DOI: 10.1002/ejoc.200901126

Carolacton – A Macrolide Ketocarbonic Acid that Reduces Biofilm Formation by the Caries- and Endocarditis-Associated Bacterium *Streptococcus mutans*

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Dedicated to Heinz G. Floss on the occasion of his 75th birthday

Keywords: Antibiotics / Structure elucidation / Molecular modeling / Natural products

The macrolide ketocarbonic acid carolacton (**1**) was isolated from the myxobacterium *Sorangium cellulosum*, strain So ce960, because of its antibiotic activity. Subsequently, carolacton (**1**) was discovered to be a highly potent agent against biofilms containing the caries- and endocarditis-associated bacterium *Streptococcus mutans*. The 2D structure of **1** was elucidated by HRMS, IR and 2D NMR spectroscopy. Initially, the stereogenic centres were determined by chemical derivatization in combination with computional methods and finally verified by X-ray analysis.

Introduction

Bacterial biofilms often evade host defenses and are inherently resistant to antimicrobial agents. These characteristics impose severe health threats, either because of biofilm growth on natural tissues or on medical devices and implants. Since chronic infectious diseases, such as endocarditis and periodontitis, or persistent nosocomial infections of implants such as stents, bone implants or artificial valves, are associated with microbial biofilms, their eradication has become a major concern in clinical treatment.[1] Because antibiotic resistance of bacteria in biofilms is found to be approximately 1000-fold higher than in planctonic form, there is an urgent need to develop new methods or tools to – ideally – selectively destroy clinically relevant biofilms.[2]

During our screening efforts to identify antibiotics from myxobacteria,[3] we discovered carolacton (**1**) in the extract of a *Sorangium cellulosum* strain in 1998,[4] because of its activity against the antibiotic-sensitive *E. coli* strain *tolC*.[5] The MIC value for **1** was determined to be 0.06 μg/mL against *E. coli tolC*, whereas other bacteria were insensitive up to a concentration of 40 μg/mL. Minor antifungal activity was observed against *Aspergillus niger*, *Pythium debaryanum* and *Sclerotina sclerotiorum* in the range of 16 to 20 μg/mL.[6] Carolacton (**1**) showed no acute toxicity in cell culture assays with L929 mouse fibroblasts.[7] The carbon skeleton of **1** was protected by a Japanese patent as a minor analog of the antifungal agent YA2-M in 2002.[8]

In a subsequent screening project based on purified metabolites from myxobacteria, carolacton (**1**) was identified as a highly potent agent that severely reduced the number of viable cells in biofilms at nanomolar concentrations. Biofilms containing the caries and endocarditis-associated bacterium *Streptococcus mutans* were sensitive at 0.005 μg/mL (at which concentration approximately 35% of the cells within the biofilm die; at 0.025 μg/mL, 66% die), while planktonic cultures were nearly insensitive to **1**.[9] Here, we report on the isolation, structure elucidation, stereochemical analysis and antibiotic activity of carolacton (**1**) produced by *S. cellulosum*, strain So ce960.

Results and Discussion

For production of carolacton (**1**), *S. cellulosum* strain So ce960, was cultivated in the presence of Amberlite XAD 16 in a medium described recently.[10] The resin from 100 L of fermentation broth was harvested by sieving and washed with aqueous 30% methanol to remove polar by-products. The crude extract (16.6 g) was collected after extraction of

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.200901126.

the XAD resin with 100% methanol, partitioning between methanol and *n*-hexane and concentration of the methanol phase. Further enrichment of the biological activity was achieved by gel chromatography on Sephadex LH-20 and by repeated RP MPLC to finally yield pure carolacton (**1**; 275 mg).

The molecular composition was determined by high-resolution MS, which matched well with the NMR spectroscopic data. From the empirical formula $C_{25}H_{40}O_8$, six double bond equivalents were calculated. Two, highly abundant carbonyl bands (ν = 1731 and 1715 cm^{-1}) in the IR spectrum of **1**, together with the corresponding carbon signals in the ^{13}C NMR spectrum at δ = 172, 175 and 213 ppm (Table 3), indicated the presence of an ester or lactone moiety, respectively, as well as a keto group. The constitution of carolacton (**1**) was finally determined by exploiting several NMR spectroscopic methods. The $^{1}H,^{13}C$ HMQC spectra revealed the correlation of 37 protons and their adjacent carbon atoms, leaving three exchangeable protons.

