Secondary Metabolites by Chemical Screening, 41[+]

Structure and Biosynthesis of Mutolide, a Novel Macrolide from a UV Mutant of the Fungus F-24′707

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Dedicated to Hans Zähner on the occasion of his 70th birthday

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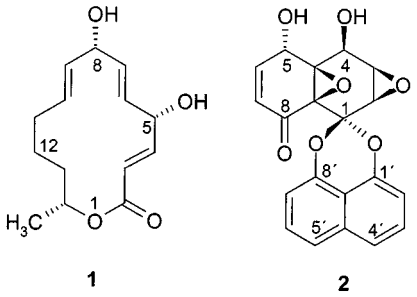
The 14-membered macrolide, mutolide (**1**), was discovered by chemical screening of the culture broth of the fungus F-24′707y, obtained after UV mutagenesis of the wild type strain, which normally produces the spirobisnaphthalene cladospirone bisepoxide (**2**). The structure of **1** was established by detailed spectroscopic analysis, X-ray analysis and derivatisation. The biogenetic origin of the carbon skeleton and the hydroxy groups was verified by feeding sodium [1-^{13}C]-acetate and $^{18}O_2$ to growing cultures of the fungus. Macrolide **1** is generated from acetate/malonate only. The unexpected change of the normal metabolite pattern of this strain is discussed, and proves the value of the OSMAC method.

Introduction

Spirobisnaphthalenes are the main polyketide metabolites of the fungus *Sphaeropsidales* sp. (strain F-24′707). Cladospirone bisepoxide (**2**)[2,3] was the first compound isolated from this strain. In the meantime, numerous further metabolites of this type have been identified[4] by making use of the OSMAC (one strain-many compounds) method.[5] Spirobisnaphthalenes are a growing group of fungal metabolites and exhibit different biological activities. Biosynthetically they are generated by the 1,8-dihydroxynaphthalene (DHN) pathway.[6] A fungal polyketide synthase is used, building the C_{10}-skeleton, that is dimerized and further transformed in the late biosynthesis. We exposed the fungus F-24′707 to UV light and isolated 25 mutants with different colony morphology in order to find even more members of these very interesting spirobisnaphthalenes. The chemical screening approach[7] was applied to the culture broth of these isolates and led to the isolation of a new metabolite, named mutolide (**1**), with an unexpected macrolide core. In this paper we present the isolation, structure elucidation and the biosynthesis of **1** from the UV mutant F-24′707y and describe the induction of the mutolide production in the parent strain using tricyclazole, an inhibitor of the cladospirone bisepoxide biosynthesis.



1 **2**

Producing Organism and Isolation

The fungus F-24′707 was described previously.[2] A total of 25 mutants, differing in colony morphology and colour compared to the black wild type, were isolated after UV mutagenesis of the parent strain. In order to examine their secondary metabolite pattern, the mutants were cultivated in two different media in a rotary shaker at 28 °C for 3 days and as a static surface culture at 28 °C for 28 days, respectively. An equal volume of ethyl acetate was added to the culture broth, the mixture was homogenised and centrifuged to remove insoluble material. The organic layers were dried and evaporated to give the crude extracts, which were dissolved in a small amount of ethyl acetate and purified by chromatography on silica gel TLC plates using chloroform/methanol (9:1) as solvent. To identify new compounds, the extracts were compared with those of the wild type strain. Only mutant F-24′707y generated a new substance that gave an intensive brown to black colour reaction with anisaldehyde/H_2SO_4 or vanillin/H_2SO_4 as staining reagent, similar to the spirobisnaphthalenes [**1**: R_f = 0.45, **2**: R_f = 0.49 (chloroform/methanol, 9:1)].

F-24′707y was cultivated in 1-L Erlenmeyer flasks containing 250 mL of medium A (oat meal/soybean meal/glucose) for 84 hours at 28 °C. Extraction of the homogenised cultures with ethyl acetate, and subsequent column chromatography on silica gel and Sephadex LH-20 led to the isolation of 100 mg/L of mutolide (**1**) and 120 mg/L cladospi-

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Table 1. ^{1}H NMR and ^{13}C NMR signals of mutolide (**1**) and specific incorporation after feeding with [1-^{13}C]acetate (*I*)

