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Stereostructure and anti-inflammatory activity of three diastereomers of ocobullenone from *Ocotea bullata*

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Abstract

A novel diastereomer of ocobullenone, designated as sibyllenone, was isolated from the stem bark of mature *Ocotea bullata* in the course of a search for anti-inflammatory compounds from this plant. The stereostructure was established by X-ray crystallography and corroborated by NOESY analysis. Ocobullenone, obtained previously, was re-isolated and crystallised successfully for X-ray analysis, thus making possible an accurate spatial comparison of ocobullenone, iso-ocobullenone and the new stereoisomer. Tested pharmacologically for cyclooxygenase-1 and 2, and 5-lipoxygenase inhibition, sibyllenone was the only compound from *O. bullata* which showed good inhibitory activity towards 5-lipoxygenase.

Keywords: *Ocotea bullata*; Lauraceae; Sibyllenone; Ocobullenone; Iso-ocobullenone; X-ray analysis; Cyclooxygenase-1 and 2; 5-Lipoxygenase

1. Introduction

The stem bark of *Ocotea bullata* (Birch) Baill. (Lauraceae) is one of the most-frequently used traditional medicines in southern Africa (Mander, 1997). *O. bullata* is a specially-protected plant in KwaZulu Natal and has become an endangered species. Its importance to the herbal medicine trade has attracted the attention of conservationists (Cunningham, 1988) and natural products chemists. To date, several new neolignans have been isolated from the bark, notably ocobullenone **1** (Sehlapelo et al., 1993), and iso-ocobullenone **2** (Drewes et al., 1995). The use of *O. bullata* bark by traditional healers for a wide-ranging list of ailments including headaches, back-ache, urinary tract problems and magical purposes, is well-documented (Hutchings, 1966). However, it has not been possible to link any of the known components with specific biological activity. Earlier, crude extracts had yielded moderate activity in the cyclooxygenase (COX-1) assay (Jaeger et al., 1996). In the light of all the above findings, we have now examined *O. bullata* bark extracts as an anti-inflammatory agent (e.g., its traditional use against pain) in terms of in vitro cyclooxygenase-1 and 2 (COX-1 and COX-2) and 5-lipoxygenase (5-LO) inhibition (Zschocke et al., 2000; Zschocke & van Staden, 2000). These enzymes (mainly) regulate the biosynthesis of prostaglandins (COX-1 and COX-2) and leukotrienes (5-LO), mediators, which are responsible for pain, inflammation and allergic reactions. The question, addressed in this paper, is whether ocobullenone **1**, its precursor **3**, iso-ocobullenone **2** and the newly isolated sibyllenone **4** contributed to the inhibitory activity described above.

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2. Results and discussion

In the course of re-isolation of the major compounds from *O. bullata* stem bark from the *n*-hexane extract, we have isolated a new, crystalline stereoisomer of ocobullenone, sibyllenone **4**, and also succeeded in growing crystals of ocobullenone **1** which were suitable for X-ray analysis. These findings have now made it possible to:

1. compare the stereostructure of all the three stereoisomers since we now have X-ray information on all of them. Previously (Drewes et al., 1995) only iso-ocobullenone had been examined by X-ray diffraction; and
2. examine the COX-1, COX-2 and 5-LO inhibitory activity, not by bark extracts, but by pure compounds of known constitution and stereochemistry.

In terms of stereochemical correlations discussed here, it should be emphasized that the findings reported reflect relative configurations and not absolute configurations. Thus ocobullenone **1** is rel.-(7*S*, 8*R*, 1′*R*,3′R)-Δ8′-5-methoxy-3,4-methylenedioxy-3′,4′-methylenedioxy-1′,2′,3′,6′-tetrahydro-6′-oxo-7.1′.8.3′-neolignan (previously erroneously given as 7*R*,8*R*,1′*R*,3′*R*) (Sehlapelo et al., 1993). Iso-ocobullenone **2** would then be rel.-(7*S*,8*S*,1′*R*,3′*R*), reflecting the opposite configuration at C-8 only. Sibyllenone **4** is designated as rel.-(7*S*,8*S*,1′*S*,3′*S*) showing identical stereochemistry at C-7 and C-8 to iso-ocobullenone but with an inverse configuration to both **1** and **2** at C-1′ and C-3′. In Fig. 1 the X-ray structures for **1** and **4** are depicted.

