular fruiting bodies containing the myxospores. Such a prokaryotic system can serve as a simple model for cell differentiation and cell positioning in multicellular organisms or tissues under the influence of pheromone-like or hormone-like chemical signals.

A pheromone activity of *Stigmatella aurantiaca* was previously described by Stephens *et al.* (14) as to be involved in the formation of fruiting bodies. In a companion paper (15) we have confirmed that diffusible signaling factor(s) are required for the aggregation of the myxobacterium *S. aurantiaca* and subsequent formation of fruiting bodies under conditions of starvation. Aggregation was abolished when factors of low

molecular weight were removed from the cells by dialysis. This finding was interpreted as proof that a pheromone is required for aggregation. Here we describe the structure elucidation of the isolated *Stigmatella* pheromone, named stigmolone, which was found by NMR spectroscopy to be 2,5,8-trimethyl-8-hydroxy-nonan-4-one [5]. The investigation was complicated by the fact that, depending on the isolation and purification procedures used, the ketone underwent intramolecular cyclization with loss of H₂O to form the six-membered heterocycle containing an enol-ether moiety, formally named 3,4-dihydro-2,2,5-trimethyl-6-(2-methylpropyl)-2H-pyran [6]. Both the ketone and dihydropyran compounds were synthesized chemically, and pheromone activity at concentrations of ca. 1 nM was unambiguously assigned to the ketone structure.

MATERIALS AND METHODS

Culturing of *S. aurantiaca* DW4/3–1 (16) and the biological assay for the activity of the pheromone were carried out as described in ref. 15.

Preparation of Natural Pheromone for NMR Analysis. The natural pheromone was collected by an elution procedure and purified as previously described (15). Briefly, for the 2- μ mol NMR sample the volatile pheromone was isolated by steam distillation of many batches of eluate collected over 5-day periods from a total of 8×10^{13} starving cells, followed by fractionation on a reversed-phase C18 column (three runs). After transfer of the active material into cyclopentane (using two 100-mg solid-phase extraction columns) the active material was subjected to normal-phase HPLC (two runs). The active fraction was evaporated to dryness under a stream of dry nitrogen at room temperature and the solvent condensed at -78°C . The residue was dissolved in 0.5 ml of CD₂Cl₂, and the CD₂Cl₂ evaporated (see above); pheromone activity in the condensate was <1% of that for the whole active material.

Analytical Procedures. The isolated natural pheromone was analyzed by (i) high-resolution MS in electron impact (EI) mode with a JMS-700 instrument (JEOL); (ii) electrospray ionization (ESI) MS with a TSQ 7000 (Finnigan-MAT, San Jose, CA); and (iii) GC-IR using a Digilab FTS-40 Fourier transform (FT) instrument equipped with a Digilab Tracer cryotrapping GC interface (Bio-Rad).

Synthetic compounds were analyzed as follows: (i) analytical TLC on precoated plates of silica gel 60 with a fluorescence indicator (Macherey & Nagel), spot detection by exposure to

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: EI, electron impact; ESI, electrospray ionization; FT, Fourier transform; 2D, two-dimensional; COSY, correlated spectroscopy; DQ, double-quantum; pt, point; DEPT, distortionless enhancement by polarization transfer; INADEQUATE, incredible natural abundance double quantum transfer experiment; HOSE, hierarchically ordered spherical description of environment; COLOC, correlation spectroscopy for long-range couplings.

[¶]To whom reprint requests should be addressed. e-mail: wplaga@sun0.urz.uni-heidelberg.de.

iodine vapor; (ii) FT-IR with an IFS 66 spectrometer (Bruker Analytik, Rheinstetten, Germany); and (iii) routine ^1H - and ^{13}C -NMR spectra at 24°C in CDCl_3 at 300 and 75.5 MHz, respectively, using a Bruker AM-300 spectrometer [chemical shifts δ in ppm relative to internal tetramethylsilane, proton-proton coupling constants (J) in Hz].

Structure Elucidation by NMR. Samples of the natural pheromone and the synthetic **5** and **6** were investigated in detail by ^1H - and ^{13}C -NMR spectroscopy at 500.13 and 125.76 MHz, respectively, on an AM-500 NMR spectrometer (Bruker Analytik) using 5-mm sample tubes (Wilmad, Buena, NJ). A variety of conventional one-dimensional and two-dimensional (2D) FT NMR experiments (17) were performed at 10°C and 30°C using the solvents CD_2Cl_2 (99.9% D, Euriso-top, Saint Aubin, France), D_2O (99.96% D, MSD Isotopes, Munich, Germany), and cyclohexane- d_{12} (99.8% D, Chemotrade, Leipzig, Germany).

For Fig. 3 the ^1H -NMR spectrum of a sample containing *ca.* 2 μmol purified pheromone in 0.4 ml CD_2Cl_2 was obtained at 30°C with 4,096 transients in 6.6 h (spectral width 3,424 Hz, time domain size 32K, repetition time 5.78 s, flip angle 77°). Detailed analysis of chemical shifts and coupling constants (Table 1) was performed with zero-filling to 64K data and resolution enhancement using Bruker's WIN-NMR software. For comparison, a sample of synthetic ketone **5** (*ca.* 10 mg) was prepared and measured in a similar way (128 transients in 17 min, repetition time 7.78 s, flip angle 29°). A conventional magnitude-mode correlated spectroscopy (COSY) 2D H-H correlation experiment was performed with the natural pheromone sample: spectral width 1,033 Hz; F_2 time domain: 2K points in 0.991 s; F_1 domain: 512 increments, 32 transients per free induction decay, 60° read pulse, average repetition time 3.24 s, total time 17 h; 2D-FT with sine-bell window functions and zero-filling in F_1 to give a $1\text{K} \times 1\text{K}$ matrix with 1.01 Hz/point (pt) digital resolution. Long-range couplings for I were confirmed by COSY and homodecoupling.