The correlations in the $^{1}H,^{1}H$ COSY NMR spectra allowed three structural units A, B, and C to be established (see Figure 1), and the interconnections between these units were determined through the relevant $^{1}H,^{13}C$ HMBC correlations. Since both oxymethine termini (at C9 and C18) of the main unit A were determined to be next neighbors to the carboxy group at C19 (δ_C = 175.91 ppm), the macrolide ring could unequivocally be established. The acylation-dependent downfield shift of 9H (δ_H = 4.76) designated the direction of ring closure. Additional correlations of C9 with the methyl and methine protons at C23 and C7 allowed the substructures A and B to be connected. Structural element B was initially elucidated from vicinal and long-range correlations in the COSY spectrum to be associated with unit C through the carbonyl group at C5, which was in agreement with the HMBC correlations (Figure 1). The HMBC spectrum also revealed correlations between the remaining methoxy (C20) and carboxyl group at C1, thus completing the carbon skeleton of carolacton (**1**). Hence, oxymethines C17 and C18, as well as the carboxy group at C1, remained as those positions bearing exchangeable protons.

The *trans* configuration of the double bonds was assigned from the vicinal coupling constant of 15.5 Hz for the $\Delta^{15,16}$ bond, while ROESY correlations between 6H and $23H_3$, as well as 7H and 9H, were utilized to determine the *E* configuration at the $\Delta^{7,8}$ bond.

The assignment of configuration for all the stereogenic centers initially relied on the chemical derivatization of **1**. To our delight, we found that treatment of **1** with *p*-bromobenzoyl chloride led to macrolactonization of the carboxy group at C1 with the C18 hydroxy group leading to bislactone **2** in very good yield. It is worth noting that this transformation resembles a Yamaguchi protocol (Scheme 1).[11] NMR analysis supported the expected overall rigid conformation, which could be employed to further elucidate the structure in combination with computational methods.

Advantageously, this transformation also protected one hydroxy group of the diol at C17 and C18 – a prerequisite

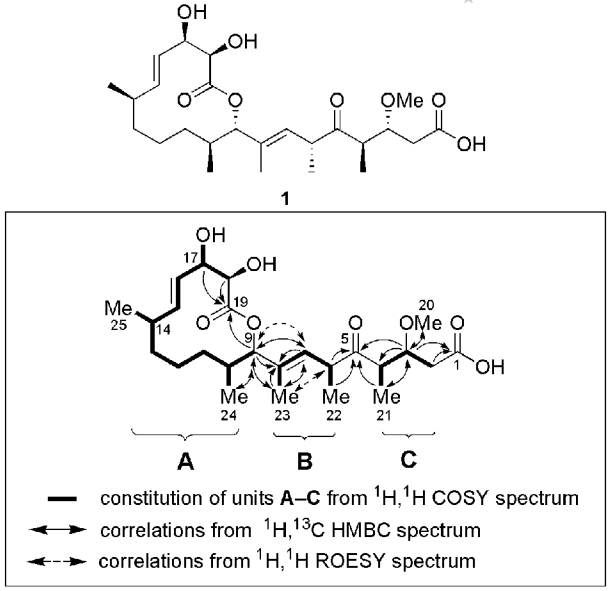
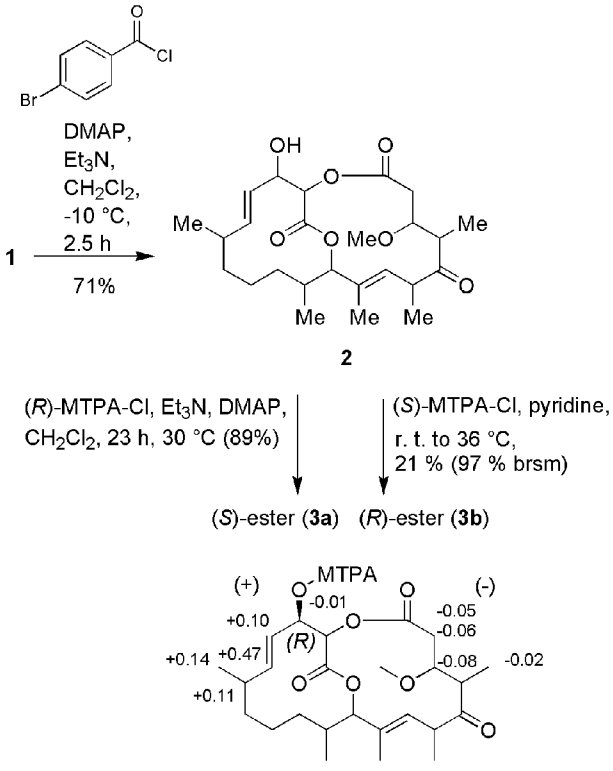


Figure 1. Structure of carolacton (**1**) and NMR assignments.