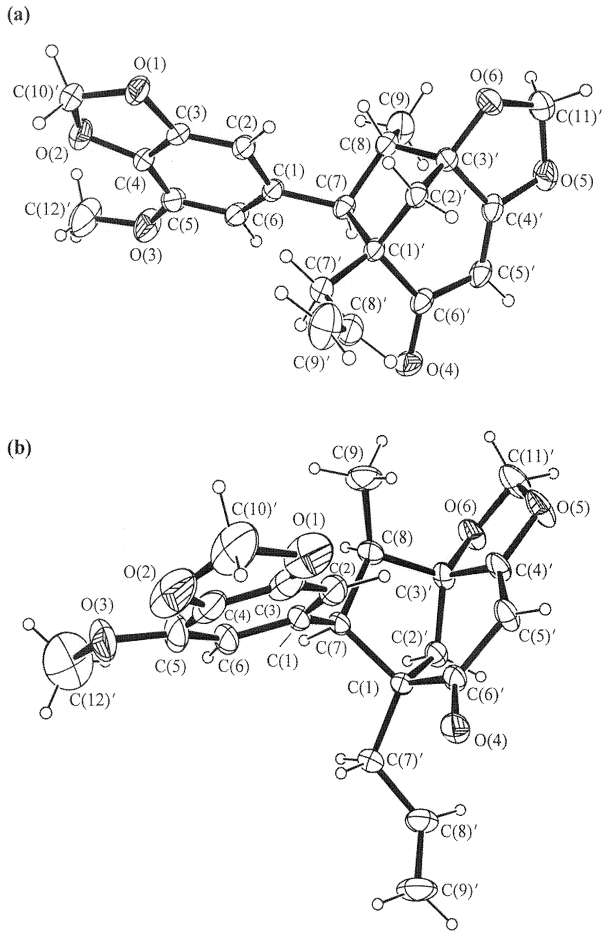


Fig. 1. X-ray structures of sibyllenone (a) and ocobullenone (b).

The X-ray structures of the three diastereomers were extremely useful in this case since ^{1}H- and ^{13}C-NMR data on their own are only of limited value in attempting to predict the overall structural detail. A comparison of ^{1}H-NMR resonances for **1**, **2** and **4** (Table 1) shows relatively few differences except that H-7 in both iso-ocobullenone (δ 2.68) and sibyllenone (δ 2.45) is well upfield of the corresponding signal (δ 3.37) in ocobullenone. This difference in chemical shift for ocobullenone probably reflects the opposite stereochemistry at the adjacent C-8 centre. Other differences were apparent in the chemical shift of H-8. This signal varies from δ 2.48 (iso-ocobullenone) to δ 2.75 (sibyllenone) to δ 2.87 (ocobullenone). The signal for the methyl proton (clear doublet) lies between δ 0.85 and δ 1.16 and this observation proved useful in establishing the relative concentrations of the three isomers in crude mixtures. From the ^{13}C spectrum the chemical shifts (see Section 3) of C-1′ and C-3′, the positions at which one might anticipate sibyllenone to exhibit a difference relatively little can be gleaned. However, the chemical shifts of the methyl groups (at C-9) for **1**–**4** were all different and provide a practical probe for assessing the composition of mixtures of the isomers.

A study of the preferred conformations (in the solid state), as revealed by X-ray analysis, provided some useful information which may be relevant to the observed biological activity (see below). Ocobullenone **1** and iso-ocobullenone **2** adopt conformations which are very similar. This is not surprising since there is only one stereochemical difference — the orientation of the methyl group on C-8. Further discussion will thus be directed at a comparison of the differences between ocobullenone **1** and sibyllenone **4** (Fig. 1).