The above sample of natural pheromone was reduced in volume to *ca.* 0.3 ml and used to obtain a ^1H -decoupled ^{13}C -NMR spectrum at 10°C with 84,000 transients in 70 h: spectral width 29,411 Hz, time domain 64K zero-filled to 128K, power-gated WALTZ-16 ^1H decoupling, repetition time 3.014 s, flip angle 30° , exponential line-broadening of 1.4 Hz. These conditions were chosen to facilitate integration of the spectrum for assignment of C_q and CH_n signals for compounds I and II shown in Table 2 (I/II ratio *ca.* 2.9). The signal-to-noise ratios ranged from *ca.* 2 for the ketone carbon of II to *ca.* 17 for CH_3 carbons of I (dihydropyran). Multiplicity analysis (CH , CH_2 , CH_3) was performed by using distortionless enhancement by polarization transfer (DEPT-90) (6 h) and DEPT-135 (23 h) experiments. Finally, a polarization-transfer CH correlation with ^{13}C detection and bilinear rotation decoupling in the F_1 domain was performed: ^{13}C domain, 5319 Hz, 4K points; ^1H domain, 1,033 Hz, 256 increments, zero-filled to 512; polarization transfer delays, 3.7 and 1.9 ms; repetition time 2.25 s, 128 transients per free induction decay, total time 21 h; magnitude-mode 2D-FT with cosine-bell window functions to give a $2\text{K} \times 512$ matrix with 2.6 Hz/pt in ^{13}C and 2.02 Hz/pt in ^1H .

A sample of the synthetic ketone **5** was prepared at *ca.* 1 M concentration in CD_2Cl_2 and exhibited a mixture of the ketone and dihydropyran forms in a ratio of *ca.* 2.5:1. A conventional ^1H -decoupled ^{13}C spectrum (1,000 transients in 100 min) gave the results summarized in Table 2.

^1H - and ^{13}C -NMR data also were obtained from a nearly pure sample of synthetic dihydropyran **6** at 15°C in cyclohexane- d_{12} (*ca.* 1 M, Tables 1 and 2). A 2D incredible natural abundance double quantum (DQ) transfer experiment (IN-ADEQUATE) was performed to establish long-range C-C connectivities (F_2 domain: 17,857 Hz, 16K data points; F_1 (DQ evolution) domain: $\pm 14,706$ Hz, 256 increments, 128 transients per free induction decay; spin-echo delay for DQ preparation 125 ms (optimal for $J_{\text{CC}} = 4.0$ Hz), relaxation delay 4.5 s, 125° read-out pulse, total time 48 h; power-mode 2D-FT with

Table 1. ^1H -NMR data for the natural pheromone preparation (compounds I and II) and for synthetic **5** and **6** (see Fig. 3)

Group	Position	δ_{H} , ppm			δ_{H} , ppm			J couplings, Hz ^e		
		in CD_2Cl_2		in D_2O	in CD_2Cl_2		in <i>c</i> -Hex.	Nat. II	Nat. I	
		Nat. II ^a	[5] ^b	[5] ^c	Nat. I ^a	[6](mix.) ^b	[6] ^d			
CH ₃	1	0.9025	0.9024	0.9184	0.8718	0.8716	0.8676	$^3J(1,2)$	6.58	6.52
	1'	0.8949	0.8947	0.9135					6.69	
CH	2	2.1125	2.1132	2.094	1.8225	1.8225	1.869 ^f	$^3J(2,3)$	7.13(3a); 6.63(3b)	7.06
CH ₂	3a	2.3345	2.3352	2.52	1.9012	1.9000	1.901 ^f	$^1J(3a,b)$	-16.54	
	3b	2.2888	2.2889	2.50				$^5J(3,6)$		1.11
								$^5J(3,10)$		0.48
CH	5	2.4523	2.4531	2.704				$^3J(5,6)$	7.08; 5.72	
CH ₂	6a	1.67 ^g	1.67 ^h	1.67 ⁱ	1.9060	1.9052	1.882 ^f	$^3J(6,7)$	n.d. ^g	6.74
	6b	1.36 ^g	1.36 ^h	1.44 ⁱ				$^4J(6,10)$		0.98
CH ₂	7a	1.38 ^g	1.38 ^h	1.48 ⁱ	1.5582	1.5577	1.5237	$^3J(6,7)$	n.d. ^g	
	7b	1.33 ^g	1.33 ^h	1.40 ⁱ						
-OH	8	1.51 ^j	2.9; 4.15 ^k							
CH ₃	9	1.1735	1.1736	1.209	1.1590	1.1563	1.1325	$^4J(9,9')$	0.21	
	9'	1.1710	1.1700							
CH ₃	10	1.0486	1.0488	1.0856	1.5807	1.5802	1.5647	$^3J(5,10)$	7.00	
rms dev. ^l			0.00046	0.114		0.0012	0.026			

^aNatural pheromone preparation (mixture of I and II), shifts relative to $\text{CHDCl}_2 = 5.32$ ppm (30°C).

^bMixture of **5** and **6** derived from synthetic **5** (30°C).

^cNearly pure **5** in D_2O , HDO = 4.75 ppm (30°C).

^dPure **6** in cyclohexane- d_{12} at 15°C (solvent = 1.38 ppm).

^e $^4J(1,3)$ for I and II, $^4J(7,9)$ for I detected by COSY.

^fDetermined from the COLOC 2D CH correlation experiment.

^gComplex second-order spin systems; δ_{H} from COSY spectrum; n.d., not determined.

^hMultiplets superimposable with those of II.

ⁱEstimated shifts assigned by analogy to II.