Scheme 1. Macrolactonization of **1**, Mosher ester formation and relevant $\Delta\delta^{SR}$ values; brsm: based on recovered starting material.

for the determination of the absolute configuration using the Mosher ester strategy.[12] Hence, bicyclic derivative **2** was treated with both enantiomeric methoxy(trifluoromethyl)phenylacetyl chlorides (MTPA-Cl) to form the diastereomeric esters **3a** and **3b** (Scheme 1). Applying the Mosher analysis rules, the absolute configuration at C17 was determined from the calculated $\Delta\delta^{SR}$ values to be (*R*). The relative configurations for all the stereogenic centers of

the bislactone **2** were initially established by a combination of extended NMR analyses and molecular modeling studies (MMFFs; see also the Supporting Information).

Particular focus was first given to the 12-membered ring of carolacton (**1**) and the $^3J_{HH}$ coupling constants between 9-H/10-H, 14-H/15-H, 16-H/17-H and 17-H/18-H. The small coupling constants (2–4 Hz) between 16-H/17-H and 17-H/18-H correlate well with a dihedral angle of about 60°, while the large coupling constants (≈ 10 Hz) between 9-H/10-H and 14-H/15-H refer to dihedral angles of about 160–180°. On the basis of these restrictions, three possible diastereomers (1st isomer: 9*S*, 10*S*, 14*R*, 17*R*, 18*R*; 2nd isomer: 9*S*, 10*S*, 14*R*, 17*R*, 18*S*; 3rd isomer: 9*S*, 10*S*, 14*S*, 17*S*, 18*S*) were selected for further analysis (Table 1).

Table 1. Dihedral angle of energy-minimized isomers of lactone ring C9–C19.

Protons	$^3J_{HH}$	Dihedral angle ϕ of isomers 9*S*,10*S*,14*R*, 17*R*,18*R*	9*S*,10*S*,14*R*, 17*R*,18*S*	9*S*,10*S*,14*S*, 17*S*,18*S*
17-H/18-H	≈ 4 Hz	54°	69°	57°
16-H/17-H	≈ 2 Hz	58°	58°	38°
14-H/15-H	≈ 10 Hz	176°	177°	176°
9-H/10-H	≈ 10 Hz	174°	178°	177°

The presence of the additional 13-membered ring in bislactone **2** paved the way for the inclusion of the side chain at C-9 of **1** into the structural analysis. NOESY spectra provided additional distance information that restricted the number of potential isomers. In the Monte Carlo searches, the dihedral angle between atoms 3-H/4-H with a large $^3J_{HH}$ coupling constant (≈ 10 Hz) was restricted to 160–180°, whereas the distances between the atom pairs 4-H/7-H and 7-H/10-H were constrained to between 2 and 4 Å, to take into account their observed NOE couplings. The calculated structures for all possible diastereomers were then analyzed for discrepancies between their configurations and their observed NMR spectroscopic data (i.e. vicinal ^{1}H coupling constants and NOE couplings). From these structural considerations, two diastereomers **2a** and **2b** emerged that only differed in the absolute configuration of one of the stereodomains (C3–C10, right-hand circle) with respect to the established domain (C11–C18, left-hand circle) (Figure 2). Importantly, the initial assignment of the stereochemistry of the lactone ring C9–C19 was thus confirmed.

In diastereomer **2b** the distance of 4 Å between 12-H_a/24-H_3 is exceptional large; in contrast, relevant NOE-contacts (Figure 2) in the second diastereomer **2a** correspond to distances smaller than 3 Å (Table 2). Assembling all the data collected, the complete relative and absolute configuration of **1** could be assigned as shown in diastereomer **2a**.

Finally, we were able to crystallize carolacton (**1**) from ethyl acetate/petroleum ether. The absolute configuration of **1**, as the refined result of the X-ray single-crystal structure determination, is depicted in Figure 2.[13,14] The relative configuration defined by the spectroscopic and chemical investigations were confirmed by this X-ray analysis. In the solid state, the packing of the molecules is characterized by

