Supporting Information

Biosynthesis of Baccharis Oxide, a Triterpene with 3,10-Oxide Bridge in A-Ring

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cDNA cloning

S. rebaudiana plant was a kind gift from Tokiwa Phytochemical Co., Ltd, Tokyo, Japan. Oligo DNAs synthesized by Nihon Bioservice (Saitama, Japan) and Invitrogen (Tokyo, Japan) were used. RNA was extracted by phenol-SDS method from roots by the same method as previously reported[8] and RNeasy Mini Kit (QUIAGEN). RNA was reverse transcribed to produce cDNAs using reverse transcriptase (Superscript III, Invitrogen) and oligo dT primer, RACE32 (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTT–3') with dNTP (0.2 mM) in a volume of 20 μL following the manufacturer's protocol. The resulting cDNA mixture was used as the template in the following PCR.

PCRs were performed in several combinations with a set of primers (1 μg each, sense primers; 351S (5'-CTI ACI MGI TGG CCI TTY AAY AAR -3') or 403S (5'- AAY GGI GAY GMI TTY AAR MRI CA -3'), and antisense primers; 493A (5'- AC IWS VCA ICC RTG RTC YTG RTC -3') or 551A (5'- WB IGS IMC IKS IGG YTC CCA IRC -3')), using Ex Taq DNA polymerase (Takara Biochemical) with dNTP (0.2 mM)

in a final volume of 100 μL following the manufacturer's protocol. PCR was carried out with a program; denature at 94˚C for 5min, 30 cycles of 94 ˚C for1 min, 42 ˚C for 2 min and 72 ˚C for 3 min, and final extension at 72 ˚C for 10 min. The PCR products (294bp) was subcloned to the plasmid vector (pT7-Blue, Novagen). Thirty-one colonies were picked up in total, and nucleotide sequence was determined. Four kinds of genes (3, 3, 24 and 1 clones each) were obtained, and named as *Str-1, 2, 3, 4*, respectively. Based on the obtained sequence, specific primers were designed to obtain the 3' and 5' end sequences of four genes. RACE PCRs were carried out following the method described in our previous paper with some modifications. [8] Despite many efforts, no extension was succeeded except *Str-3*. Used primers in RACE for *Str-3* were as follows. For 3'-RACE; ST3-454S (5′-AAA GGA CAT GAC TTC ATT AAA GCC-3′), ST3-476S (5′-AGC ATG TAT CGC CAT ATT ACG AAG-3′). For 5'-RACE; ST3-204A (5′-ACA TGC ACC ATT AAG ACC GCC GTC-3′), ST3-241A (5′-TGG ATT GCA TCC TTC CCA CTC ATG-3′), ST3-417A (5′-ACC CAA ATA TAA TCC GGG ATT CTG-3′), ST3-442A (5′-TAG AGC TTG AAT GGC AAG ACT TGC-3′) and ST3-452A (5′-GTT CAA TGT CAT GGT TTA GAT CGG-3′).

To obtain the full-length clones, PCR was carried out with ST3-Eco-N (5'-AGT ATA **GAA TTC** ATG TGG AGG TTA AAG ATA GCA GAT GGG-3', *EcoRI* site in bold face) and ST3-Xho-C (5'-CCA TGG **CTC GAG** TTA AAC CTT TGT GGG TTG AGG TAG CAC-3' *Xho* I site in bold face). PCR was carried out using Fusion™ DNA polymerase (FINNZYMES) with the program; denature at 98˚C for 30 sec, 30 cycles of 98 ˚C for 10 sec, 69 ˚C for 15 sec and 72 ˚C for 50 sec, and final extension at 72 ˚C for 5 min. The 2.3-kb PCR product was digested with *Eco* RI and *Xho* I and ligated into the corresponding sites of pYES2 (Invitrogen) to construct the plasmid pYES2-Str-3. The obtained full length cDNA clone, *Str-3* was sequenced. This sequence is available under accession number: AB455264 in DDBJ sequence-database.