Comparison of the solid state conformations of **1** and **4** is dominated by the observation that the sp^2 carbon at C-5 in **1**, which forms part of the cyclohexenone ring, is directed "upwards"; whereas in **4**, the same carbon points "downwards". In both structures the cyclohexanone ring adopts a five-point coplanar conformation and the methylene group at C-2′ in both cases is the "out of plane" atom, e.g. in sibyllenone the out of plane angle is 39°. Also, in sibyllenone **4**, the plane of the substituted benzene ring, if regarded

to be in the plane of the paper, bisects the cyclopentane ring almost perfectly.

Clearly, the difference in overall shape of **1** and **4**, results from the fact that sibyllenone **4**, has opposite configurations at chiral centres C-1′ and C-3′ when compared with ocobullenone **1** and iso-ocobullenone **2**. One obvious consequence of the above arrangement in sibyllenone **4** is the close proximity of one of the methylene hydrogens on C-2′ to H-2 on the aromatic ring. To probe this situation a detailed NOE analysis was performed on sibyllenone and the results are presented (see Section 3). The results confirm the predictions and are in complete accord with the X-ray analysis.

The results of the pharmacological investigation show that none of the four compounds under investigation had any inhibitory effect in either the COX-1 or the COX-2 test system up to an upper concentration of 500 μM.

On the other hand, two of the neolignans, ocobullenone **1**, and sibyllenone **4**, showed a distinct inhibition of 5-LO. The inhibition values, (at test concentration of 100 μg/ml) for **1**–**4** were 47.9 ± 6.9, 17.8 ± 3.2, 37.6 ± 17.2 and $97.7 \pm 1.5\%$, respectively. The IC_{50} value of 18.6 μM, for sibyllenone **4** indicates that it is a good inhibitor of 5-LO. Ocobullenone **1** and precursor **3** with IC_{50} values of ca. 100 μM, and iso-ocobullenone **2** with a value greater than 100 μM posses only moderate inhibitory activity. Further structure–activity studies are required before unambiguous conclusions can be drawn from the available data.

In our earlier investigations (Zschocke et al., 2000), the volatile fraction of the bark extract proved to exhibit very good inhibitory activity on COX-1 and 5-LO and it was, therefore, suggested as the active principle of *O. bullata* bark extracts. Our present study shows that sibyllenone, and probably also ocobullenone, contribute to the inhibitory activity of *O. bullata* bark extracts. This is, therefore, the first meaningful structure–activity study of the main compounds (as opposed to purified fractions) in *O. bullata* for which the conclusions can be extrapolated back to a traditional use of the bark.

3. Experimental

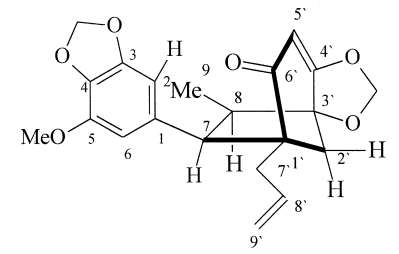
Mps. uncorrected ^{1}H- and ^{13}C-NMR spectra were recorded at 200, 500 MHz and 50, 125 MHz, respectively. NOESY experiments were carried out at 300 MHz. Solvent used is $CDCl_3$. Plant material was collected from a felled, mature tree grown in the Knysna forest, Western Cape province. A voucher specimen is deposited at the herbarium of the University of Natal.

3.1. Extraction and isolation

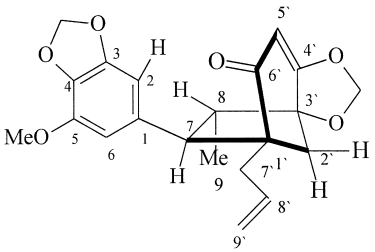
Dried, ground stem bark (600 g) was extracted three times with 3 l *n*-hexane in a Soxhlet apparatus for 12 h

Table 1
^{1}H-NMR spectral data in ppm (in $CDCl_3$, 500 MHz) for sibyllenone, iso-ocobullenone and ocobullenone

