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Terpenes from *Otostegia integrifolia*

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Abstract

The essential oil and chloroform extract of air-dried leaves of *Otostegia integrifolia* Benth. were investigated for the first time using analytical and preparative gas chromatography (GC), GC-mass spectrometry (MS) and NMR techniques. A total of 40 constituents including monoterpenes, sesquiterpenes, diterpenes and their derivatives were identified. A prenylbisabolane type diterpene, 1-methyl-4-(5,9-dimethyl-1-methylene-deca-4,8-dienyl)cyclohexene was identified as a major component. The chloroform extract of the leaves yielded two labdane type diterpenoids, 15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene and 9(13),15(16)-diepoxy-3α-hydroxy-16-dihydrolabda-14-ene, a saturated hydrocarbon, pentatriacontane, and stigmasterol. The structures of the isolated compounds were established by spectroscopic methods.

Keywords: *Otostegia integrifolia* Benth.; Lamiaceae; Essential oil; Furanolabdane diterpenes; Otostegindiol; Preotostegindiol; (+)-Axinyssene; Pentatriacontane; Stigmasterol

1. Introduction

Otostegia integrifolia Benth. belongs to the Lamiaceae (Labiatae) family (Fichtl and Adi, 1994). It is one of the plants used in traditional medicine in Ethiopia. The plant has insecticidal properties and is often used as fumigant for pots and houses. The roots are used for treating lung diseases (Fichtl and Adi, 1994). No previous phytochemical investigation of the plant has been reported. However, there are reports on the chemical investigation of *Otostegia* species. Thymol, γ-terpinene and *p*-cymene were reported as major constituents in the essential oil of *O. fruticosa* analysed by gas chromatography–mass spectrometry (GC–MS) (Aboutabl et al., 1995). Furthermore, from aerial parts of the same plant, isolation of three new and five known labdane diterpenes together with an iridoid glucoside was reported. These were otostegin A (**1**), otostegin B (**2**), 15-*epi*-otostegin B (Al-Musayeib et al., 2000), preleoheterin, leoheterin (**3**) (Hon et al., 1993), and related compounds leopersin C, 15-*epi*-leopersin C (Tasdemir et al., 1998), ballonigrin (Brian and James, 1977), vulgarol (Popa and Pasechnik, 1975) and 8-*O*-acetylharpagide (Scarpati et al., 1965) (Fig. 1).

Our investigation of air-dried leaves of *O. integrifolia* resulted in the isolation of a prenylbisabolane type diterpene, axinyssene, from the essential oil and two new furanolabdane type diterpenes, otostegindiol and preotostegindiol, a saturated hydrocarbon, pentatriacontane, and stigmasterol from the chloroform extract.

2. Results and discussion

The essential oil of air-dried leaves of *O. integrifolia* was analysed by GC and GC–MS. Mass spectra and retention indices on a non-polar stationary phase (CPSil-5) of the components were compared with a library of mass spectra of authentic compounds established under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2002): *trans*-2-hexenal, *trans*-hex-3-ene-1-ol, 1-hexanol, α-thujene,α-pinene, thuja-2,4(10)-diene, 1-octene-3-ol, β-pinene,

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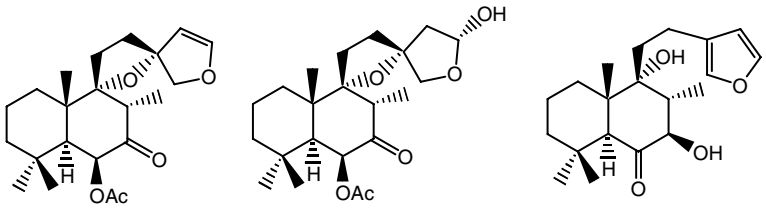
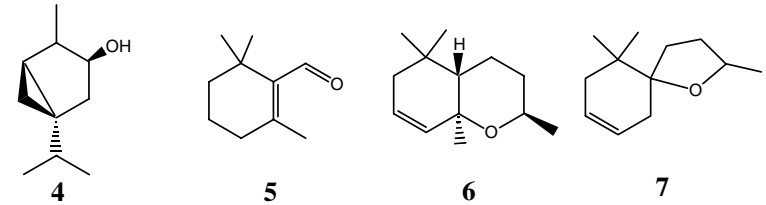