Expression in *Saccharomyces cerevisiae* and product analysis

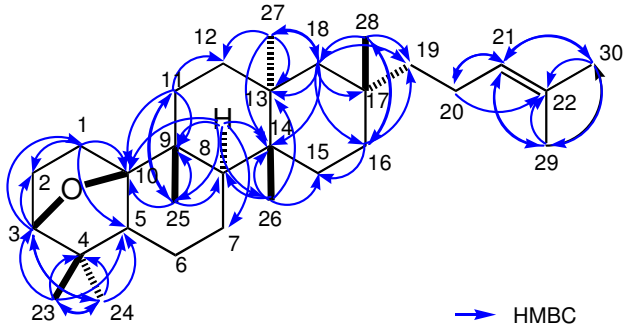
The plasmid pYES2-PNA was transferred to *S. cerevisiae* strain GIL77[9] using Frozen-EZ Yeast Transformation II^{TM} kit (ZYMO RESEARCH). The transformant was inoculated in 20 mL synthetic complete medium without uracil (SC-U), containing ergosterol (20 μg/mL), hemin chloride (13 μg/mL) and Tween 80 (2.5 mg/mL), and incubated at 30 ˚C for 3 days. The obtained culture were transferred into 2-L of the same medium, and incubated at 30 ˚C for 3 days. Cells were collected by centrifugation, and transferred into SC-U medium (2-L) with the same supplements and 2% galactose in place of glucose. Cells were collected and resuspended into 0.1 M potassium phosphate buffer (pH 7.0, 1-L) supplemented with 2% glucose and hemin chloride (13 μg/mL) and further incubated for 24 hours at 30˚C. Cells were collected and refluxed with 200 mL of 20% KOH/50% EtOH *aq*. for 2 h. After extraction with hexane (150 mL x 3), the extract was washed with 100 mL each of 1N HCl, 1M $NaHCO_3$, saturated *aq*. NaCl, successively, concentrated, and checked by TLC (Merck #5715) which was developed with hexane:ethyl acetate=93:7 and visualized with heating after sprayed with 10% sulfuric acid (Fig. 2).

The concentrated extract was applied on a silica gel column (Wako C-200 silica gel, 100 g) with benzene as an eluent. Fractions (50 mL each) were collected after 300 mL collection of the first fraction. Fractions 1-5 containing the product corresponding to spot **a** were combined and concentrated. Amorphous solids in the concentrated solution were collected and recrystallized from ethyl acetate, to yeild **2**, (10.2 mg; mp. 148-149 ºC, $[\alpha]_D^{25}$ +43.7 (c=0.47, $CHCl_3$); literature values[11]: mp. 148-149 ºC, $[\alpha]_D^{25}$ +42 (c=2.16, $CHCl_3$)). Combined fractions 8-12 corresponding to spot **b** were concentrated and analyzed by

GCMS (Fig. S4-E) and NMR (data not shown). Fractions 13-22, corresponding to spot **c,** were combined, concentarated and analyzed by GC/MS (Fig. S4-D).

^{1}H- and ^{13}C-NMRs were measured in $CDCl_3$ (JEOL ECA-500). NMR data and HMBC correlation are presented in Fig. S1. ^{1}H- and ^{13}C-NMR spectra were shown in Fig. S2 and S3, respectively. GC/MS analysis was carried out using a Shimadzu (Kyoto, Japan) GCMS-QP2010 with a Restec Rtx-5MS glass capillary column (30 m in length, 0.25 mm in diameter, 0.25 μm film thickness) and He as a carrier gas (45 cm/sec)) by the temperature raising program; 250 ºC for 2 min, raising the temperature at the rate of 20 ºC/min until 330 ºC, and 330 ºC for 5min. The temperature of the ionization chamber was 250 ºC, and ionization was by electron impact at 70 eV. All authentic specimens used were from our laboratory stocks.