C-Atom	δ_H [ppm][a]	δ_H [ppm] [b]	δ_C [ppm][b]	*I* [c]
2			168.6	**7.7**
3	5.83 (dd, 16.0, 1.0)	5.81 (dd, 16.0, 1.0)	118.8	0.0
4	6.73 (dd, 16.0, 7.0)	6.70 (dd, 16.0, 7.0)	152.9	**9.0**
5	4.83 (m)	4.89 (m)	70.8	0.0
6	5.64 (dd, 16.0, 4.5)	5.41 (m)	131.1	**7.9**
7	5.78 (ddd, 16.0, 6.0, 1.0)	5.77 (ddd, 16.0, 7.0, 1.0)	135.0	0.0
8	4.61 (m)	4.62 (s, br)	73.0	**8.9**
9	5.59 (dd, 16.0, 5.0)	5.57 (m)	135.6	0.1
10	5.46 (m)	5.41 (m)	132.6	**8.1**
11	1.98 (m)	1.94 (m)	31.7	0.1
12	1.51 (m) 1.33 (m)	1.40 (m)	25.0	**9.1**
13	1.51 (m)	1.52 (m)	35.5	0.2
14	4.97 (m)	4.98 (m)	72.8	**8.7**
15	1.23 (d, 7.0)	1.18 (d, 6.5) 4.50 (s, br, 5-OH) 3.96 (s, br, 8-OH)	18.9	0.2

[a] CD_3OD. – [b] [D_6]Acetone. – [c] According to Scott,[10] specific incorporations were normalized to the peak intensity of the C-5 signal.

rone bisepoxide (**2**). Addition of tricyclazole, an inhibitor of the DHN biosynthesis,[8,9] to a final concentration of 1 mg/L to growing cultures of the wild-type F24′707 resulted in a complete loss of the cladospirone bisepoxide biosynthesis.[6] Instead, 70 mg/L of **1** could be isolated. Compound **1** was not detectable in the tricyclazole untreated cultures.

Structure Elucidation

The molecular formula $C_{14}H_{20}O_4$ results from both a HREI mass spectrum (*m/z* = 252.1361, M^+) and elemental analysis, and indicates five double bond equivalents. The presence of hydroxyl and carbonyl groups as well as double bonds is shown by IR absorption bands at $\tilde{\nu}$ = 3308, 1708, and 1640 cm^{-1}, respectively. As expected, the ^{1}H-NMR spectrum shows signals for 20 protons (Table 1), two of which (δ_H = 3.96 and 4.50) are readily exchangeable with D_2O pointing to hydroxy groups. In addition, signals of a methyl group (δ_H = 1.18, *J* = 6.5 Hz) attached to a methine group at δ_H = 4.98, of three methylene groups (δ_H = 1.30–1.47, 1.48–1.56, 1.88–2.00), of two methine groups (δ_H = 4.62, 4.89) attached to oxygen and of six olefinic methine groups between δ_H = 5.41 and 6.70 are seen. The ^{13}C-NMR spectrum exhibits 14 carbon signals (Table 1).

Besides the signals of the proton-attached carbon atoms, that of the quaternary carbon atom at δ_C = 167.0 is observable, indicating an ester group. Proton and carbon signal assignments resulted from a ^{1}H-^{13}C correlation spectrum. Proton connectivities arose from a ^{1}H-^{1}H COSY experiment and led to a chain of 14 carbon atoms. This was confirmed by $^nJ_{C,H}$ couplings observed in an HMBC experiment. Although a *trans* coupling constant of *J* = 16 Hz ([D_6]acetone) could be established only from three of the six double bond methine groups, the *all-trans* configuration of **1** was confirmed by ^{1}H-NMR spectroscopy in CD_3OD, where the *trans* coupling constants of five olefinic methine protons became detectable (Table 1). The requirement of a ring closure follows from the molecular formula. In order to prove this result and to obtain information about the stereogenic centres of **1**, an X-ray analysis was envisaged from a crystal obtained from acetone. The structure including the relative configuration of **1** is shown in Figure 1.