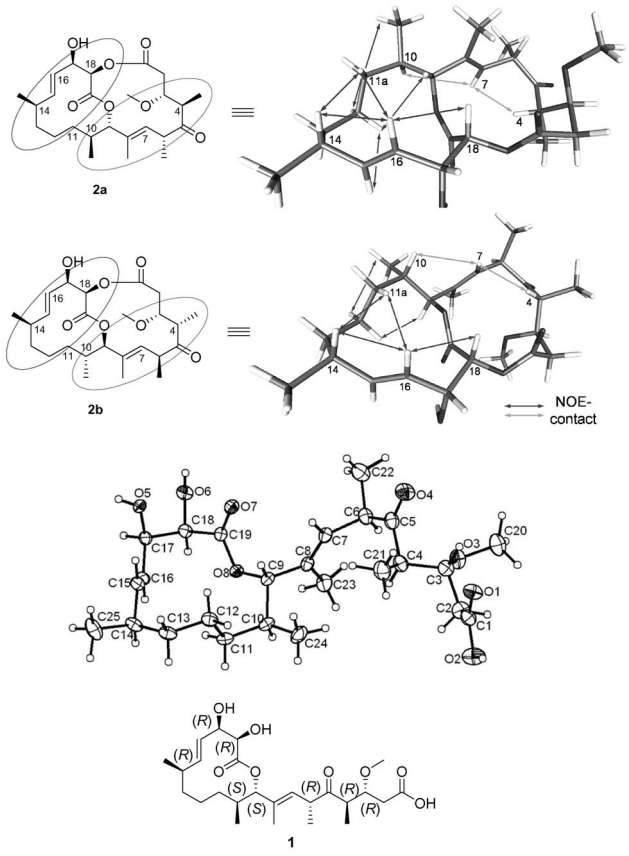


Figure 2. Isomers **2a** and **2b** resulting from modelling studies on bislactone **2** and selected NOE-contacts (numbering refers to Figure 1). X-ray analysis[12] and fully assigned structure of carolacton (**1**).

Table 2. Relevant coupling constants and NOE-contacts of diastereomers **2a** and **2b**.

Protons	$^3J_{HH}$	Dihedral angle **2a**	**2b**	Protons	Distances [Å] **2a**	**2b**
17-H/18-H	≈ 3 Hz	61°	61°	16-H/18-H	2.6	2.7
16-H/17-H	≈ 2 Hz	54°	46°	14-H/16-H	2.4	2.4
14-H/15-H	≈ 10 Hz	171°	177°	16-H/11-H_a	2.9	2.9
9-H/10-H	≈ 12 Hz	176°	159°	15-H/12-H_b	2.7	2.8
6-H/7-H	≈ 10 Hz	153°	178°	14-H/11-H_a	2.4	2.3
3-H/4-H	≈ 10 Hz	178°	175°	12-H_a/24-H_3	2.4	4.0
				12-H_b/9-H	2.2	3.1
				10-H/7-H	2.7	3.1
				7-H/4-H	2.3	2.6

intermolecular hydrogen bridges linking the carboxy groups O1 and O2 with the hydroxy groups at O5 and O6 of the lactone ring (see Figure 2). Interestingly, our conformational analysis of **1** utilizing NMR spectroscopic data combined with molecular modelling, reveal that the conformation of **1** is close to identity both in solution and in the crystal. Diagnostic data in solution are the expected large vicinal coupling constants between the *trans*-oriented proton pairs 3H/4H, 6H/7H, 9H/10H, and 14H/15H.[15] In addition, the largest NOEs in the ROESY spectrum were observed for the proton pairs 9H/7H, 6H/23H_3 and 3H/20H_3 as well as 10H/23H_3, and 9/12b.

Conclusions

In conclusion, we disclose the isolation and structure elucidation of carolacton (**1**), a metabolite produced by the myxobacterium *Sorangium cellulosum* strain So ce960, by using NMR spectroscopy combined with molecular modeling, chemical derivatization and X-ray analysis. Importantly, carolacton (**1**) shows promise as a lead structure for the development of compounds that can reduce the number of viable cells in biofilms of the caries- and endocarditis-associated bacterium *Streptococcus mutans*.

Ongoing work in our laboratories is aimed at developing carolacton analogs with superior activity, and at elucidating the mode of action of this fascinating novel class of natural products.

Experimental Section

General: Optical rotations were measured with a Perkin–Elmer 241 MC polarimeter. UV/Vis spectra were recorded with a Shimadzu UV/Vis scanning spectrometer UV-2102 as solutions in methanol [Uvasol, (Merck)]. IR spectra were recorded with a Nicolet FT-IR spectrometer 20 DXB. NMR spectra were obtained with a Bruker spectrometer DMX 600 (^{1}H: 600.1 MHz, ^{13}C: 150.9 MHz), DRX-500 (^{1}H: 5400 MHz, ^{13}C: 125 MHz) or DPX-400 (^{1}H: 400 MHz, ^{13}C: 100 MHz); internal standard was the solvent signal. MS (EI at 70 eV; DCI with isobutane) were recorded with a Finnigan spectrometer MAT 95; resolution $M/\Delta M$ = 1000; high-resolution data from peak matching ($M/\Delta M$ = 10000). MIC values were determined by serial dilution according to published procedures.[6]

Analytical thin-layer chromatography was performed with silica gel 60 F_{254} plates (Merck, Darmstadt); the spots were visualized either with UV light at 254 nm, or by staining with vanillin/H_2SO_4 in methanol or $KMnO_4$ in water. RP-HPLC was performed using an RP18 column with water/methanol gradient elution.

All compounds prepared were isolated by RP-HPLC chromatography (LiChrospher RP18 with water/methanol gradient elution) as pure homogeneous substances according to TLC and NMR analyses, and were identified by appropriate spectroscopic methods (^{1}H and ^{13}C NMR, HRMS).