Atom(s)	Sibyllenone	Iso-ocobullenone	Ocobullenone
Aryl OCH_2O	5.98 (*dd*, J = 1.5 Hz)	5.90, 5.92 (*dd*, J = 1.4 Hz)	5.88 (*dd*, J = 1.5 Hz)
Alkyl OCH_2O	5.68 (*d*, J = 5.0 Hz)	5.46, 5.73 (*dd*, J = 0.3 Hz)	5.65 (*dd*, J = 0.3 Hz)
1	–	–	–
2	6.31–6.46 (*m*)	6.24 (*dd*, J = 1.6 Hz)	6.16 (*dd*, J = 1.7 Hz)
3	–	–	–
4	–	–	–
5	–	–	–
6	6.31–6.46 (*m*)	6.26 (*dd*, J = 1.6 Hz)	6.16 (*dd*, J = 1.7 Hz)
7	2.45 (*d*, J = 7.4 Hz)	2.68 (*d*, J = 5.9 Hz)	3.37 (*d*, J = 11.9 Hz)
8	2.75 (*q*, J = 6.8 Hz)	2.48 (*dq*, J = 13.7 Hz)	2.87 (*dq*, J = 11.9, 7.4 Hz)
9 (Me)	1.06 (*d*, J = 6.7 Hz)	1.16 (*d*, J = 6.9 Hz)	0.85 (*d*, J = 7.4 Hz)
1′	–	–	–
2′a	2.14 (*d*, J = 10.9 Hz)	2.08 (*dd*, J = 10.8, 1.28 Hz)	2.06 (*dd*, J = 10.5 Hz)
2′b	2.32 (*d*, J = 10.9 Hz)	2.34 (*d*, J = 10.8 Hz)	2.26 (*dd*, J = 10.5 Hz)
3′	–	–	–
4′	–	–	–
5′	5.47 (*s*)	5.43 (*s*)	5.57 (*s*)
C═O	–	–	–
7′a	1.32 (*dd*, J = 8.7 Hz)	2.07 (*dd*, J = 14.2, 8.9 Hz)	2.05 (*dd*, 14.2, 8.9 Hz)
7′b	2.40–2.51 (*m*)	2.67 (*dddd*, J = 13.9, 5.8, 1.3 Hz)	2.56 (*dddd*, J = 14.1, 5.8, 1.3, 1.4 Hz)
8′	5.49–5.70 (*m*)	5.66–5.85 (*m*)	5.75 (*m*)
9′	4.84–4.93 (*m*)	5.07–5.18 (*m*)	5.06 (*m*)
OMe	3.90 (*s*)	3.86 (*s*)	3.83 (*s*)



1



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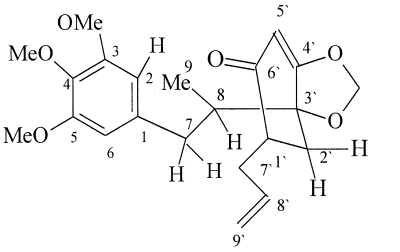

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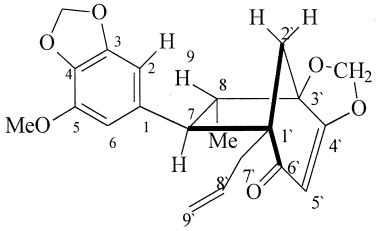

4

in total, yielding 65 g crude extract (=10.8%). One portion (4.8 g) of the *n*-hexane extract was fractionated by vacuum liquid chromatography on silica gel 0.04–0.063 mm (Merck) in a Buchner funnel (13cm × 5.7cm, 150 g), using mixtures of *n*-hexane with increasing amounts of ethyl acetate (400 ml per fraction). Sibyllenone (**4**), rel.-(7*S*,8*S*,1′*S*,3′*S*)-Δ8′-5-methoxy-3,4-methylenedioxy-3′4′-methylenedioxy-1′,2′,3′,6′-tetrahydro-6′-oxo-7.1′.8.3′-neolignan, (32 mg) crystallized from fr. IX (80% *n*-hexane), ocobullenone (**1**) (25 mg) and iso-ocobullenone (**2**) (10 mg) from fr. X (75% *n*-hexane), precursor (Δ^8-3,4,5-trimethoxy-3′,6′-dihydro-3′4′-methylene-dioxy-6-oxo-8,3′-neolignan) (**3**) (78 mg) from fr. XI (50% *n*-hexane). The known compounds (**1**–**3**) were identified by comparison of ^{1}H-NMR data with literature data and reference compounds (Sehlapelo et al., 1993; Drewes et al., 1995).