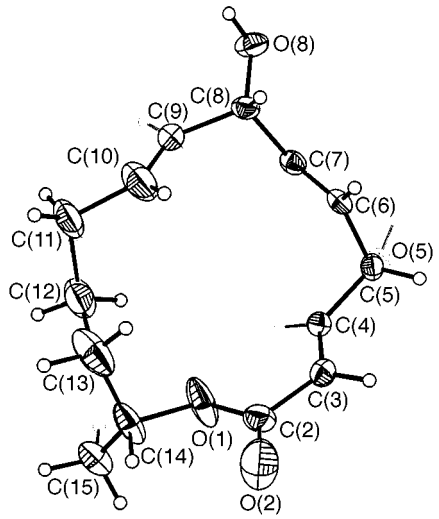


Figure 1. Perspective view of mutolide (**2**)

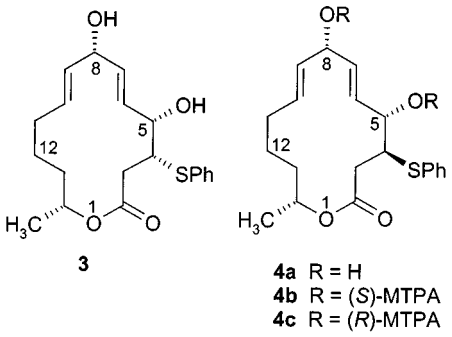
The elucidation of the absolute stereochemistry of **1** using the advanced Mosher's ester methodology[11] failed with the standard reaction conditions for unknown reasons. Therefore we transformed mutolide by adding thiophenol, which reacts with the double bond of the α,β-unsaturated lactone.[12] The resultant diastereomers (**3**/**4a**, 1:3) were separated by silica gel chromatography using dichloromethane/acetone (97:3) as eluent, and their structure was confirmed by NMR spectroscopy. The relative stereochemistry of C-4 for derivative **3** and **4a** was deduced from a comparison of the coupling constants in the ^{1}H-NMR spectra (**3**: $J_{4,5}$ = 2.0 Hz; **4a**: $J_{4,5}$ = 8.5 Hz). The absolute stereochemistry of both carbinol centres of compound **4a** was elucidated as (5*S*) and (8*S*), by using the advanced Mosher's ester methodology (Table 2). The configuration of C-14 as (*R*) follows in connection with the X-ray data of **1** (Figure 1). Thus the

stereochemistry of mutolide (**1**) was confirmed as (5*S*), (8*S*), and (14*R*).

Table 2. Selected ^{1}H-NMR data for the Mosher ester **4b** and **4c**, δ_H in $CDCl_3$ (*J* in Hz)

H atom	**4b**	**4c**	Δδ **4b**–**4c**
2a	2.60 dd (18.0, 3.5)	2.53 dd (18.0, 3.0)	+0.07
2b	2.70 dd (18.0, 5.0)	2.69 dd (18.0, 5.0)	+0.01
3	3.32 ddd (8.5, 5.0, 3.5)	3.18 ddd (8.0, 5.0, 3.0)	+0.14
4	5.89 m	5.98 t (8.0)	*S* [a]
5	5.50 ddd (16.0, 8.0, 1.5)	5.60 m	–0.10
6	5.98 dd (16.0, 4.0)	5.89 dd (16.0, 4.0)	+0.09
7	5.89 m	5.88 m	*S* [a]
8	5.36 dd (16.0, 6.5)	5.46 dd (16.0, 6.5)	–0.10
9	5.54 dd (16.0, 6.5)	5.65 dd (16.0, 6.5)	–0.11
10a	1.89 m	1.90 m	–0.01
10b	2.13 m	2.20 m	–0.07

[a] Absolute configuration of the centers of chirality.



Feeding Experiments

Feeding of sodium [1-^{13}C]acetate resulted in a signal enhancement of seven carbon atoms in mutolide (C-2, C-4, C-6, C-8, C-10, C-12, C-14) as shown in Table 1. As expected, the carbon skeleton of **1** is derived from seven acetate/malonate units. Cultivation of the strain in a closed vessel in a [$^{18}O_2$]/[$^{16}O_2$] atmosphere[6] resulted in **1** (16% ^{18}O incorporation), with significant α-isotopic shifts in the ^{13}C-NMR spectrum for C-5 (17 ppb) and C-8 (19 ppb) indicating the incorporation of both oxygens by mono-oxygenases. The DCI mass spectrum shows the expected isotopic pattern of single and double labelled mutolide (**1**) {*m/z* (%) = 287 (100) [M ($^{16}O_2$) + NH_3 + NH_4^+], 289 (10) [M (^{16}O+^{18}O) + NH_3 + NH_4^+], 291 (5) [M ($^{18}O_2$) + NH_3 + NH_4^+]}. Following the usual polyketide pathway for macrolides, we assume that **5** is the intermediate that is released from the polyketide synthase (PKS). The structure of **5** is consistent with the observation that most double bonds in macrolides are positioned between malonyl- or methylmalonyl-CoA units by the dehydratase activity of the PKS. Compound **5** is further modified by two post-polyketide oxygenations, likely to give bisepoxide **6**. Further transformations would result in the formation of **1**, as shown in Figure 2 (Path A). It is, however, possible that only one of the double bonds, C-5/C-6 or C-7/C-8, of **5** is converted into the corresponding epoxide, that is reduced immediately. The second hydroxy group could be introduced by a mechanism similar to the biosynthesis of lipoxins, from polyunsaturated carboxylic acids (Figure 2, Path B).[13] As a result of these oxygenation steps the C-6/C-7 double bond is found in a position not determined by the PKS.