Molecular modeling studies were performed using Macromodel (version 7.5) and the MMFF94S force field, together with the generalized Born/Surface area (GB/SA) chloroform solvent model. Structures were subjected to a minimization procedure to the nearest local minimum prior to the generation of new local energy conformers by Monte Carlo searching.

Isolation: Fermentation (100 L) of *S. cellulosum* So ce960 was performed in the presence of 1.5 L Amberlite XAD 16. The resin was harvested by filtration in a process filter (210 μm), washed with H_2O to remove adherent cells, with 30% MeOH in H_2O to remove polar constituents, and finally eluted with MeOH (8 L). After evaporation of the MeOH, the residual water layer was extracted three times with EtOAc. The organic layer was dried with anhydrous Na_2SO_4, filtered, concentrated in vacuo, redissolved in MeOH and extracted with *n*-hexane. After removal of the hexane layer, the MeOH was evaporated to give a crude extract (16.6 g).

Further enrichment was achieved by chromatography on Sephadex LH-20 (column 8 × 79 cm, eluent MOH, flow 28 mL/min). Based on TLC analyses (UV visualization) a fraction (8.0 g) containing carolacton (**1**) was collected and separated further by RP chromatography [Merck Prepbar 100 chromatography system; column 10 × 40 cm, ODS AQ, 120 Å, 15 μm (YMC); solvent: MeOH/ammonium acetate buffer adjusted to pH 5, 57:43; flow rate: 170 mL/min; UV detection: 210 nm]. The fraction containing carolacton (1.3 g) was further purified by preparative RP MPLC [column: 3 × 48 cm, ODS AQ, 120 Å, 16 μm (Kronlab); solvent: MeCN/0.05 M ammonium acetate buffer adjusted to pH 5, 65:35; flow rate: 17 mL/min; UV detection at 206 nm] yielding carolacton (275 mg) after evaporation.

Carolacton (1): $C_{25}H_{40}O_8$ (468.58). M.p. 142 °C (CH_2Cl_2/petroleum ether). Optical rotation: $[\alpha]_D^{22}$ = –205 (*c* = 1.07, in MeOH). IR (KBr): $\tilde{\nu}$ = 3419 (w), 2962 (m), 2931 (m), 1731 (s), 1715 (s), 1456 (w), 1375 (w), 1196 (m), 1127 (w), 1089 (m) cm^{-1}. UV (MeOH): λ_{max} (lg ε) = 204 (4.06), 259 (2.35), 290 (2.58) nm. TLC (silica gel 254 nm): EtOAc/MeOH/H_2O, 65:30:10 (R_f = 0.62). HPLC [column: 2 × 125 mm, Nucleosil 120 5 μm C18 (Macherey–Nagel); flow rate: 0.3 mL/min; R_t = 12.8 min]: solvent A [95:5, H_2O/MeCN + 5 mM NH_4Ac, pH5.5], solvent B [5:95, H_2O/MeCN + 5 mM NH_4Ac, pH 5.5], gradient from 10% B to 100% B in 30 min, 10 min 100% B. For ^{1}H und ^{13}C NMR spectroscopic data, see Table 3. ESI-HRMS: calcd. for $[C_{25}H_{40}O_8 + H]^+$ 469.2796; found 469.2806.