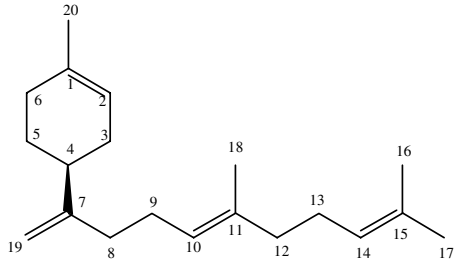
1 **2** **3**

Fig. 1. Selected labdane diterpenes from *Otostegia fruticosa*.

3-octanol, phenylacetaldehyde, limonene, (*Z*)-β-ocimene, linalool, *trans*-sabinol (**4**), mentha-1,5-diene-8-ol, terpinene-4-ol, α-terpineol, β-cyclocitral (**5**), nerol, geraniol, vinylguajacol, dihydroedulan (**6**), theaspirane (**7**), eugenol, α-ylangene, β-bourbonene, *E*-β-caryophyllene, geranylacetone, α-humulene, β-ionone, γ-muurolene, germacrene D, 4,5-di-*epi*-aristolochene, α-muurolene, δ-amorphene, *E*-nerolidol, spathulenol, caryophyllenoxide, and β-eudesmol were identified. The major component which could not be identified by this method was isolated by preparative GC and its structure was established as (+)-1-methyl-4-(5,9-dimethyl-1-methylene-deca-4,8-dienyl)-cyclohexene (**8**) from its MS, 1D and 2D NMR data. The dried and pulverised leaves of the plant were also soaked in chloroform at room temperature for 48 h. Unlike the essential oil, the chloroform extract contained mainly non-volatile substances. Part of the extract was subjected to repeated column chromatography on silica gel and Sephadex LH-20 columns and two new furanolabdane type diterpenes, **9** and **10** (Fig. 2), a saturated hydrocarbon pentatriacontane, and stigmasterol were isolated.



4 **5** **6** **7**



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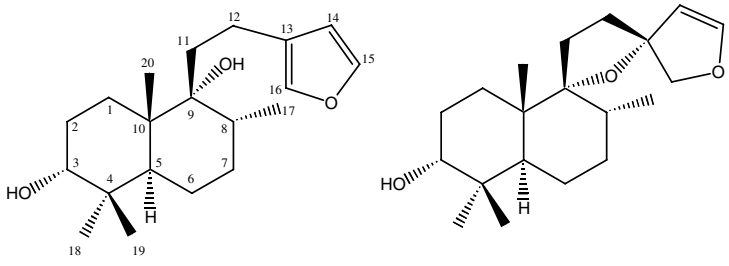

9 **10**

Fig. 2. Structures of selected constituents from *O. integrifolia* leaves.

2.1. (+)-1-Methyl-4-(5,9-dimethyl-1-methylene deca-4,8-dienyl)cyclohexene (**8**)

Compound **8** was isolated as an oil by preparative GC from the essential oil of the dried leaves of the plant. It exhibited a positive optical rotation and its mass spectrum showed a molecular ion peak at m/z 272. Its ^{1}H (Table 1) and HMQC NMR data indicated the presence of four allylic methyl singlets, three olefinic and one aliphatic methine signals, one exocyclic methylene singlet and seven aliphatic methylene multiplets. The ^{13}C NMR (Table 1) contained signals due to four olefinic quaternary carbons in addition to the signals observed in the ^{1}H NMR spectrum. The ^{1}H and ^{13}C NMR data in combination with the mass spectrum confirmed the elemental composition $C_{20}H_{32}$, a diterpenoid hydrocarbon with five degrees of unsaturation. Analysis of connectivities in the ^{1}H–^{1}H COSY and HMBC spectra confirmed that four of the five unsaturations were due to double bonds and the fifth was due to a ring.