^jResidual water.

^kFor pure synthetic **5** as neat liquid.

^lrms deviation of all shifts (except 8-OH) relative to natural compound.

Table 2. ¹³C-NMR data for the natural pheromone preparation and the synthetic compounds **5** and **6**

Position	δ_c , ppm ^a		Predictions ^b		δ_c , ppm ^a			Predictions ^b				
	in CD ₂ Cl ₂ , 10°C		ketone [5]		in CD ₂ Cl ₂		in c-Hex.		Dihydropyran [6]		enol [5b]	
	Nat. II	[5](mix).	S. Tool	S. Edit	Nat. I	[6](mix.)	[6]	S. Tool	S. Edit	S. Tool	S. Edit	
1	22.643	22.796	21.8	22.0 (4)	22.382	22.554	22.880	22.7	23.0 (3)	22.7	23.0 (3)	
1'	22.571	22.720										
2	24.350	24.426	22.4	25.0 (3)	27.099	27.155	27.478	22.9	27.0 (2)	22.6	27.0 (2)	
3	50.377	50.511	47.7	43.0 (2)	39.655	39.775	40.299	41.6	26.0 (2)	43.5	26.0 (2)	
4	214.404	214.795	211.3	213.0 (2)	145.456	145.585	146.526	149.8	149.0 (2)	149.4	162 (1)	
5	46.902	47.069	47.4	44.0 (2)	100.677	100.599	99.719	100.8	137 (1)	105.9	137 (1)	
6	27.531	27.682	21.8	27.0 (2)	25.280	25.394	25.942	27.2	31.0 (2)	21.0	31.0 (2)	
7	41.380	41.473	43.0	34.0 (2)	33.612	33.733	34.320	43.5	34.0 (2)	43.8	34.0 (2)	
8	70.677	70.544	68.7	71.0 (3)	72.118	72.157	72.041	69.4	76.0 (2)	69.1	71.0 (3)	
9	29.261	29.413	30.1	29.0 (4)	26.501	26.651	26.970	28.0	28.0 (3)	30.2	29.0 (4)	
9'	29.210	29.227										
10	16.480	16.657	13.7	15.0 (3)	17.945	18.086	18.244	12.9	20.0 (2)	12.9	20.0 (2)	
rms dev. ^c		0.172	2.43	3.20		0.118	0.642	4.21		4.98		

^aComparing the natural isolated mixture I + II (*ca.* 5 mM) with a synthetic mixture derived from **5** (*ca.* 1 M) in CD₂Cl₂ at 10°C (CD₂Cl₂ = 53.80 ppm), and with pure **6** (*ca.* 1 M) in cyclohexane-d₁₂ at 15°C (solvent = 26.40 ppm); assignments for natural material obtained by CH correlation, including discrimination of 1,1' and 9,9' for the ketone.

^bShift predictions made with SPECTOOL and with WIN-SPECEDIT based on (*n*) spheres of HOSE code; bold highlights data where SPECTOOL predictions favor dihydropyran **6**.

^crms deviation of all shifts relative to those for the natural material.

cosine-bell window functions and zero-filling to give a 512 × 16K matrix with 1.1 Hz/pt in F₂, 59.2 Hz/pt in F₁). The expected one-bond C-C correlations were detected as pairs of doublets at the corresponding DQ frequencies in F₁. Pairs of correlation signals for 21 long-range correlations over 2–4 bonds (¹J_{CC} < 4 Hz) also were detected at the correct DQ frequencies, but only in five cases could the coupling be resolved as a signal splitting.

Finally, two long-range CH correlation experiments, correlation spectroscopy for long-range couplings (COLOC), were performed for **6** with the following parameters: F₂ domain, 17,857 Hz, 8K points; F₁ domain, 680 Hz, 128 increments, (a) 64 or (b) 96 transients per free induction decay; polarization/rephasing delays of (a) 125/62.5 ms (optimal for J_{CH} = 4.0 Hz) or (b) 300/150 ms (optimal for J_{CH} = 1.7 Hz), repetition time (a) 4.92 or (b) 5.18 s, total time (a) 12 h or (b) 18 h; magnitude-mode 2D-FT with sine-bell window functions (shifted π/6) to give a 256 × 4K matrix and 4.26 Hz/pt in F₂, 2.66 Hz/pt in F₁.

¹H and ¹³C chemical shift predictions for **5**, **5b**, and **6** were made using: (i) the SPECTOOL software (version 2.1, Chemical Concepts, Weinheim, Germany) based on empirical shift increment rules; (ii) the WIN-SPECEDIT software (version 961001, Bruker-Franzen, Bremen, Germany) based on data libraries for 1,000 (¹H) or 10,000 (¹³C) compounds and using hierarchically ordered spherical description of environment (HOSE) codes to define atomic environments in spheres.

Molecular modeling of **5**, **5b**, and **6** was performed by using the MM2+SCF force field of CHEM3D (version 3.2, CambridgeSoft, Cambridge MA).

Syntheses. *5-Methyl-hexan-3-ol* [**2**]. A standard Grignard reaction was performed by using magnesium turnings (12.2 g, 0.5 mol) and ethyl iodide (78.4 g, 0.5 mol) in diethyl ether (130 ml). After addition of isovaleric aldehyde **1** [34.6 g, 0.4 mol in diethylether (43 ml)], the reaction mixture was refluxed for 2 h and allowed to cool. Ice (50 g) was added, and the mixture was acidified using 6 M HCl until a clear, homogeneous solution was obtained. The organic layer was separated, and the aqueous layer was extracted twice with 50 ml of diethyl ether. The combined organic layers were washed once with a saturated Na₂S₂O₅ solution, twice with a saturated NaHCO₃ solution, and finally dried with MgSO₄. Evaporation of the solvent afforded a residue, which was purified by distillation under reduced pressure (54°C/2.1 kPa) to yield **2** as a colorless liquid (40 g, 86%).