Fig. S1 NMR analysis of baccharis oxide



	ST-3 Product-1		Reported in ref.10	
No.	^{13}C	^{1}H	^{13}C	^{1}H
1	32.1	1.96	32.0	1.94
2	24.8		24.8	
3	84.3	3.75(d, *J* = 5.5 Hz)	84.3	3.75(d, *J* = 5.7Hz)
4	43.4		43.3	
5	53.2		53.1	
6	19.7		19.6	
7	20.2		20.1	
8	39.2	1.42	39.1	1.4
9	37.4		37.3	
10	93.8		93.8	
11	29.8	2.24(td, *J* = 14, 5.5 Hz)	29.8	2.23(td, *J* = 14, 5.3 Hz)
12	32.3		32.3	
13	36.6		36.5	
14	39.2		39.1	
15	29.5		29.4	
16	34.6	1.53	34.6	1.54
17	31.8		31.8	
18	44.2	1.17 , 1.14	44.2	1.27 , 1.20
19	43.1		43.0	
20	23.1	2.00 , 1.85	23.0	2.00 , 1.80
21	125.2	5.09(m)	125.3	5.09(t, *J* = 7.0 Hz)
22	130.8		130.8	
23	22.9	0.89(s)	22.8	0.88(s)
24	24.2	1.01(s)	24.2	1.00(s)
25	22.1	1.23(s)	22.0	1.23(s)
26	15.4	1.03(s)	15.4	1.02(s)
27	20.0	1.04(s)	20.0	1.04(s)
28	32.86	0.89(s)	32.8	0.89(s)
29	17.60	1.60(s)	17.5	1.59(bs)
30	25.74	1.68(s)	25.7	1.67(bs)