The connectivity within the molecule was established by interpretation of the 2D ^{1}H–^{1}H COSY and HMBC spectra. In the HMBC spectrum, it was observed that two of the allylic methyl groups (H_3-16) (δ values Table 1) and (H_3-17) were correlated with each other and also with H-14. In addition, these methyls as well as the methine (H-14) were coupled to the olefinic quaternary carbon (C-15). This indicated that the two methyls were geminal and connected to the olefinic quaternary carbon that itself was connected to the olefinic methine carbon. C-15 also exhibited coupling in the HMBC to H_2-13 which in the ^{1}H–^{1}H COSY exhibited coupling to H-14 as well as to H_2-12. The latter was coupled to H_3-18 whose carbon (δ 26.4, C-18) was correlated with H_2-12 in the HMBC spectrum. Further in the ^{1}H–^{1}H COSY, H_2-12 exhibited allylic coupling to H-10. The latter was coupled to H_2-9 which was itself coupled to H_2-8. H_2-8 displayed allylic coupling with the exocyclic olefinic methylene (H_2-19). This constituted the 5,9-dimethyl-1-methylene-4,8-decadienyl side chain of the compound. On the other hand, H-4 was coupled to each of the H_a-5 and H_b-5 multiplets. The latter were coupled to H_a-6 and H_b-6. H-4 was also coupled to H_a-3 and H_b-3. The latter were coupled to the olefinic methine signal (H-2) that itself exhibited allylic coupling to H_3-20. This constituted the 1,4-disubstituted cyclohexene ring moiety of **8**. This diterpene hydrocarbon is the first of its kind to be isolated from the genus *Otostegia* or any other plant. But its optical antipode, named (−)-axinyssene, was recently reported as a constituent of a Japanese marine sponge and exhibited anti-tumor activity (Kodama et al., 2003).

2.2. Otostegindiol (**9**)

Compound **9** was obtained as a white solid upon repeated column chromatography on silica gel and Sephadex LH 20 columns. Its mass spectrum exhibited a molecular ion peak at m/z 320, which in combination with its ^{1}H and ^{13}C NMR data (Table 1) led to the elemental composition of $_{20}H_{32}O_3$, an oxygenated diterpenoid with five degrees of unsaturation. Fragment peaks

Table 1
^{1}H and ^{13}C NMR data of compounds **8**, **9** and **10**

Atom no.	**8**			**9**			**10**		
	δ ^{1}H	m (J)	δ ^{13}C	δ ^{1}H	m (J)	δ ^{13}C	δ ^{1}H	m (J)	δ ^{13}C
1		–	133.8	1.25	*m*	25.1	1.24	*m*	25.1
				1.93	*m*		1.63	*m*	
2	5.41	*bs*	121.6	1.64	*m*	25.6	1.65	*m*	25.2
				1.96	*m*		1.93	*m*	
3	1.97	*m*	32.1	3.38	*bs*	76.3	3.37	*bs*	76.1
	2.15	*m*							
4	2.14	*m*	40.6	–	–	43.2	1.87	–	42.4
5	1.48	*m*	28.9	1.90	*m*	39.8		*m*	39.8
	1.79	*m*							
6	1.86	*m*	31.2	1.34	*m*	21.6	1.35	*m*	21.2
	1.95	*m*		1.50	*m*		1.47	*m*	
7	–	–	154.4	1.35	*m*	31.4	1.32	*m*	31.6
				1.46	*m*		1.46	*m*	
8	2.11	*m*	35.6	1.79	*m*	37.1	1.79	*m*	37.3
9	2.22	*m*	27.5	–		77.5		–	93.3
10	5.29	*t* (7.0)	125.1	–		37.8		–	37.7
11	–	–	135.4	1.67	*m*	35.5	1.66	*m*	32.0
				1.91	*m*		1.91	*m*	
12	2.09	*m*	40.1	2.46	*m*	21.9	1.81	*m*	35.6
13	2.17	*m*	27.5	–		126.1	–	–	93.0
14	5.22	*t* (7.0)	125.2	6.27	*bs*	111.3	5.14	*d* (2.8)	107.6
15	–	–	131.5	7.34	*bs*	143.2	6.43	*d* (2.8)	147.8

at m/z 81 and 95 in the mass spectrum indicated the presence of a β-monosubstituted furan ring. This was confirmed by the ^{1}H NMR spectrum which showed typical signals of a β-monosubstituted furan ring at δ 6.27 (1H, *bs*, H-14),7.34 (1H, *bs*, H-15) and 7.22 (1H, *bs*, H-16). This indicated that three of the unsaturations were due to the furan ring. The remaining two should be due to two rings since no signal arising from multiple bonds was present in any of the spectra. Moreover, the resonances of several methylene protons connected to the same carbon at different chemical shifts substantiated the presence of rings in the molecule. The ^{1}H NMR (Table 1) also contained three methyl singlets, a methyl doublet and a methine multiplet. Presence of a methine carbinol group (H-3) was also observed. The remaining proton signals were overlapping in the range of δ 1.24–2.00. These include six methylene and a methine protons.