3.2. Sibyllenone (*4*)

Colourless crystals, mp 160°C $[\alpha]_D^{23} = 0°$ ($CHCl_3$; *c*.0021). UV: λ_{max} (nm) 208, 241; λ_{min} (nm) 227. ^{1}H-NMR in $CDCl_3$ (Table 1). ^{13}C-NMR: δ 15.6 (C9) [14.2 for ocobullenone, 16.9 for iso-ocobullenone and 13.3 for the precursor], 36.4 (C-7′), 44.6 (C-2′), 48.7 (C-8), 55.8 (C-1′) (59.8 for ocobullenone and 58.2 for iso-ocobullenone), 56.3 (C-7), 56.8 (OMe), 89.9 (C-3′) (91.2 for ocobullenone and 87.4 for iso-ocobullenone), 96.5 (C-5′), 101.3 (alkyl OCH_2O), 101.5 (aryl OCH_2O), 109.7 (C-2), 109.7 (C-6), 117.0 (C-9′), 133.6 (C-1), 134.5 (C-4), 135.3 (C-8′), 143.2 (C-5), 149.1 (C-3), 176.1 (C-4′), 201.1 (C-6′, C=O). EI-MS *m*/*z* (rel. int.): 370 (M^+, 8), 193 (12), 178 (100), 137 (10), 89 (9). NOE experiments show correlations between C-9 (Me) and H-7, between H-6 and H-7, H-8 (weak), between H-2, H-6 and H-2′b, H-7′b, between H-8 and H-2, between H-9 and the aliphatic OCH_2O.

3.3. Crystallization of *1* and *4*

In order to grow crystals suitable for X-ray analysis ocobullenone **1**, had to be recrystallised four times (EtOAc–hexane) at a controlled temperature of 5°C. For sibyllenone **4**, the original crystals proved to be unsatisfactory (twinned crystals) for X-ray diffraction. However, by using petroleum ether (40–60C°) and ethyl acetate in a two-phase system which was kept at room temperature for an extended period, suitable crystals were grown.

3.4. X-ray analysis

Diffraction data were collected on a Nonius CAD 4 diffractometer with graphite monochromated MoK radiation. The unit cell parameters were determined by a least squares fit. Structures were solved by direct methods.

For sibyllenone $C_{21}H_{22}O_6$: Final *R* value was 0.0405. Crystals were monoclinic with cell parameters [Å], *a* = 16.147 (3), *b* = 6.7760 (15) and *c* = 16.956 (2), *V* [Å^3] = 1841.0 (6), space group P21/*n*, *Z* = 4 (Fig. 1).

For ocobullenone $C_{21}H_{22}O_6$: Final *R* value was 0.1132. Crystals were triclinic with cell parameters [Å], *a* = 6.6587 (7), *b* = 7.9795 (8), *c* = 9.6202 (10), *V* [Å^3] = 460.75(8), space group P1, *Z* = 1 (Fig. 1).

3.5. Bioassays

The COX-1 assay was performed as described (White and Glassman, 1974) with slight modifications (Jäger et al., 1996); the COX-2 assay was performed according to the published procedures (Noreen et al., 1998), slightly modified (Zschocke et al., 2000). Inhi-

bition refers to reduction of PGE_2 formation in comparison to an untreated sample. Positive control measurements were carried out with indomethacin IC_{50} = 3.1 μM (COX-1) and 188 μM (COX-2). The 5-LO was performed as described (Kuhl et al., 1984), modified by Zschocke et al. (1997). Inhibition refers to reduction of 5-HETE formation in comparison with a blank sample. Positive control measurements were carried out with nordihydroguaiaretic acid (NDGA, IC_{50} = 0.5 μM). All results are the mean of at least three experiments. IC_{50} values were determined by regression analysis of the results at three different concentrations of the sample.

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