Fig. S2 ^{1}H-NMR of baccharis oxide

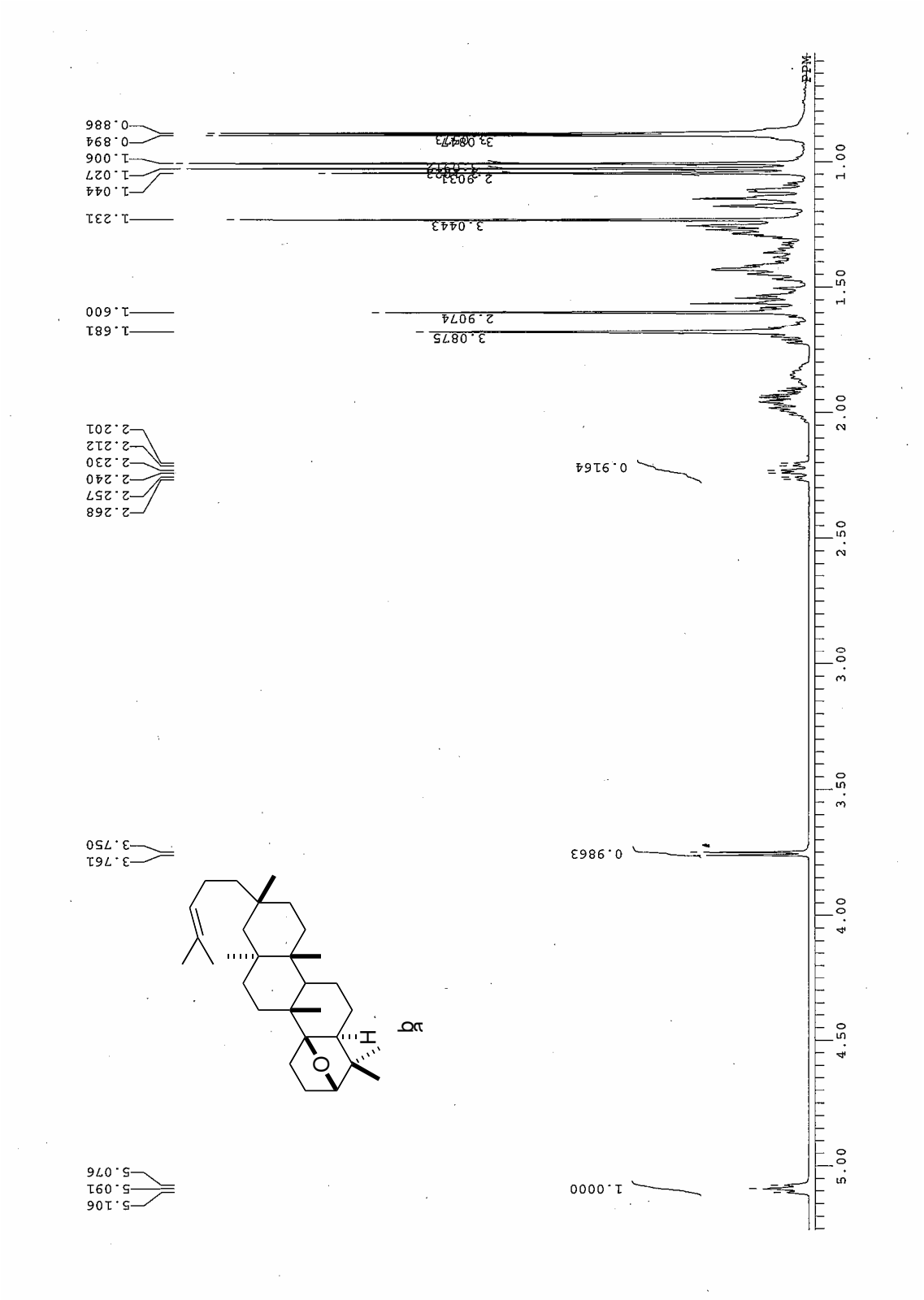


Fig. S3 ^{13}C-NMR of