The ^{13}C NMR of **9** contained signals due to twenty carbon atoms, four primary, six secondary, six tertiary and four quaternary carbons. This was also confirmed by a PENDANT experiment (Homer and Perry, 1994). Among these, two carbons were oxygenated. One was a methine carbinol at δ 76.3 and the second was a quaternary carbinol at δ 77.5.

In the ^{1}H–^{1}H COSY spectrum of compound **9** (Fig. 3), several key couplings indicating a furanolabdane diterpene skeleton were observed. Similarly, in the HMBC spectrum (Fig. 4) key couplings were observed that substantiated the connectivity found in the ^{1}H–^{1}H COSY. The fact that two of the primary methyl groups (H_3-18) and (H_3-19) were geminal was indicated by couplings between the two groups in the HMBC spectrum. Furthermore, the methine carbinol exhibited couplings to each of these geminal methyl proton singlets as well as to H_2-1 and H_2-2. This indicated that the secondary hydroxyl group has to be at C-3 on the A-ring

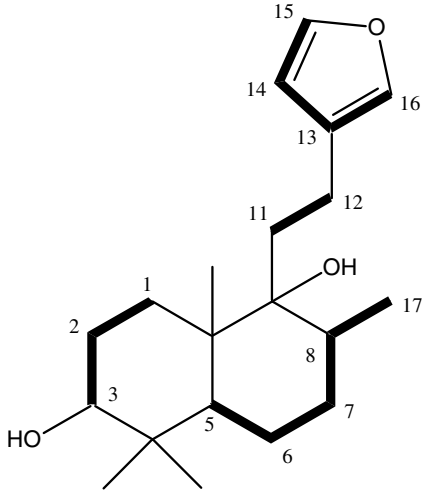

Fig. 3. Key ^{1}H–^{1}H COSY couplings (bold face bonds) observed for compound **9**.

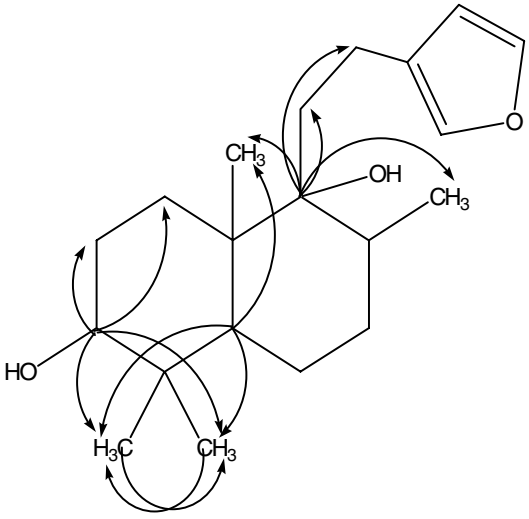

Fig. 4. Key HMBC couplings observed for compound **9**.

of the molecule. Another key structural information that indicated that the tertiary alcohol should be at C-9 was obtained from the couplings observed between the latter and the methyl proton doublet (H_3-17), the methyl proton singlet (H_3-20), the methylene proton multiplets (H_2-11) and (H_2-12).

The relative stereochemistry of the chiral centres at C-3, C-5, C-8, C-9 and C-10 was deduced from the NOESY spectrum of the compound and the *J*-values. In the NOESY of **9** (Fig. 5) couplings were seen between H-3/H_3-18, H-3/H_3-19 and H-3/H_a-2 and H-3/H_b-2. This indicated that H-3 must be equatorial and on the β-side of the molecule. This was also supported by the small *J*-values for the equatorial–axial and equatorial–equatorial couplings between H-3/H_a-2 and H-3/H_b-2.