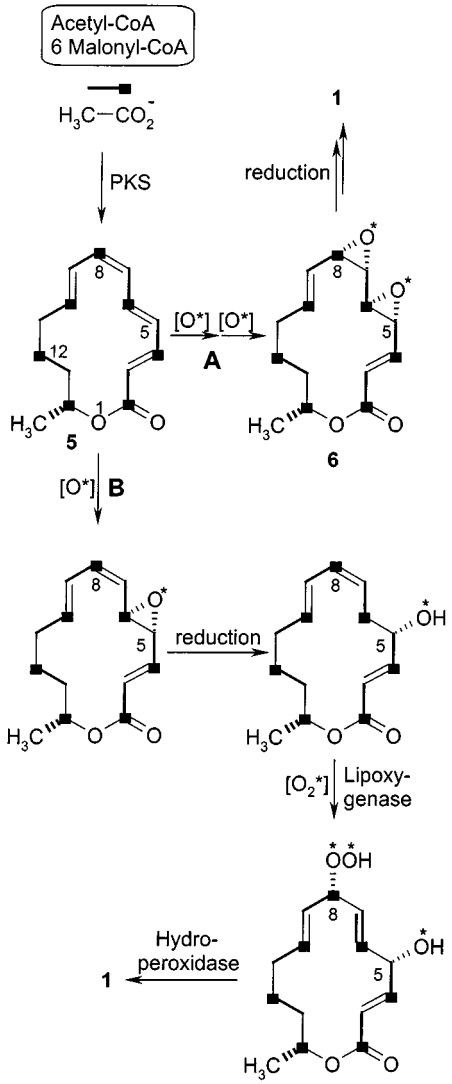
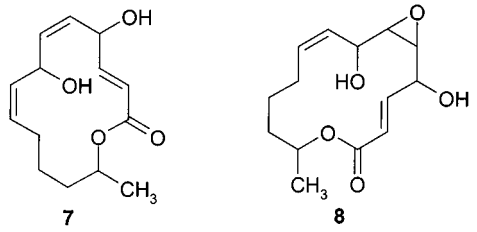


Figure 2. Biosynthesis of the carbon skeleton of mutolide (**1**), and tentative steps in the late biosynthesis

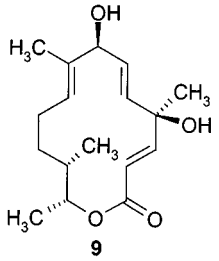
Conclusions

A novel 14-membered macrolide, named mutolide (**1**), was detected as a metabolite of the mutant fungus F-24′707y, together with cladospirone bisepoxide (**2**), the usually produced spirobisnaphthalene. Macrolide **1** shows only weak antibacterial activity (inhibition zone diameter 10 mm at 1 mg/mL in agar diffusion assay) against *B. subtilis* and *E. coli*. Nigrosporolide[14] (**7**), isolated from the mould *Nigrospora sphaerica*, and seiricuprolide[15] (**8**) from *Seiridium cupressi*, are so far the only known examples of microbial 14-membered macrolides exclusively derived from acetate, and both show phytotoxic activity. Other fungal 10- (e.g. decarestricines[16]), 12- (e.g. cladospolides[17]) and 16-mem-

bered macrolides (e.g. brefeldin A[18]) generated by acetate/malonate only are known. 14-membered macrolides of bacterial origin have been investigated in detail in the past, and all of them are derived from both, acetate and propionate [e.g cineromycin B (**9**)[19]].