Bislactone 2: To a solution of carolacton (**1**; 7.5 mg, 16 μmol, 1 equiv.) and a catalytic amount of DMAP in CH_2Cl_2 (0.8 mL), was added dropwise a solution of *p*-bromobenzoyl chloride (3.5 mg, 16 μmol, 1 equiv.) in CH_2Cl_2 (0.8 mL) at –19 °C. After addition of three drops of Et_3N, the solution was stirred for 2.5 h at –10 °C. Subsequently, the reaction was terminated by adding saturated ammonium chloride solution at –10 °C. After warming to r.t., the solution was washed with brine (1.5 mL), the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 2 mL). The combined organic layers were concentrated in vacuo and purified directly by semi-preparative HPLC (RP-18; H_2O/MeCN, 50:50 → 0:100 in 30 min; **2** R_t = 19 min). Bislactone **2** was collected as a colorless solid (5.1 mg, 11.3 μmol, 71%). R_f = 0.72 (CH_2Cl_2/MeOH = 10:1). $[\alpha]_D^{20}$ = –32.2 (*c* = 0.32, CH_2Cl_2). ^{1}H NMR (C_6D_6, 500 MHz): δ = 5.63 (br. s, 1 H, 18-H), 5.59 (ddd, *J* = 15.4, 10.0, 2.0 Hz, 1 H, 15-H), 5.54 (dd, *J* = 9.5, 1.5 Hz, 1 H, 7-H), 5.09 (dd, *J* = 15.4, 2.7 Hz, 1 H, 16-H), 4.91 (d, *J* = 11.1 Hz, 1 H, 9-H), 4.52 (ddd, *J* = 9.9, 2.7, 2.0 Hz, 1 H, 17-H), 4.11 (d, *J* = 9.9 Hz, 1 H, OH), 3.32 (dq, *J* = 9.5, 7.2 Hz, 1 H, 6-H), 3.33–3.24 (m, 1 H, 3-H), 3.10 (dq, *J* = 9.5, 6.8 Hz, 1 H, 4-H), 3.03 (s, 3 H, 20-H_3), 2.49 (dd, *J* = 12.4, 5.3 Hz, 1 H, 2-H_a), 2.19–2.09 (m, 1 H, 14-H), 1.95–1.87 (m, 1 H, 10-H), 1.84 (dd, *J* = 12.4, 0.8 Hz, 1 H, 2-H_b), 1.59 (d, *J* = 1.5 Hz, 3 H, 23-H_3), 1.24–1.12 (m, 2 H, 13-H_2), 1.12–1.04 (m, 1 H, 12-H_a), 0.98 (d, *J* = 7.2 Hz, 3 H, 22-H_3), 0.96 (d, *J* = 6.8 Hz, 3 H, 21-H_3), 0.95–0.87 (m, 1 H, 12-H_b), 0.91 (d, *J* = 6.6 Hz, 3 H, 25-H_3), 0.77–0.69 (m, 1 H, 11-H_b), 0.70 (d, *J* = 7.2 Hz, 3 H, 24-H_3) ppm. ^{13}C NMR (C_6D_6, 125 MHz): δ = 211.3 (s, C5), 171.8 (s, C1), 167.8 (s, C19), 139.7 (s, C8), 136.6 (d, C15), 127.7 (d, C16), 126.4 (d, C7), 81.8 (d, C3), 78.5 (d, C9), 73.2 (d, C18), 71.2 (d, C17), 57.1 (q, C20), 49.2 (d, C6), 43.0 (d, C4), 38.1 (d, C10), 35.9 (d, C14), 35.4 (t, C13), 33.3 (t, C2), 29.7 (t, C11), 22.6 (q, C25), 18.7 (q, C23), 18.6 (t, C12), 17.7 (q, C22), 15.6 (q, C21), 15.2 (q, C24) ppm. HRMS (ESI): *m*/*z* calcd. for $C_{25}H_{38}O_7Na$ $[M + Na]^+$ 473.2531; found 473.2515.

(*S*)-Mosher Ester 3a: To a solution of bicyclic derivative **2** (2.1 mg, 4.7 μmol, 1 equiv.) and a catalytic amount of DMAP in CH_2Cl_2 (0.4 mL), was added successively (*R*)-(–)-MTPA-Cl (9 μL, 48.0 μmol, 10 equiv.) and Et_3N (10 μL, 70 μmol, 15 equiv.) at r.t. After stirring for 23 h at 30 °C, the reaction mixture was concentrated in vacuo and purified directly by semi-preparative HPLC (RP-18; H_2O/MeCN = 50:50 → 0:100 in 30 min; **3a** R_t = 28 min)

Table 3. ^1H and ^{13}C NMR spectroscopic data of carolacton (**1**) in $CDCl_3$.[a]

H	δ_H	Multiplicity	*J* [Hz]	ROESY[b]	C	δ_C	Multiplicity
–	–	–	–	–	1	175.54	s
$2H_a$	2.67	dd	15.7, 4.2	(3) > 20	2	35.81	t
$2H_b$	2.45	dd	15.7, 5.9	(3) 21			
3H	3.75	ddd	8.5, 5.9, 4.2	(2ab, 4) 20, 21	3	80.06	d
4H	3.00	dq	8.5, 6.9	(3), 23, 6 >7	4	47.27	d
–	–	–	–	–	5	213.29	s
6H	3.50	dq	10.2, 6.8	(22, 7) 23 >4, 3	6	47.40	d
7H	5.38	dd	10.2, 1.5 (br.)	9 > (6), 4 >10, 22	7	129.36	d
–	–	–	–	–	8	135.80	s
9H	4.76	d	11.3	7 >23, 12b, 24	9	83.25	d
10H	2.06	ttd	11.5, 7.0, 2.6	(24) 23 >7	10	33.33	d
$11H_a$	1.77	tt	13.5, 4.0	(11a) 14, 16	11	28.37	t
$11H_b$	1.00	m	_[c]	_[c]			
$12H_a$	1.27	m	_[c]	_[c]	12	18.81	t
$12H_b$	1.00	m	_[c]	_[c]			
$13H_a$	1.39	tt	12.8, 4.0	(14) >25	13	34.39	t
$13H_b$	1.27	m	_[c]	_[c]			
14H	2.33	dddq	11.1, 9.8, 4.5, 6.6	(25) 16, >(15) 11b, 13a	14	35.66	d
15H	5.47	ddd	15.5, 9.8, 1.9	> 25, 17	15	134.45	d
16H	5.54	dd	15.5, 2.5	18, (17) >14	16	125.41	d
17H	4.50	dt	3.4, 2.5	18, (16) >15	17	72.92	d
18H	4.18	d	3.4	(17), 16	18	73.66	d
–	–	–	–	–	19	171.91	s
$20H_3$	3.32	s	–	3 > 2a, 4	20	58.01	q
$21H_3$	0.94	d	6.8/7	(4) > 3 >2b	21	12.77	q
$22H_3$	1.12	d	6.6	(6) >7 >23, 20	22	15.27	q[c]
$23H_3$	1.72	d	1.1	6>>9, 10 >4, 24	23	12.95	q
$24H_3$	0.78	d	7	23, (10), 9 >12a	24	15.27	q[c]
$25H_3$	0.99	d	6.6	15 >13a/b	25	21.64	q