The presence of NOESY couplings between H-5/H_3-18 indicated that H-5 had to be α-oriented like the C-18 methyl. NOESY correlations between H-8/H_3-20, H-8/H-11, H-11/H_3-20 and H_3-19/H_3-20 indicated the close

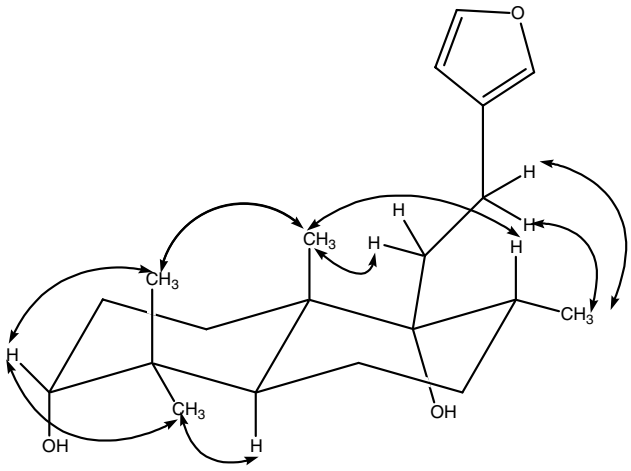

Fig. 5. Key NOESY correlations observed for **9**.

proximity of these groups and their β-disposition. Therefore, the C-17 methyl group had to be equatorial and α-oriented. Thus, all the NMR data of the compound are in agreement with the proposed structure of 15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene (**9**).

2.3. Preotostegindiol (*10*)

Compound **10** was obtained as a white solid. Its ^{1}H NMR (Table 1) was similar to that of **9**, except that the furan ring signals were replaced by signals typical of a β,β-disubstituted dihydrofuran ring. These signals appeared at δ 6.43 (1H, *d*, $J = 2.8$, H-15), 5.14 (1H, *d*, $J = 2.8$, H-14) and a two proton AB system at δ 4.53 (1H, *d*, $J = 10.1$) and 4.05 (1H, *d*, $J = 10.1$) corresponding to the methylene group of the dihydrofuran ring (H_2-16). This was further confirmed by the signals in the ^{13}C NMR spectrum at δ 93.3 (C-9) and 93.0 (C-10), which were joined by the ether linkage of the 9(13)-epoxy group and the appearance of an oxygenated methylene signal at δ 81.5 (C-16) instead of the olefinic methine signal at δ 138.9 in **9**. The remaining NMR data of **10** resembled those of **9**. The conversion from **10** to **9** under mildly acidic conditions was further proof for the structure of **10**.

Prefuranic and furanic labdane diterpenoids are commonly encountered in many species of the Lamiaceae family, such as *O. fruticosa* (Al-Musayeib et al., 2000), *Leonurus heterophyllus* (Hon et al., 1993), *L. persicus* (Tasdemir et al., 1998) *Marrubium vulgare* (Bergeron et al., 1995), and *Ballota aucheri* (Rustaiyan et al., 1992). *O. integrifolia* is the only species, so far, in which C-3 hydroxylated prefuranic and furanic labdanes are found. In addition, unlike the labdanes from other species, these two labdanes from *O. integrifolia*, are missing C-6-, C-7- or C-8-oxygenations.

3. Experimental

3.1. General

The plant material used in this study was collected near the town of Ambo, Ethiopia. Melting points were measured with an Electrothermal Melting Point apparatus. The NMR spectra were recorded on a Bruker WM 400 or 500 MHz spectrometer in either deuterated benzene or deuterated chloroform. The chemical shift values are reported with reference to TMS and the coupling constants are given in hertz. Optical rotations were measured as solutions in methanol or benzene on a Perkin–Elmer 341 polarimeter at 589 nm and 20 °C.