7 **8**



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Inhibition of the 1,8-dihydroxynaphthalene (DHN) biosynthesis resulted in the complete loss of the cladospirone bisepoxide (**2**) production, but activation of that of **1**. This experiment is a good example for the ability of enzyme inhibitors to change biosynthetic pathways, one is suppressed but others can be induced. Due to the putative intracellular cross-talk of different metabolic and biosynthetic pathways, the production of a new metabolite is not obvious, but can never be excluded. Both UV mutagenesis and addition of tricyclazole apparently activates a silent gene cluster responsible for the mutolide biosynthesis. The appertaining polyketide synthase (PKS) generates a reduced polyketide similar to those from bacterial type I PKS. Thus at least two different PKSs are existent in the same strain, leading to basically different structures of the generated secondary metabolites, demonstrating the value of the OSMAC-concept.[5] Further experiments to investigate the "biosynthetic switch" are in progress.

While the biosynthesis of bacterial macrolides is investigated in some detail,[20,21] little is known about fungal PKS for the production of macrolides and other reduced polyketides.[22,23] Investigations of the genetic organisation of strain F-24′707 would be of large interest, because **1** resembles other products of typical modular PKS. This result extends the view on the inherent diversity of secondary metabolite pathways in microorganism, and gives further ideas to use these enzymes as a putative tool for combinatorial biosynthesis.[24]

Experimental Section

General Remarks: Melting points: Reichert hot-stage microscope (not corrected). – ^{1}H- and ^{13}C-NMR spectra: Bruker AMX 300 (300 MHz), Varian Unity 300 (300 MHz), Varian Inova 500 (500 MHz). Chemical shifts are expressed in δ values (ppm) using the solvent as internal reference. The multiplicities of the ^{13}C-NMR values were assigned by attached proton test (APT). – MS: Varian MAT 731 and Finnigan MAT 311 A (EI 70 eV, direct insert, high resolution with perfluorokerosine as standard). – Elemental analysis: Mikroanalytisches Labor des Institutes für Organische Chemie, Universität Göttingen. – IR spectra: Perkin–Elmer Model 298 (KBr discs). – UV spectra: Kontron Uvikon 860. – Optical Rotation value: Perkin–Elmer 343. – X-ray analysis: Siemens-STOE AED2 diffractometer, data collection with SHELXS-97.[26] – Fermentation: Braun BS4 (250 rpm, 28 °C), Universität Göttingen (100 spm, 28 °C). – Homogenization: Ultra-Turrax, Janke Kunkel KG. Centrifugation: Sigma 5 K. – TLC: Silica gel plates (HPTLC ready-to-use plates, silica gel 60 F_{245} on aluminium foil or glass, Merck). – LC: Silica gel ICN SiliTech 32–63 (0.032–0.063 mm, ICN Biomedicals GmbH), Sephadex LH-20 (Pharmacia). – Degreased soybean meal: Heuselwerk-Magstadt. – Oat meal: Hafergold (Neuform). – Malt extract: Merck. – Yeast extract: Gibco BRL. – Sodium [1-^{13}C]acetate and [$^{18}O_2$]: Campro Scientific.

UV Mutagenesis and Isolation of Mutants: 10 mL of a 0.9% sodium chloride solution was added to a 10 d old agar plate of *Sphaeropsidales* sp. (strain F-24′707), and the aerial mycelium was scraped off with a sterile needle. The liquid was transferred into sterile Eppendorf reaction valves, diluted, and the closed reaction valves exposed to UV light for 3 min. Aliquots of the mixtures were plated on LCSB agar[2] and incubated at 28 °C until the detection of single colonies. Colonies with other than wild type morphology were isolated, grown on LCSB agar and stored in liquid nitrogen for further experiments.

Screening: A 1-cm^2 piece of agar from 10 d old cultures (LCSB agar) was used to inoculate a 300-mL Erlenmeyer flasks containing 50 mL of medium A [oat meal 2%, degreased soy meal 2%, and glucose 2%] and medium B [glucose 0.4%, yeast extract 0.4%, and malt extract 1%], respectively, and to inoculate P (*Penicillium*) flasks containing 1 L of medium A or B, respectively. The Erlenmeyer flasks were cultivated for 3 d at 28 °C in a rotary shaker (250 rpm), the P flasks were cultivated as a static surface culture for 28 d at 28 °C. These cultures were used for TLC analysis in the routine screening.