[a] $^1H/^{13}C$ at 600/150 MHz; internal reference $CDCl_3$ signal at $\delta_{H/C}$ = 7.27/77.0 ppm, coupling constants after Gauss multiplication. [b] Numbers of correlating protons, > denotes lower signal intensity, coupling vicinal proton numbers given in brackets. [c] Overlapping signals.

to afford (*S*)-Mosher ester **3a** as a colorless oil (2.8 mg, 4.3 μmol, 89%). R_f = 0.24 (PE/Et_2O = 5:1). ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.65–7.62 (m, 2 H, Ar-H), 7.42–7.38 (m, 3 H, Ar-H), 5.97 (ddd, *J* = 3.2, 3.2, 1.4 Hz, 1 H, 17-H), 5.61 (d, *J* = 3.2 Hz, 1 H, 18-H), 5.55 (dd, *J* = 15.5, 3.2 Hz, 1 H, 16-H), 5.46 (ddd, *J* = 15.5, 9.4, 1.4 Hz, 1 H, 15-H), 5.32 (dq, *J* = 9.5, 1.2 Hz, 1 H, 7-H), 4.78 (d, *J* = 11.2 Hz, 1 H, 9-H), 3.59 (s, 3 H, OMe), 3.55–3.45 (m, 1 H, 3-H), 3.24 (dq, *J* = 9.5, 7.2 Hz, 1 H, 6-H), 2.92 (s, 3 H, 20-H_3), 2.95–2.86 (m, 1 H, 4-H), 2.79 (dd, *J* = 12.6, 5.3 Hz, 1 H, 2-H_a), 2.37–2.26 (m, 1 H, 14-H), 2.26 (dd, *J* = 12.6, 1.8 Hz, 1 H, 2-H_b), 1.98–1.89 (m, 1 H, 10-H), 1.78–1.72 (m, 1 H, 11-H_a), 1.61 (d, *J* = 1.2 Hz, 3 H, 23-H_3), 1.39–1.23 (m, 3 H, 12-H_a + 13-$H_{a,b}$), 1.17 (d, *J* = 7.2 Hz, 3 H, 22-H_3), 1.04 (d, *J* = 6.8 Hz, 3 H, 21-H_3), 0.95–0.87 (m, 2 H, 11-H_b + 12-H_b), 0.92 (d, *J* = 6.7 Hz, 3 H, 25-H_3), 0.86 (d, *J* = 7.0 Hz, 3 H, 24-H_3) ppm. HRMS (ESI): *m*/*z* calcd. for $C_{35}H_{45}O_7Na$ $[M + Na]^+$ 689.7114; found 689.7131.