baccharis oxide

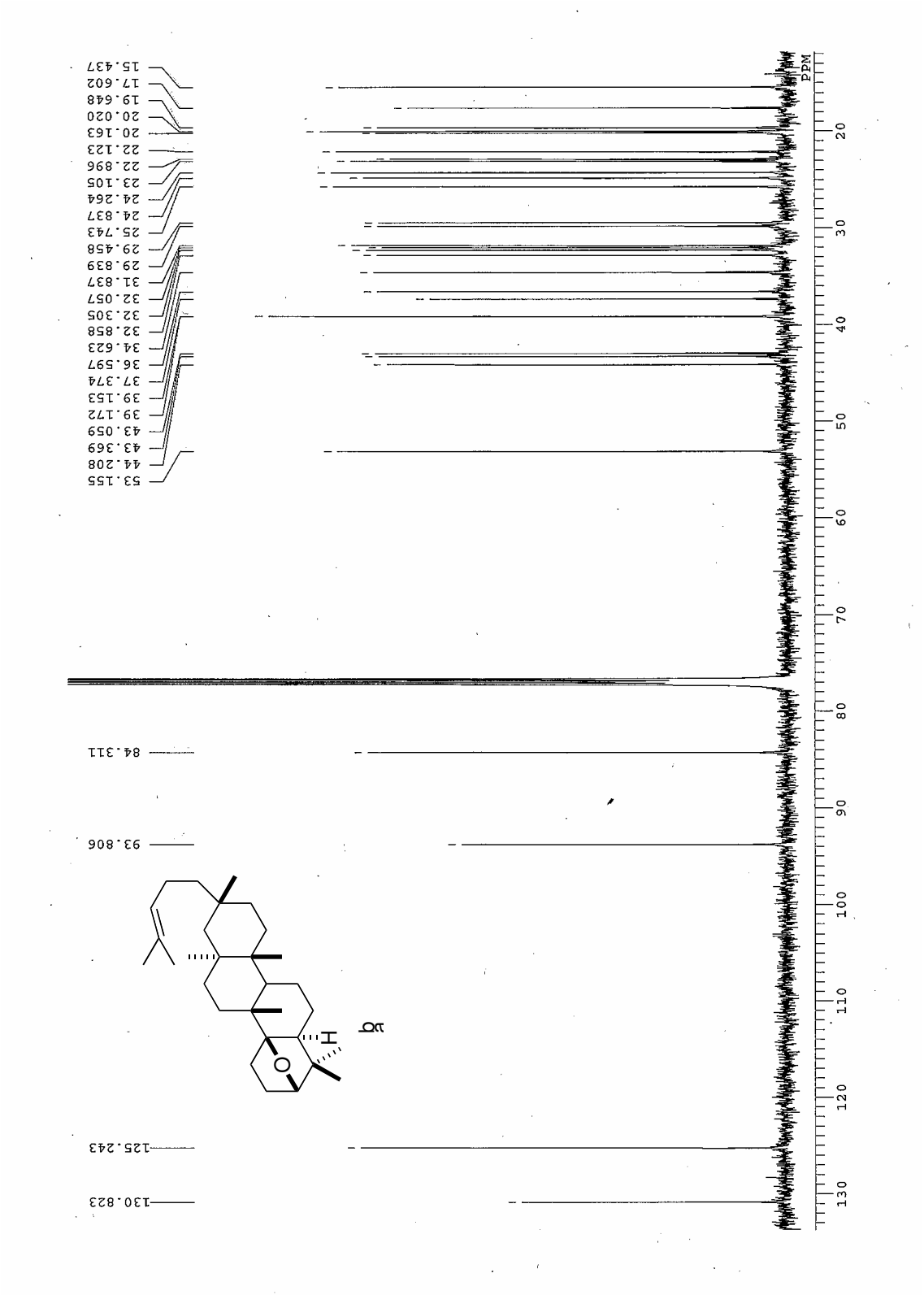
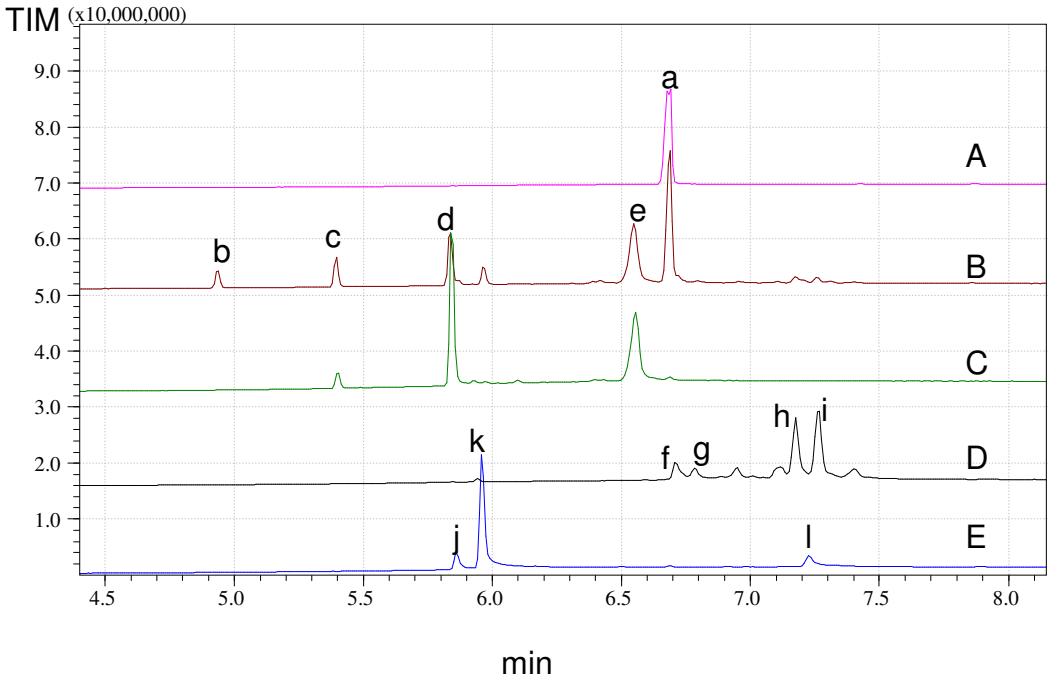