Fermentation of F-24′707y: A 5-cm^2 piece of agar from 10 d old cultures grown on LCSB agar was used to inoculate 1-L Erlenmeyer flasks containing 250 mL of medium A. The flasks were cultivated for 84 h at 28 °C and 100 spm. The wild type was cultivated in a similar manner, but with the addition of 1 mg/L of tricyclazole from the beginning of the fermentation.

Isolation and Purification: After harvesting, 2.5 L of ethyl acetate was added to the culture broth (about 2.25 L from 9 1-L Erlenmeyer flasks), and the mixture was homogenised for 5 min. After addition of Celite (500 g) the mixture was centrifuged for 30 min and the pellet was discarded. The liquid phases were separated, and the aqueous layer was extracted once with 500 mL of ethyl acetate. The combined organic phases were dried with Na_2SO_4, and evaporated to dryness. The obtained dark brown crude material was purified by chromatography on silica gel (column 30 × 4 cm), by using petroleum ether/ethyl acetate (1:2) as the eluent. Further purification of the cladospirone bisepoxide/mutolide mixture was carried out by gel permeation chromatography on Sephadex LH-20 (column 100 × 2.5 cm) in MeOH, to yield pure amorphous **1** (100 mg/L) and **2** (150 mg/L).

Feeding Experiments: Feeding of sodium [1-^{13}C]acetate (10 mmol, 99% ^{13}C atom purity) was carried out with the fungus F-24′707y

at 28 °C in a 2-litre fermentor, using 900 mL of medium A. The fermentor was inoculated with 10 vol-% of a pre-culture grown for 72 h in a rotary shaker at 250 rpm and 28 °C. The precursor was added to the culture as a sterile aqueous solution, adjusted to pH 6.5, during the 24th and 30th h, and the culture was harvested after 36 hours. The experiment for the incorporation of [$^{18}O_2$] follows the procedure described previously.[25] During the cultivation of the strain in medium A, 1260 mL of $^{18}O_2$ and 400 mL of $^{16}O_2$ were taken up.[6] Workup as described before yielded labelled **1** in the following amounts: 23 mg/L ([1-^{13}C]acetate), 10 mg/L ([$^{18}O_2$] experiment).

Mutolide [(3*E*,5*S*,6*E*,8*S*,9*E*,14*R*)-5,8-Dihydroxy-14-methyloxacyclotetradeca-3,6,9-trien-2-one] (1): Soluble in methanol or acetone; insoluble in *n*-hexane or water. M.p. 168 °C. – R_f value: 0.45 ($CHCl_3$/MeOH, 9:1). – IR (KBr): $\tilde{\nu}$ = 3308 cm^{-1} (OH), 1708 (C=O), 1640 (C=C). – UV (CD_3CN): λ_{max} (lg ε) = 192 (4.21), 234 (sh) (3.68). – $[\alpha]_D^{20}$ = –61 (*c* = 1.79 in CD_3CN). – CD (CD_3CN): $\lambda_{extr.}$ ($[\Theta]^{22}$) = 234 (–39300). – ^{1}H NMR (300 MHz, [D_6]acetone): see Table 1.; (300 MHz, CD_3OD): see Table 1. – ^{13}C NMR (75.5 MHz, [D_6]acetone): see Table 1. – EI-MS (70 eV): *m*/*z* (%) = 252.1361 (3) [M^+, calcd. for $C_{14}H_{20}O_4$ and found], 234 (3) [$M^+ - H_2O$], 151 (43), 109 (71), 95 (75), 81 (96), 55 (100). – $C_{14}H_{20}O_4$ (252.3): calcd. C 66.65, H 7.99; found C 66.34, H 8.21.

X-Ray Crystal-Structure Analysis of 2:[26] Compound **2** (molecular formula $C_{14}H_{20}O_4$, M_r 252.3) was crystallized by concentrating a saturated solution of **1** in acetone at 4 °C. Crystal size 0.15 × 0.2 × 0.2 mm, orthorhombic, space group $P2_12_12_1$, *a* = 523.9(1), *b* = 759.9(1), *c* = 3430.2(7) pm, α = β = γ = 90°, *V* = 1.366 nm^3, *Z* = 4, $D_{calcd.}$ = 1.227 Mg/m^3, μ = 0.089 mm^{-1}, Stoe–Siemens–Huber diffractometer coupled to a Siemens CCD area detector with graphite-monochromated Mo-K_α radiation (λ = 0.71073), –140 °C, Θ range = 2.37–25.08°, 25168 reflections measured, 2406 unique. Structure solved by direct methods using SHELXS-97[27] and refined against F^2 on all data by full-matrix least squares with SHELXL-97.[28] A riding model with idealised hydrogen geometry was employed, the anisotropic refinement converged at R_1 = 0.0449 for $F > 2\sigma(F)$ and wR_2 (F^2) = 0.1184 for all reflections.