IR(film): $\bar{\nu}$ (cm⁻¹) = 3,345 (s, broad), 2,958 (vs.), 2,926 (vs.), 2,871 (s), 1,467, 966.

¹H-NMR (CDCl₃): δ = 0.78–0.90 (m; 9 H, CH₃), 1.09–1.49, 1.62–1.78 [m; 5 H, CH₂, (CH₃)₂CH], 2.01 (s; 1H, OH), 3.50 (mc; 1 H, CHOH).

¹³C-NMR (CDCl₃): δ = 9.8, 22.1, 23.5 (all q, CH₃), 24.7 [d, (CH₃)₂CH], 30.8, 46.3 (both t, CH₂), 71.3 (d, CHOH).

5-Methyl-hexan-3-one [**3**]. To a solution of the alcohol **2** (11.6 g, 0.1 mol) in diethyl ether (50 ml), a solution of Na₂Cr₂O₇·2H₂O (9.98 g) and H₂SO₄ (7.5 ml) in water (50 ml) was added at 25°C. After 2 h stirring at room temperature, the organic layer was separated, and the aqueous layer was extracted twice with 25 ml of diethyl ether. The combined organic layers were washed with a saturated NaHCO₃ solution (50 ml), then with water (50 ml), and dried (MgSO₄). The solvent was evaporated and the residue was purified by distillation under reduced pressure (48°C/4.0 kPa) to give the ketone **3** as a colorless liquid (6.8 g, 60%).

IR(film): $\bar{\nu}$ (cm⁻¹) = 2,959 (vs.), 2,874 (s), 1,714 (vs.), 1,464 (s), 1,412, 1,368 (s), 1,105.

¹H-NMR (CDCl₃): δ = 0.90 (d, J = 6.6 Hz; 6 H, CH₃), 1.03 (t, J = 7.3 Hz; 3 H, CH₃), 2.13 (mc; 1 H, CH), 2.27 (d, J = 6.9 Hz; 2 H, CH₂CH), 2.39 (q; J = 7.3 Hz; 2 H, CH₂CH₃).

¹³C-NMR (CDCl₃): δ = 7.8, 22.6 (both q, CH₃), 24.7 [d, (CH₃)₂CH], 36.4, 51.5 (both t, CH₂), 211.5 (s, CO).

2,5,8-Trimethyl-7-nonen-4-one [**4**]. A solution of diisopropylamine (1.95 g, 19.3 mmol) in tetrahydrofuran (20 ml) was stirred under nitrogen at –5°C. A solution of butyllithium (13.15 ml, 1.6 M in hexane, 21 mmol) was added. A solution of the ketone **3** (2 g, 17.5 mmol) in tetrahydrofuran (10 ml) was added dropwise at –70°C under nitrogen, and stirring was continued for 15 min. Then a solution of 3,3-dimethylallyl bromide (2.6 g, 17.5 mmol) in tetrahydrofuran (5 ml) was added at –70°C, and the mixture was stirred for 40 min under nitrogen without further cooling. The resulting mixture was added to ice/water (50 ml) and acidified by addition of 2 M HCl. The organic layer was separated, and the aqueous layer was extracted with 3 × 50 ml CH₂Cl₂. The combined organic layers were washed with a saturated NaHCO₃ solution and dried (MgSO₄). The solvent was evaporated under reduced pressure to afford the crude product, which was purified by flash chromatography in CH₂Cl₂ on silica gel 60 (230–400 mesh; Macherey & Nagel). The ketone **4** was obtained as a colorless oil (1.7 g, 53%).

TLC analysis: R_f value, CH₂Cl₂, 0.63.

¹H-NMR (CDCl₃): δ = 0.88 [d, J = 6.6 Hz; 6 H, (CH₃)₂CH], 1.02 (d, J = 6.9 Hz; 3 H, CH₃CH), 1.39, 1.66 (br. s; 3 H each, allylic CH₃), 1.95–2.60 (m; 6 H, CH, CH₂), 5.02 (mc; 1 H, vinyl-H).

¹³C-NMR (CDCl₃): δ = 15.9 (q, CH₃), 17.7 (q, CH₃), 22.58, 22.60 (q, CH₃; d, CH), 24.2, 25.7 (q, CH₃), 31.4 (t, CH₂), 46.8 (d, CH), 50.5 (t, CH₂), 121.6 (d, vinyl-CH), 133.4 (s, vinyl-C), 214.2 (s, CO).

2,5,8-Trimethyl-8-hydroxy-nonan-4-one [5]. Trifluoroacetic acid (95% vol/vol; 3.4 ml) was added to the olefin **4** (500 mg, 2.7 mmol) at 0°C. After stirring for 40 min at room temperature, 5 M KOH (17.5 ml) was added at 0°C. After stirring for 15 h at room temperature the resulting mixture was extracted with 3 × 15 ml CH₂Cl₂. The combined organic layers were washed twice with 20 ml of water, dried (Na₂SO₄), and evaporated under reduced pressure. The residue was purified by flash chromatography [hexane/ethyl acetate 6:4 (vol/vol)]. The ketone **5** was obtained as a colorless oil (330 mg, 60%) and contained <2% of the dihydropyran **6** as assessed by NMR.

TLC analysis: R_f values, CH₂Cl₂, 0.06; hexane/ethyl acetate 6:4 (vol/vol), 0.32.

NMR data for **5** are presented in Tables 1 and 2.