Fig. S4 GC/MS analysis of products



Each chromatogram indicates; A: purified baccharis oxide, B: crude extract of transformant, C: crude extract of transformant with void vector, D: fraction corresponding to spot **c** on TLC, and E: fraction corresponding to spot **b** on TLC. MS fragmentation patterns of each peaks (a and f-l) and authentic specimens were shown in Fig. S5. Yeast metabolites (peaks b-e) were identified as squalene, oxidosqualene, dioxidosqualene and ergosterol, respectively, by comparing with the authentic specimens (data not shown).

Fig. S5 MS fragmentation patterns of products (1)

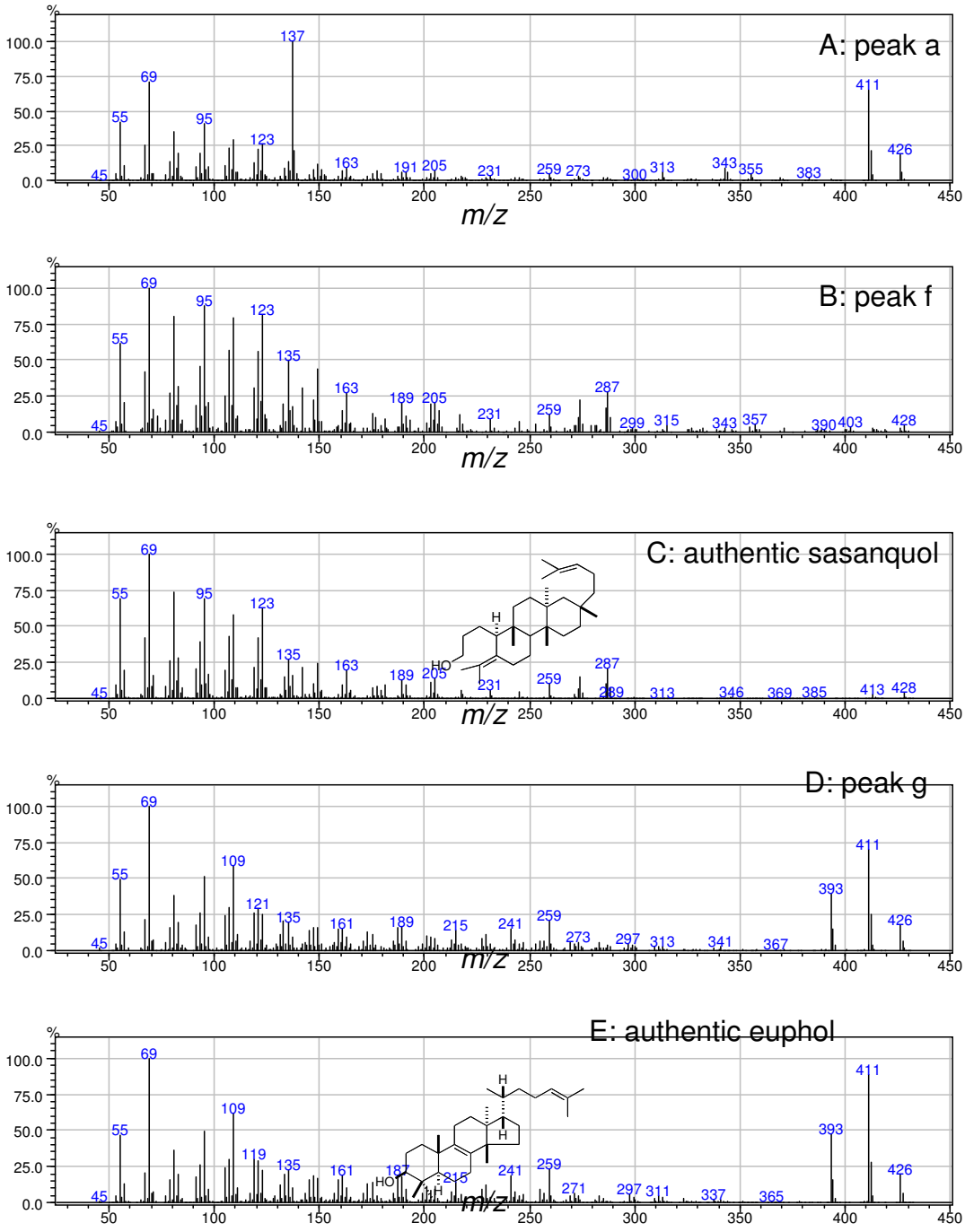


Fig. S5 MS fragmentation patterns of products (continued (2))