(*R*)-Mosher Ester 3b: To a solution of bicyclic derivative **2** (2.0 mg, 4.4 μmol, 1 equiv.) in pyridine, was added dropwise (*S*)-(+)-MTPA-Cl (7 μg, 37.4 μmol, 8.5 equiv.) at r.t. and the solution was stirred for 19 h. After further dropwise addition of (*S*)-(+)-MTPA-Cl (7 μL, 37.4 μmol, 8.5 equiv.) the solution was stirred for 24 h at 36 °C. The reaction mixture was diluted with a saturated solution of copper sulfate (0.5 mL) and extracted with Et_2O (2 × 1 mL). The combined organic layers were washed with H_2O (1 mL) and brine (1 mL), dried with anhydrous $MgSO_4$ and concentrated in vacuo. The residue was purified by semi-preparative HPLC (RP-18; H_2O/MeCN = 50:50 → 0:100 in 30 min; **3b** R_t = 28 min) to afford (*R*)-Mosher ester **3b** as a colorless oil (0.6 mg, 0.9 μmol, 21% or 97% brsm). R_f = 0.24 (PE/Et_2O = 5:1). ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.68–7.65 (m, 2 H, Ar-H), 7.39–7.45 (m, 3 H, Ar-H), 5.95 (ddd, *J* = 3.2, 3.0, 1.8 Hz, 1 H, 17-H), 5.62 (d, *J* = 3.0 Hz, 1 H, 18-H), 5.45 (dd, *J* = 15.4, 3.2 Hz, 1 H, 16-H), 5.33 (dq, *J* = 9.2, 1.3 Hz, 1 H, 7-H), 4.99 (ddd, *J* = 15.4, 10.2, 1.8 Hz, 1 H, 15-H), 4.75 (d, *J* = 11.0 Hz, 1 H, 9-H), 3.79 (s, 3 H, OMe), 3.62–3.53 (m, 1 H, 3-H), 3.27 (dq, *J* = 9.5, 7.1 Hz, 1 H, 6-H), 3.09 (s, 3 H, 20-H_3), 2.89 (dq, *J* = 9.1, 6.8 Hz, 1 H, 4-H), 2.84 (dd, *J* = 12.7, 5.3 Hz, 1 H, 2-H_a), 2.32 (dd, *J* = 12.7, 2.0 Hz, 1 H, 2-H_b), 2.26–2.17 (m, 1 H, 14-H), 1.93–1.89 (m, 1 H, 10-H), 1.76–1.67 (m, 1 H, 11-H_a), 1.63 (d, *J* = 1.3 Hz, 3 H, 23-H_3), 1.35–1.20 (m, 3 H, 12-H_a + 13-$H_{a,b}$), 1.18 (d, *J* = 7.1 Hz, 3 H, 22-H_3), 1.06 (d, *J* = 6.8 Hz, 3 H, 21-H_3), 0.90–0.75 (m, 2 H, 11-H_b + 12-H_b), 0.85 (d, *J* = 7.0 Hz, 3 H, 24-H_3), 0.78 (d, *J* = 6.7 Hz, 3 H, 25-H_3) ppm. HRMS (ESI): *m*/*z* calcd. for $C_{35}H_{45}O_9Na$ $[M + Na]^+$ 689.7114; found 689.7139.

Supporting Information (see also the footnote on the first page of this article): Tables of NMR spectroscopic data of **2**, **3a**, and **3b**. X-ray analysis of **1**: crystal data and structure refinement, bond lengths and angles, X-ray structure. Also included are the molecular modeling data, selected 1D and 2D NMR spectra, and MIC values of carolacton **1**.

Acknowledgments

This work was supported by the Fonds der Chemischen Industrie. We thank W. Kessler for large-scale fermentation, K. Schober and S. Reinecke for technical assistance, C. Kakoschke and B. Jaschok-Kentner for recording the NMR spectra.

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[13] X-ray data presented by Diamond, Crystal Impact Software.
[14] X-ray structure determination. A distinctly block-shaped colorless crystal ($0.34 \times 0.57 \times 0.9$ mm^3) was selected for data collection. The first data set was gathered at 153 K on a X8-Apex Bruker-AXS diffractometer (Mo-K_α radiation). Of the 21641 reflections collected in the range from 1.5 to 32.4° θ, there were 9514 independent reflections (R_{int} = 0.023). We determined the acentric orthorhombic space group $P2_12_12_1$ with a unit cell of a = 6.2270(2) Å, b = 14.5886(6) Å, c = 29.339(1) Å. The structure was solved by direct methods,[16] and showed the position of all carbon and oxygen atoms. Subsequent refinement revealed the position of all hydrogen atoms in Difference Fourier maps. Full-matrix least-squares refinement against F_o^2 with anisotropic thermal parameters and free refinement of the hydrogen positions (458 parameter) resulted in $R1$ = 0.048 and $wR2$ = 0.12 with $I>2\sigma_I$. There was one remaining difference peak of around one electron (the other peaks were of around 0.3 e and placed at atom bonds) resulting from a slight disorder of O(2). This may be reasonable because of a possible alternative hydrogen bridge to O(7) in the crystal packing. To determine the absolute structure, we collected a second data set with Cu-K_α radiation. This was performed on a 4-circle Stoe Stadi4 diffractometer at 297 K. 6041 reflection were collected in the range from 3 to 50° θ, including 2796 (R_{int} = 0.02) independent reflections. An empirical absorption correction on psi-scans was applied. To keep the reflex to parameter ratio reliable, we reduced the number of parameters to 324 using rigid hydrogen position in the full-matrix least-squares refinement. We also applied a split position to O2 and obtained residuals of $R1$ = 0.04 and $wR2$ = 0.106 for the final refinement. The Flack's absolute structure parameter was determined to 0.0(3). In conjunction with the spectroscopic and chemical investigation results, there is no doubt that the determined structure represents the correct enantiomeric form. CCDC-735883 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
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Received: October 3, 2009
Published Online: January 26, 2010