In a pilot experiment, the following HPLC procedure was used instead of flash chromatography. Crude material (80 mg) was suspended in 0.3 ml of 70% methanol/water, filtered through a 100-mg Bakerbond spe* C18 solid-phase extraction column, and diluted to 60% methanol/water. Portions of 15- μ l (corresponding to ca. 1.9 mg of crude material) were loaded onto a reversed-phase column (Ultropac TSK ODS-120T, 5 μ m, 25 × 4.6 mm; LKB) and eluted with 60% methanol/water at 1 ml/min (retention time for **5** was 14–17 min). The material from several runs was extracted with 5 × 20 ml of cyclopentane, pooled, dried (Na₂SO₄), and evaporated to give **5**. This procedure resulted in a lower yield and partial conversion of **5** to **6** (53%).

3,4-Dihydro-2,2,5-trimethyl-6-(2-methylpropyl)-2H-pyran [6]. To a stirred solution of the ketone **5** (250 mg, 1.25 mmol) in cyclohexane (2 ml), *p*-toluenesulfonic acid monohydrate (ca. 5 mg) and MgSO₄ (220 mg) were added at room temperature. Stirring was continued for 45 min, and the resulting mixture was purified by flash chromatography [hexane/ethyl acetate 6:4 (vol/vol)] to give the dihydropyran **6** as a colorless oil (140 mg, 62%).

TLC analysis: R_f values, CH₂Cl₂, 0.62; hexane/ethyl acetate 6:4 (vol/vol), 0.74.

ESI-MS, in acetonitrile (positive-ion mode): m/z = 182.8 [M + H]⁺; high-resolution EI-MS, in cyclohexane-d₁₂: m/z = 182.1678 [M⁺] corresponds to C₁₂H₂₂O with a measured minus exact mass difference of Δm = 0.73 mDa.

NMR data are presented in Tables 1–3.

RESULTS

Structure Elucidation of the Natural Pheromone. The purification procedure described in detail elsewhere (15) yielded

Table 3. C-C connectivities in the dihydropyran **6**

Sequential	¹ J _{CC} , Hz	Long-range	Bonds (<i>n</i>)	ⁿ J _{CC} , Hz
1, 2	35.2	3, 5	2	3.5
2, 3	33.8	3, 6	3	<3
3, 4	53.0	3, 8	3	<2
4, 5	83.8	4, 6	2	<3
5, 6	40.9	4, 7	3	2.5
6, 7	32.9	4, 8	2	<2
7, 8	35.8	4, 9	3	<2
8, 9	40.4	4, 10	2	3
5, 10	48*	5, 8	3	3.7

Couplings determined from DQ-2D correlation matrix; digital resolution 1.1 Hz/pt; bold marks long-range connectivities that can only occur in a cyclic structure.

*Echo time 15 ms, digital resolution 4.4 Hz/pt.

an active pheromone preparation that gave a single peak upon GC analysis. The mass spectrum (EI) of this GC peak was identical with the mass spectrum obtained from the original preparation (before GC separation, data not shown), suggesting that the preparation was homogenous. The positive-ion ESI-MS spectrum of the purified pheromone in pentane/methyl acetate/isopropanol (4:1:5) showed a single predominant peak at m/z = 223.1, assigned to [M + Na]⁺ and confirmed by collision-induced dissociation with release of a daughter ion with m/z = 23.0 [Na⁺]. Thus, the nominal molecular mass was determined to be 200 Da. High-resolution mass analysis of the two largest fragments observed in the conventional EI-MS spectrum gave masses of 185.1558 ([M – CH₃]⁺ = C₁₁H₂₁O₂, Δm = 1.6 mDa) and 182.1647 ([M – H₂O]⁺ = C₁₂H₂₂O, Δm = –2.4 mDa). The FT GC-IR spectrum (Fig. 1) exhibited a carbonyl band at 1,703 cm^{–1} and a hydroxyl group absorption at 3,380 cm^{–1}. These data indicated that the pheromone is a hydroxy ketone with the molecular formula C₁₂H₂₄O₂ (molecular mass = 200 Da).

Initial ¹H-NMR studies were performed with a small quantity (ca. 0.2 μ mol) of purified pheromone. The one-dimensional ¹H-NMR spectrum showed signals only in the narrow range of 0.8–2.6 ppm, indicative of aliphatic CH protons without geminal oxygen neighbors. Integration of the spectrum revealed that, unexpectedly, two substances were present in a ratio of 3:1. The major component, called compound I, was found by COSY 2D NMR to be composed of the following two fragments or spin systems: –C-CH₂-CH(CH₃)₂ with three unfilled valences and CH₃-C-CH₂-CH₂-C-(CH₃)₂ with three unfilled valences. The sum of these fragments is C₁₂H₂₂, indicating that two –OH groups were not accounted for, but resonances for hydroxyl protons were not detected because of the relatively large amount of residual water present (δ = 1.505 ppm). All chemically equivalent protons were also magnetically equivalent, suggesting that compound I was an open-chain molecule. Thus, the enol **5b** (Fig. 2) was our first hypothesis for I.

The minor compound II exhibited a total of 23 nonexchangeable protons and several of the structural features found for I. However, the spin systems were complex with nonequivalence of methylene protons and isopropyl methyls. Because of the small sample quantity, the COSY data for II were inconclusive, and no detectable ¹³C-NMR spectrum could be obtained in a 24-h acquisition. After 3 months of storage at 4°C the ratio I/II had changed from initially 3.0 to 0.88, clearly indicating the interconvertibility of the two forms.