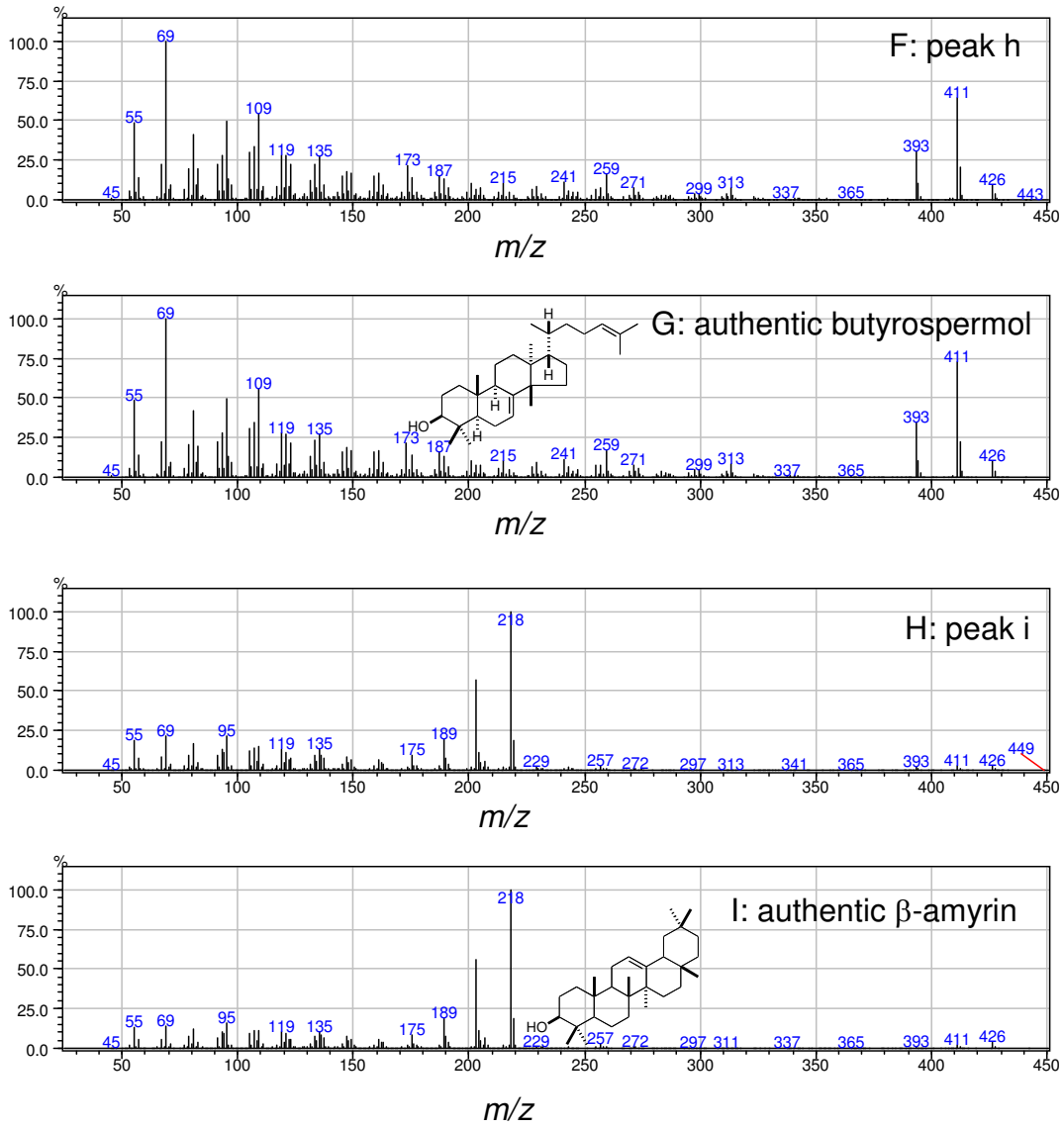


Fig. S5 MS fragmentation patterns of products (continued (3))