3.2. Hydrodistillation, extraction and isolation procedure

About 500 g of cleaned, air dried and pulverised leaves of *O. integrifolia* were divided into two equal portions. The first part was homogenised and hydrodistilled in a Clevenger type apparatus for 2.5 h and a slightly greenish oil was collected in HPLC grade hexane. The oil was analysed on an Orion capillary GC with FID detector, containing two columns, a 25 m/0.25 mm i.d. non-polar CPSil-5-CB and a slightly polar CPSil-19-CB column of identical dimensions. The oven temperature was programmed from 50 to 230 °C at a rate of 3 °C/min. The injector and detector temperatures were kept at 200 and 250 °C, respectively. The oil was then further analysed using GC-MS on a HP 5890 GC coupled to a VG Analytical 70-250S mass spectrometer with electron impact (70 eV) ionisation. Retention indices and mass spectra of the components were compared with library spectra generated under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2002). Some high boiling components, including **8**, were apparently unknown. Then the oil was fractionated into 12 fractions on a modified Varian 1400 preparative gas chromatograph, equipped with a stainless steel column (1.85 m×4.3 mm) packed with 10% polydimethylsiloxane SE 30 on Chromosorb W-HP. Then **8** was isolated from fraction 9 using the same preparative GC equipped with a column (2 m×4.3 mm) packed with the slightly polar SE 52 stationary phase.

The second part of the dried pulverised leaves of *O. integrifolia* was soaked in chloroform at room temperature for 48 h. The extract was filtered and the chloroform was removed under vacuum to give a dark oily residue. Part of the extract was subjected to column chromatography on a silica gel column with hexane as eluent containing increasing amounts of ethyl acetate. Fourteen fractions were collected. Fractions 1 and 2 eluted with pure and 5% hexane in ethyl acetate, respectively, and yielded pentatriacontane. Fractions 7, 8 and 10 gave impure solids upon evaporation of the solvents under vacuo. Each of these was re-chromatographed separately on silica gel columns using *n*-hexane with increasing amounts of ethyl acetate (20–30%, v/v) as eluent, followed by purification from chlorophyll on a Sephadex LH-20 column using chloroform/methanol (2:1, v/v) as eluent. By this method, fraction 7 gave stigmasterol, fraction 8 afforded compound **10** and fraction 10 yielded compound **9**.

3.3. (+)-Axinyssene (*8*)

Colourless oil, $RI_{CPSil5} = 1142$, sense of optical rotation (benzene): (+); MS (EI, 70 eV), m/z (rel. inten.): 272 (5), 257 (2), 229 (3), 187 (12), 175 (4), 159 (4), 147 (4), 133 (5), 119 (13), 107 (20), 93 (36), 81 (50), 69 (100), 55 (25), 41 (75). ^{1}H and ^{13}C NMR data, see Table 1.

3.4. Otostegindiol (*9*)

White crystals from hexane; m.p. 124–125 °C; $[\alpha]_{20}^{589} = (+)\ 25$ ($c = 0.01$, methanol); ^{1}H and ^{13}C NMR

(see Table 1); MS (EI, 70 eV), m/z (rel. inten.): 320 [M^+] (33), 302 (20), 284 (3), 259 (6), 241 (8), 207 (10), 189 (10), 179 (14), 165 (53), 150 (88), 135 (34), 123 (58), 109 (26), 95 (56), 81 (100), 69 (34), 55 (22), 43 (35).

3.5. Preotostegindiol (*10*)

White crystals. NMR data, see Table 1. **10** rearranged completely to **9** when submitted to GC or GC-MS.

3.6. Pentatriacontane

MS (EI, 70 eV), m/z (rel. inten.): 492 [M^+] (1), 464 (8), 436 (7), 365 (6) 351 (6), 337 (6), 323 (6), 309 (6), 295 (6), 281 (7), 267 (7), 253 (7), 239 (7), 225 (8), 211 (9), 197 (9), 183 (10), 169 (11), 155 (12), 141 (14), 127 (17), 113 (20), 99 (28), 97 (18), 85 (69), 83 (19), 71 (85), 69 (17), 57 (100), 55 (16), 43 (48). ^{1}H NMR: δ 0.95 (6H), δ 1.45 (66H); ^{13}C NMR: 14.6 (2q), 23.4 (t), 30.1 (t), 30.4 (30t), 32.6 (t).

3.7. Stigmasterol

White solid, MS (EI, 70 eV), [M^+] at m/z 412, elemental composition: $C_{29}H_{48}O$. ^{1}H NMR same as authentic sample.

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