Considerable effort was then made to obtain sufficient material from 8 × 10¹³ cells for ¹³C-NMR. The resulting NMR sample contained about 2 μ mol with which it was possible to perform detailed ¹H-NMR, COSY 2D, ¹³C-NMR, ¹³C-DEPT, and CH 2D correlation experiments (see *Materials and Methods*, Fig. 3 *Lower*, Tables 1 and 2). The analysis showed initially a 4:1 ratio for I/II and confirmed that I must be either the enol **5b** or possibly the cyclic dihydropyran **6**. Compound II was

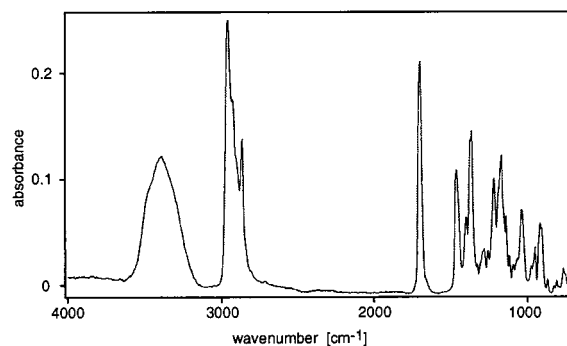


Fig. 1. The FT GC-IR spectrum of the cryotrapped pheromone stigmolone isolated from *S. aurantiaca*.

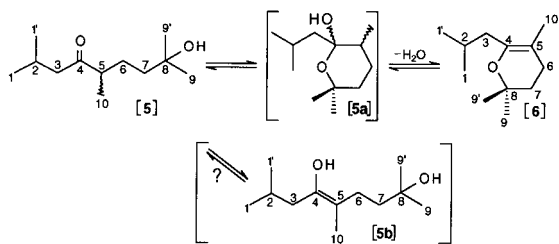


FIG. 2. Possible equilibria involving the ketone **5** (stigmolone), the hemiacetal **5a**, the enol **5b**, and the dihydropyran **6**. The numbering scheme used for NMR assignments is shown, and wavy bonds indicate indeterminate stereochemistry (mixture of enantiomers).

unequivocally identified as the open-chain ketone **5** with tertiary hydroxyl function. The magnetic nonequivalence observed at all methylene and methyl sites for this molecule is caused by the presence of the ketone function (diamagnetic susceptibility effects) at C4 next to the asymmetric center C5.

The assignment of compound I to the enol **5b** or the dihydropyran **6** could not be made with certainty because both substances feature the same basic spin systems for nonexchangeable ^1H and ^{13}C . The simplicity of the spectrum favored the open-chain form **5b**, but chemically **6** made more sense, e.g., if the purification and drying procedures using organic solvents drives the equilibrium from **5** toward **6** (Fig. 2). ^{13}C chemical shift predictions provided evidence favoring the dihydropyran **6**, especially for C3, C5, C6, and C9 (Table 2), whereas ^1H predictions were equivocal. Molecular modeling showed that the dihydropyran ring can adopt two conformations of equal energy with either the 9 or the 9' methyl in a pseudo-axial position. Rapid interconversion of these conformers and the axial and equatorial positions at C6, C7, C8

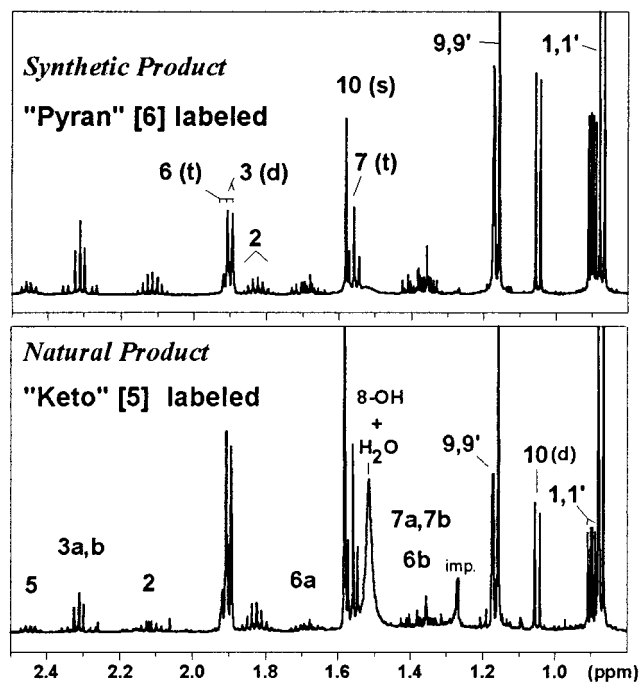


FIG. 3. Comparison of the 500-MHz ^1H -NMR spectra of the natural pheromone stigmolone (Lower, ca. 2 μmol in 0.4 ml CD_2Cl_2 at 30°C) and the synthetic ketone **5** (Upper, 50 μmol in 0.4 ml CD_2Cl_2 at 30°C). Both spectra show a mixture of species: I/II (6/5) ratio = 4.0 (natural product) or 1.15 (synthetic), as a result of a partial conversion of the ketone **5** (spin system II labeled in the lower spectrum) to the dihydropyran **6** (spin system I labeled in the upper spectrum) during purification (see Fig. 2).

would be necessary to create a pseudo plane of symmetry, resulting in the simple spectrum observed for compound I.

To clarify the structure of I and to determine unambiguously whether I or II is in fact the pheromone, authentic compounds **5** and **6** were prepared via organic synthesis.

Synthesis and NMR Analysis of 2,5,8-Trimethyl-8-hydroxynonan-4-one [5]. The ketone **5** was synthesized via the procedure outlined in Fig. 4 (see *Materials and Methods*). The Grignard addition of ethyl magnesium iodide to isovaleric aldehyde **1** gave the racemic alcohol **2**, which then was oxidized to the ketone **3**. Enolate formation under kinetic control at -70°C and subsequent reaction with 3,3-dimethylallyl bromide gave the desired olefin **4**, with almost perfect regioselectivity of the alkylation. For the *Markovnikov* hydration of the olefin, **4** was treated with trifluoroacetic acid (yielding initially the corresponding trifluoroacetate), followed by alkaline hydrolysis to the tertiary alcohol **5**.