Preparation of 3 and 4a: 55 mg of **2** in 2 mL of acetone were cooled to –78 °C, and added to a solution of 24 mg of thiophenol and 22 mg of triethylamine in 1 mL of acetone at –78 °C. The mixture was stirred for 30 min and allowed to warm up to room temperature. After stirring for additional 2 h the solvent was evaporated in vacuo. The oily residue was purified by chromatography on silica gel [column 40 × 1.5 cm, CH_2Cl_2/acetone (97:3)] to yield 18 mg (22%) of **3** and 58 mg (73%) of **4a**.

(4*R*,5*S*,6*E*,8*S*,9*E*,14*R*)-5,8-Dihydroxy-14-methyl-4-phenylthiooxacyclotetradeca-6,9-dien-2-one (3): M.p. 145 °C. – R_f = 0.88 ($CHCl_3$/MeOH, 9:1). – IR (KBr): $\tilde{\nu}$ = 3417 cm^{-1}, 3253, 1722, 1658, 1628, 1582. – UV (CH_3CN): λ_{max} (lg ε) = 206 nm (sh) (4.35), 256 (3.79). – $[\alpha]_D^{20}$ = –50 (*c* = 1.03 in MeOH). – CD (CH_3CN): $\lambda_{extr.}$ ($[\Theta]^{22}$) = 212 nm (–48100), 230 (2400), 254 (-9400). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.14 (d, $J_{15,14}$ = 6.0 Hz, 15-H_3), 1.48 (m, 12-H_2), 1.51 (m, 13-H_a), 1.60 (m, 13-H_b), 1.94 (m, 11-H_a), 2.13 (m, 11-H_b), 2.25 (ddd, $J_{3a,3b}$ = 18.0 Hz, $J_{3,4}$ = 2.0 Hz, $J_{3,5}$ = 1.0 Hz, 3-H_a), 2.77 (dd, $J_{3a,3b}$ = 18.0 Hz, $J_{3,4}$ = 9.5 Hz, 3-H_b), 3.94 (ddd, $J_{4,3b}$ = 9.5 Hz, $J_{4,3a}$ = 2.0 Hz, $J_{4,5}$ = 2.0 Hz, 4-H), 4.27 (m, 5-H), 4.49 (t, $J_{8,7}$ = $J_{8,9}$ = 7.0 Hz, 8-H), 4.70 (m, 14-H), 5.48 (ddd, $J_{6,7}$ = 15.5 Hz, $J_{6,5}$ = 4.0 Hz, *J* = 1.0 Hz, 6-H), 5.53 ($J_{9,10}$ = 15.0 Hz, $J_{9,8}$ = 7.0 Hz, 9-H), 5.64 ($J_{10,9}$ = 15.0 Hz, $J_{10,11}$ = 7.0 Hz, 10-H), 5.85 (ddd, $J_{7,6}$ = 15.5 Hz, $J_{7,8}$ = 8.0 Hz, $J_{7,5}$ = 2.0 Hz, 7-H), 7.24–7.33 (m, 3 aromatic H), 7.44–7.47 (m, 2 aromatic H). – ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 19.4 (14-Me), 23.6 (C-12), 30.9 (C-11), 32.3 (C-3), 33.8 (C-13), 48.4 (C-4), 69.2 (C-5), 71.1 (C-14), 74.1 (C-8), 127.9, 129.3 (× 2), 132.2 (× 2), 133.1 (phenyl C), 130.2 (C-6), 131.8 (C-7), 132.5 (C-10), 133.0 (C-9), 171.3 (C-2). – EI-MS (70 eV): *m*/*z* (%) = 362.1551 (22) [M^+, calcd. for $C_{20}H_{26}O_4S$ and found], 344 (4) [$M^+ - H_2O$], 252 (7), 182 (100), 180 (58), 163 (33), 135 (21), 109 (33), 91 (19), 84 (25), 55 (23).