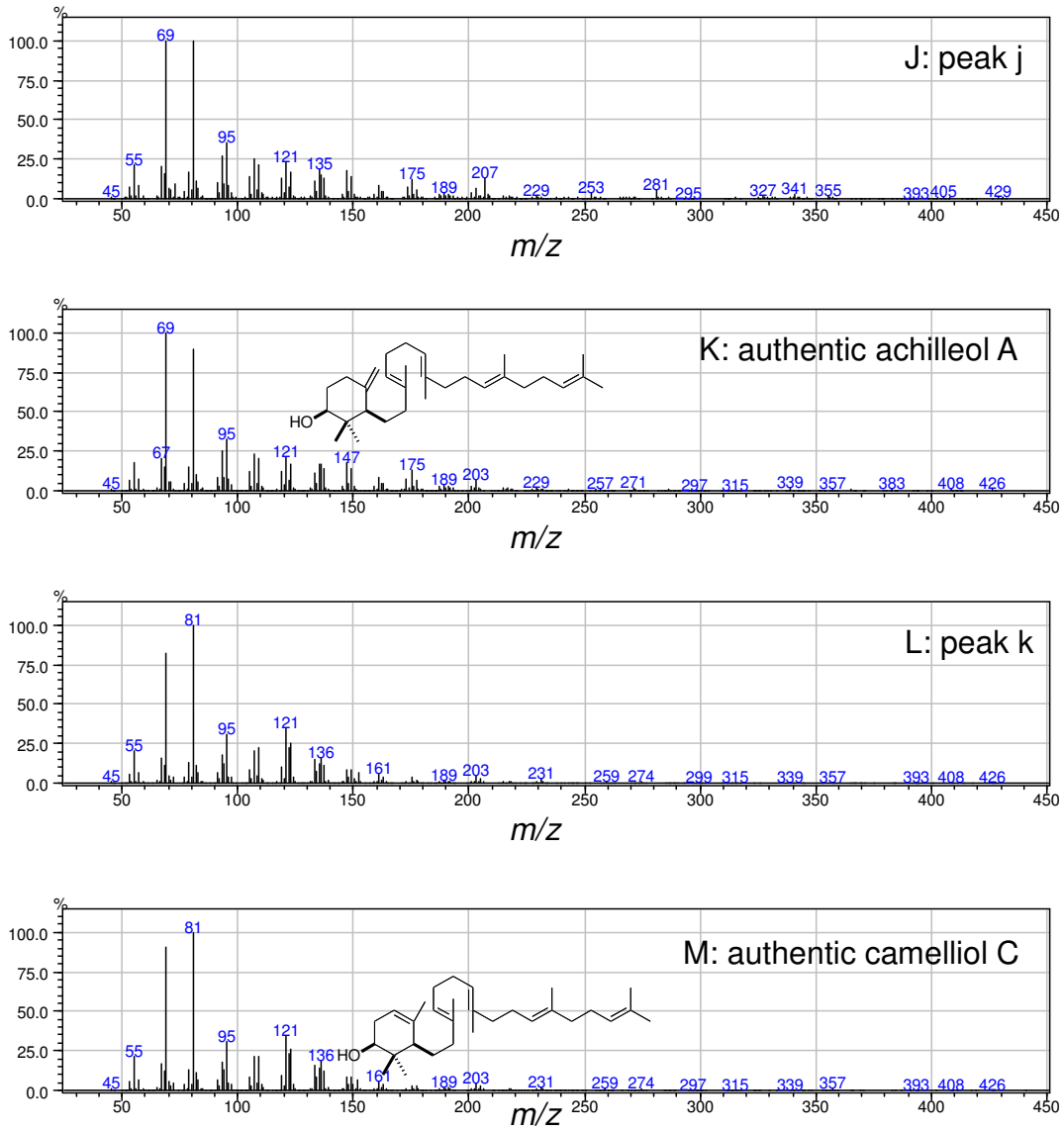


Fig. S5 MS fragmentation patterns of products (continued (4))

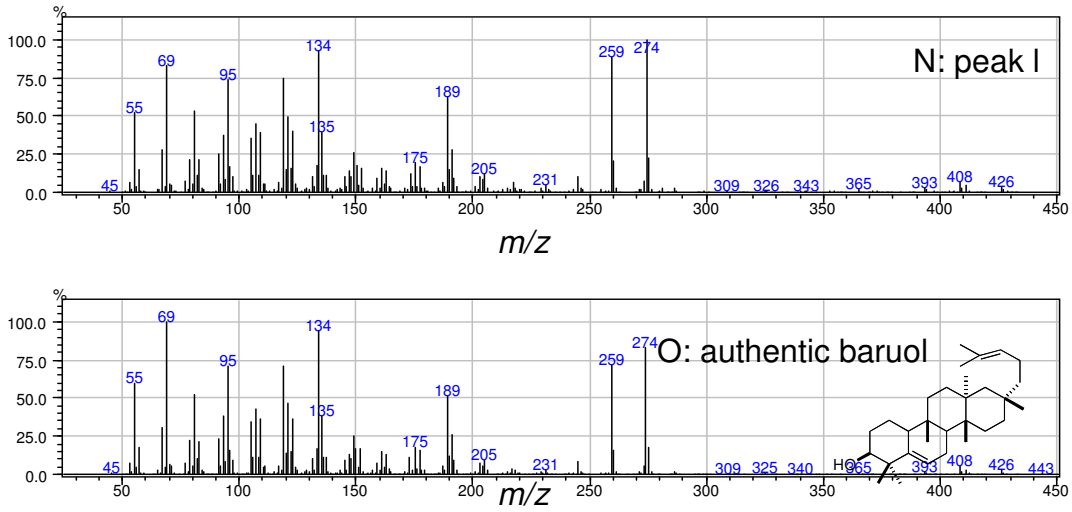


Fig. S6 Phylogenetic analysis of some OSCs from higher plants