A 10-mg sample of synthetic **5**, purified by RP-HPLC (pilot experiment, see *Materials and Methods*) and dissolved in 0.4 ml of CD_2Cl_2 , gave the ^1H -NMR spectrum shown in Fig. 3 (Upper). Again, compounds I (dihydropyran) and II (ketone) were observed, and their signals were essentially superimposable on those obtained from the natural product (see Tables 1 and 2). The only difference was that the ratio I/II (1.15) differed from that of the natural isolated product. Positive-ion ESI-MS analysis of the NMR sample diluted in chloroform/methanol (1:1) or acetonitrile gave major peaks at $m/z = 423.3 [2\text{M}_I + \text{Na}^+]$, $223.1 [\text{M}_I + \text{Na}^+]$, and $183.0 [\text{M}_{II} + \text{H}^+]$ or $[\text{M}_I - \text{OH}]^+$.

The synthesis strategy chosen should have produced only the ketone **5**, but we suspected that the work-up procedure was causing a partial conversion to **6** (Fig. 2). Therefore, a second, larger batch of synthetic **5** was purified by flash chromatography. A sample of the neat liquid gave a ^1H -NMR spectrum (not shown), which clearly corresponded to the spectrum of compound II with <2% of **6** present. In addition, a one-proton signal from the 8-OH group was observed at 4.15 ppm. A ca. 130-mg sample of the synthetic material was dissolved in CD_2Cl_2 to a volume of 0.38 ml. The ^1H -NMR spectrum (10°C) showed again the characteristic spectrum of **5** with the 8-OH

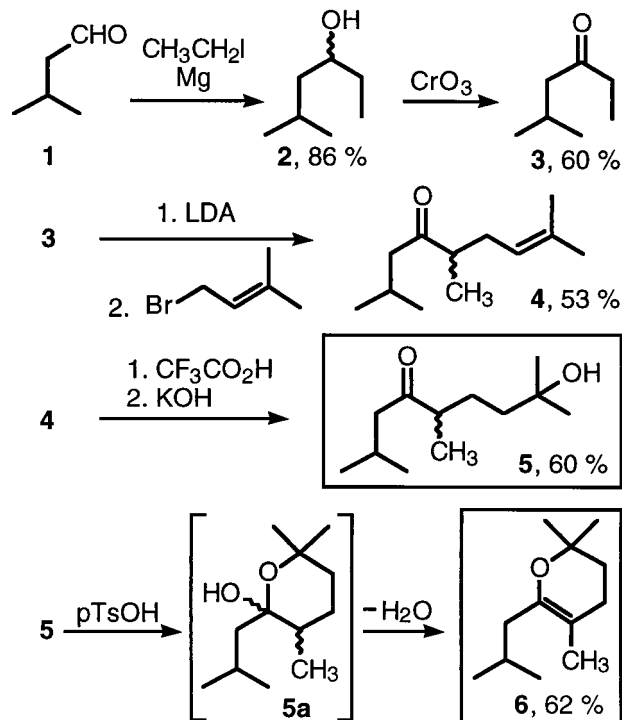


FIG. 4. Chemical synthesis of the *Stigmatella* pheromone stigmolone **5** and the dihydropyran **6**.

signal appearing at 2.9 ppm. Initially, **6** was not detectable (<0.5%), but after 3 days reached a level of *ca.* 2.5%.

An aliquot of this NMR sample (*ca.* 50 μ l) was extracted with 0.5 ml of D₂O. The ¹H-NMR spectrum of the D₂O phase (not shown) corresponded to the ketone **5** (II) with some minor differences in chemical shifts (Table 1). At pH 5 I increased to *ca.* 7% after 7 days. The pH then was lowered to 1.9 (trifluoroacetic acid); the H5 signal for the ketone **5** decreased to 17% of its nominal intensity after 3.5 h (30°C) and 4% after 6 h (deuterium exchange); the H10 methyl group appeared as a singlet. The dihydropyran **6** was reduced to 1–2%, indicating that the acid-catalyzed equilibrium strongly favored **5** over **6**.

Synthesis and NMR Analysis of 3,4-Dihydro-2,2,5-trimethyl-6-(2-methylpropyl)-2H-pyran [6]. To verify dihydropyran **6** as the structure of compound I a large quantity of this material was synthesized unambiguously by cyclization of **5**, most likely via **5a**, in the presence of catalytic amounts of acid plus a dehydrating agent. The purified dihydropyran **6** was found to be more stable in cyclohexane than in methylene chloride. A concentrated solution of **6** in cyclohexane-d₁₂ (*ca.* 50 mg in 0.35 ml) gave a ¹H-NMR spectrum characteristic for I with only minor differences in chemical shifts (solvent effects, Table 1, rms deviation = 0.026 ppm). The ¹³C-NMR spectrum of **6** agreed reasonably well with the data for I, despite differences in concentration, solvent, and temperature (Table 2); ketone **5** was not detected (<1%).

INADEQUATE DQ-2D experiments were performed for analyzing C-C connectivities over 1–4 bonds (Table 3) and COLOC 2D experiments examined long-range CH correlations. A total of 21 long-range C-C connectivities were detected with ¹J_{CC} < 4 Hz. The two-bond connectivity between C4 and C8 and the three-bond connectivities C3,C8 and C4,C9 can occur only in the cyclic dihydropyran structure **6** and not in the alternative enol structure **5b**. A total of 26 long-range CH connectivities were detected with COLOC, including the ⁴J_{HCCOC} between H9 and C4, which is unique for structure **6**. Finally, the solution used for NMR was submitted to ESI-MS and high-resolution EI-MS, confirming the molecular formula of C₁₂H₂₂O for **6**.