(4*S*,5*S*,6*E*,8*S*,9*E*,14*R*)-5,8-Dihydroxy-14-methyl-4-phenylthiooxacyclotetradeca-6,9-dien-2-one (4a): M.p. 124 °C. – R_f = 0.86 ($CHCl_3$/MeOH, 9:1). – IR (KBr): $\tilde{\nu}$ = 3301 cm^{-1}, 1737, 1672, 1641, 1581. UV (CH_3CN): λ_{max} (lg ε) = 206 nm (sh) (4.32), 255 (3.81). – $[\alpha]_D^{20}$ = +92 (*c* = 2.51 in MeOH). – CD (CH_3CN): $\lambda_{extr.}$ ($[\Theta]^{22}$) = 208 nm (sh) (30900), 228 (–4300), 255 (5600). – ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.15 (d, $J_{15,14}$ = 7.0 Hz, 15-H_3), 1.38–1.53 (m, 12-H_2, 13H_a), 1.53–1.68 (m, 13-H_b), 1.86–2.00 (m, 11-H_a), 2.06–2.10 (m, 11-H_b), 2.60 (dd, $J_{3a,3b}$ = 17.0 Hz, $J_{3a,4}$ = 6.5 Hz, 3-H_a), 2.77 (dd, $J_{3a,3b}$ = 17.0 Hz, $J_{3b,4}$ = 3.5 Hz, 3-H_b), 3.10 (br, OH), 3.24 (ddd, $J_{4,5}$ = 8.5 Hz, $J_{4,3a}$ = 6.5 Hz, $J_{4,3b}$ = 3.5 Hz, 4-H), 4.42 (t, $J_{4,5}$ = $J_{6,5}$ = 8.0 Hz, 5-H), 4.63 (m, 8-H), 4.83 (m, 14-H), 5.45 (dd, $J_{9,10}$ = 15.0 Hz, $J_{9,8}$ = 6.5 Hz, 9-H), 5.56 (dd, $J_{10,9}$ = 15.0 Hz, $J_{10,11}$ = 6.5 Hz, 10-H), 5.71 (ddd, $J_{6,7}$ = 15.0 Hz, $J_{6,5}$ = 7.5 Hz, *J* = 1.0 Hz, 6-H), 5.88 (dd, $J_{7,6}$ = 15.0 Hz, *J* = 4.0 Hz, 7-H), 7.25–7.33 (m, 3 aromatic H), 7.46 (m, 2 aromatic H). – ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 18.7 (14-Me), 21.8 (C-12), 30.2 (C-11), 32.7 (C-13), 37.7 (C-3), 52.6 (C-4), 70.8 (C-14), 71.0 (C-8), 73.8 (C-5), 126.4 (C-6), 127.5, 129.1 (× 2), 132.2 (× 2), 134.1 (phenyl C), 131.0 (C-10), 132.3 (C-9), 136.9 (C-7), 170.0 (C-2). – EI-MS (70 eV): *m*/*z* (%) = 362.1551 (22) [M^+, calcd. for $C_{20}H_{26}O_4S$ and found], 344 (3) [$M^+ - H_2O$], 252 (5), 182 (100), 180 (64), 163 (32), 135 (23), 109 (25), 91 (14), 84 (24), 55 (16).

Preparation of Mosher's Esters:[11] To 5 mg of **4a** in 2 mL of CH_2Cl_2 were sequentially added 0.2 mL pyridine, 3 mg 4-(dimethylamino)-pyridine, and 20 mg of (*R*)-(–)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*R*)-MTPA-Cl]. The mixture was stirred for about 12 h at room temperature, checked with TLC to make sure that the reaction was complete, and passed through a disposable pipette (0.4 × 5 cm) containing silica gel (ICN Silica 32–63), and eluted with 10 mL of CH_2Cl_2. The oily residue was dried in vacuo and purified by chromatography on Sephadex LH-20 (acetone) to give the (*S*)-MTPA Mosher ester **4b** (10 mg). With (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride the (*R*)-MTPA Mosher ester **4c** was obtained (11 mg). Both yields were >95%. For partial ^{1}H-NMR assignments of **4b** and **4c** see Table 2.

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[26] Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-136543. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44–1223/336-033; E-mail: deposit@ccdc.cam.ac.uk).
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