Biological Activity of Compounds 4, 5, and 6. The pheromone activity of **4**, **5**, and **6** was tested in our standard bioassay. Both the isolated natural pheromone and **5** were active at concentrations of about 1 nM (15). The enone **4** was inactive over the range 1 nM to 1 μ M. The dihydropyran **6** was inactive at 10 nM but showed essentially full activity at 100 nM, most likely because of the presence of a small amount of ketone **5** formed in aqueous solution (Fig. 2).

DISCUSSION

Synthetic **5** and **6** have been shown by NMR to be identical to compounds II and I, respectively, which were detected in CD₂Cl₂ solutions of concentrated, purified natural pheromone. The possibility that I was the enol **5b** could be ruled out as follows: (i) no enol-OH signal was detected for I, (ii) the NMR properties of synthetic **6** closely matched those of I, and (iii) the dihydropyran ring for **6** was confirmed conclusively by long-range connectivities.

An unexpected aspect of this work was the discovery that the procedures used to purify the natural pheromone resulted in significant conversion of II (**5**) to I (**6**) in organic solvents. In fact, pure synthetic **5** could be prepared only when flash chromatography was used without a final drying step. The equilibrium of Fig. 2 is quite slow in organic solvents or neutral aqueous solution but has a half-life for exchange of H5 of only a few hours at pH 2. The acid-catalyzed hydrolysis of dihydropyran derivatives to the corresponding hydroxy ketones was described earlier (18). The predominance of **5** in dilute aqueous solution can be explained by a shift of the equilibrium from **6** toward **5** caused by the high concentration of water. It is reasonable that the hemiacetal **5a** and/or the enol **5b** serve as

intermediates in this process, and they may be responsible for some of the impurities detected by NMR at a level of 1–3%.

The synthetic ketone **5** and the natural isolated pheromone exhibit the same specific activity in the bioassay, whereas **4** and **6** are inactive. This finding suggests that the ketone and hydroxyl functional groups of **5** may be important for its interactions with a putative receptor (specificity). We have named the hydroxy ketone **5** stigmolone, because of its biological origin and the functional groups present. Stigmolone is a new type of prokaryotic pheromone. Up to now, aliphatic (hydroxy) ketones and alcohols as signal substances have been found only in insects (19).

Note that stigmolone contains one asymmetric center at C5; however, the configuration of the natural material and the possible stereospecificity of a putative receptor are unknown. The synthetic material is racemic, and the extensive isolation and purification procedures for obtaining natural material most likely also lead to racemization via the equilibrium of Fig. 2. Rapid epimerization under mild conditions at an equivalent position in a similar hydroxy ketone, 4,6-dimethyl-7-hydroxynonan-3-one, has been studied (20, 21). Thus, it is not surprising that natural and synthetic materials have the same biological activity.

With the chemical synthesis presented here, large amounts of the highly active pheromone stigmolone now can be made available, even in isotopically labeled forms. Therefore, we hope to be able to identify the stigmolone receptor in the near future and are confident that other possible physiological effects of stigmolone can be investigated.

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1. Karlson, P. & Lüscher, M. (1959) *Nature (London)* **183**, 55–56.
2. Konijn, T. M., Van De Meene, J. G. C., Bonner, J. T. & Barkley, D. S. (1967) *Proc. Natl. Acad. Sci. USA* **58**, 1152–1154.
3. Butenandt, A., Beckmann, R. & Stamm, D. (1961) *Hoppe-Seyler Z. Physiol. Chemie* **324**, 84–87.
4. Wirth, R., Muscholl, A. & Wanner, G. (1996) *Trends Microbiol.* **4**, 96–103.
5. Dunny, G. M. & Leonard, B. A. B. (1997) *Annu. Rev. Microbiol.* **51**, 527–564.
6. Jaenicke, L. (1991) in *Progress in Botany*, eds. Behnke, H., Esser, K., Kubitzki, K., Runge, M. & Ziegler, H. (Springer, Berlin), Vol. 52, pp. 138–189.
7. Starr, R. C., Marnier, F. J. & Jaenicke, L. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 641–645.
8. Boland, W. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 37–43.
9. Kelly, D. R. (1996) *Chem. Biol.* **3**, 595–602.
10. Rasmussen, L. E. L., Lee, T. D., Roelofs, W. L., Zhang, A. & Daves, G. D. (1996) *Nature (London)* **379**, 684.
11. Swift, S., Bainton, N. J. & Winson, M. K. (1994) *Trends Microbiol.* **2**, 193–198.
12. Shimkets, L. J. (1990) *Microbiol. Rev.* **54**, 473–501.
13. Dworkin, M. (1996) *Microbiol. Rev.* **60**, 70–102.
14. Stephens, K., Hegeman, G. D. & White, D. (1982) *J. Bacteriol.* **149**, 739–747.
15. Plaga, W., Stamm, I. & Schairer, H. U. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 11263–11267.
16. Qualls, G. T., Stephens, K. & White, D. (1978) *Dev. Biol.* **66**, 270–274.
17. Hull, W. E. (1994) in *Two-Dimensional NMR Spectroscopy*, eds. Croasmun, W. R. & Carlson, R. M. K. (VCH, New York), 2nd Ed., pp. 67–456.
18. Boeckman, R. K. & Bruza, K. J. (1977) *Tetrahedron Lett.* **48**, 4187–4190.
19. Mori, K. (1996) *Biosci. Biotech. Biochem.* **60**, 1925–1932.
20. Chan, P. C.-M., Chong, J. M. & Kousha, K. (1994) *Tetrahedron* **50**, 2703–2714.
21. Mori, K. & Watanabe, H. (1985) *Tetrahedron* **41**, 3423–3428.