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Phytochemistry 66 (2005) 887–899
PHYTOCHEMISTRY
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Biosynthesis of benzofuran derivatives in root cultures of *Tagetes patula* via phenylalanine and 1-deoxy-D-xylulose 5-phosphate

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Received 21 January 2005; received in revised form 21 February 2005
Available online 28 March 2005

Abstract

Root cultures of *Tagetes patula* L. cv. Carmen were grown with a mixture of unlabeled glucose and [U-$^{13}C_6$]glucose or [1-$^{13}C_1$]glucose as carbon source. Isoeuparin and (−)-4-hydroxytremetone were isolated by solvent extraction of the cultured tissue, purified by chromatography and analysed by ^{1}H and ^{13}C NMR spectroscopy. Amino acids obtained by hydrolysis of protein from the same experiments were used for the reconstruction of the labelling patterns in central metabolic intermediates. These labelling patterns were used for the prediction of isotopolog compositions in the benzofuranone derivatives via different hypothetical pathways. Comparison with the experimentally observed isotopolog distributions showed that the benzenoid ring and the acetoxy group are exclusively or predominantly (>98%) derived from phenylalanine and not from acetyl-CoA via a polyketide-type biosynthesis. The isopropylidene side chain and two carbon atoms of the furan and dihydrofuran moiety, respectively, originate from an isoprenoid building block obtained exclusively or predominantly (>98%) via the deoxyxylulose phosphate pathway. The exomethylene atom of the isopropylidene side chain is biosynthetically equivalent to the (*Z*)-methyl group of dimethylallyl diphosphate. The data indicate that isoeuparin and (−)-4-hydroxytremetone are assembled from 4-hydroxyacetophenone and dimethylallyl diphosphate via prenyl-substituted 4-hydroxyacetophenone and dihydrobenzofurans as intermediates.

Keywords: *Tagetes*; Retrobiosynthesis; Isoeuparin; 4-Hydroxytremetone; ^{13}C-glucose

1. Introduction

Benzofuran and benzodihydrofuran derivatives are found in several species of higher plants including Asteraceae, Rutaceae, Liliaceae, and Cyperaceae. The vast majority of the few hundred naturally occurring benzofurans is detected in Asteraceae (for review, see Proksch and Rodriquez, 1983).

6-Hydroxytremetone (**3**, Fig. 1) was first isolated from *Tagetes patula* by Bohlmann and Zdero (1979). More recently, isoeuparin (**1**) and compounds **4**–**7** (Fig. 1) were also identified in the aerial parts and the roots of *T. patula* plants, as well as in hairy root cultures of the same plant (Sütfeld et al., 1985; Tang et al., 1987; Parodi et al., 1988; Menelaou et al., 1993).

Some of the naturally occurring benzofurans act as photosensitisers (Proksch and Rodriquez, 1983; Downum and Rodriguez, 1986). 6-Hydroxytremetone (**3**, Fig. 1) and dehydrotremetone (**6**) were shown to exhibit antimicrobial properties and toxicity against fish (Cagniant and Cagniant, 1975; Proksch and Rodriquez, 1983).

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doi:10.1016/j.phytochem.2005.02.022

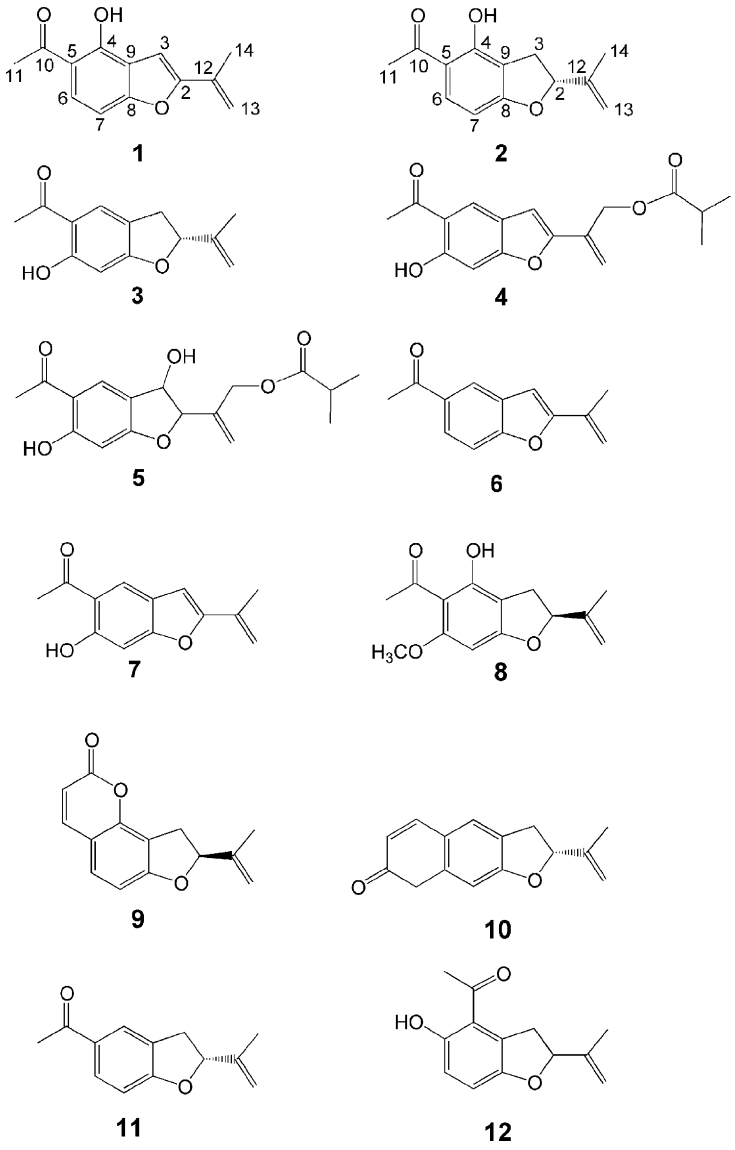

Fig. 1. Some compounds with dihydrobenzofuran or benzofuran moieties from plants. Isoeuparin (**1**) and (−)-4-hydroxytremetone (**2**) were found in root culture extracts from *Tagetes patula* (this study). (+)-Remirol (**8**) was isolated from *Remirea maritima* (Cyperaceae) (Allan et al., 1969) and (+)-angenomalin (**9**) was isolated from *Angelika anomala* (Apiaceae) (Hata et al., 1967) showing (*S*)-configuration at C-2 (Allan et al., 1970; Abu-Mustafa et al., 1971). In contrast, (+)-isoangenomalin (**10**) isolated from *Scaevola frutescens* (Goodeniaceae) (Kikuchi et al., 1974), (−)-tremetone (**11**) isolated from *Helichrysum stoechas* (Kawase et al., 1980) or *Isocoma wrightii* (Asteraceae) (Zalkow et al., 1979), and (−)-6-hydroxytremetone (**3**) from *Eupatorium urticaefolium* (Asteraceae) (Bonner et al., 1964; Harada et al., 1968) showed (*R*)-configuration. 4-Acetyl-2-isopropenyl-2,3-dihydrobenzofuran-6-ol **(12)** was isolated from *Lasiolanea morii* (Asteraceae) (Yamaguchi et al., 1986).

Plant growth inhibitory activity (Cespedes et al., 2002), anti-HIV activity (Piacente et al., 1994) and allergenic activity (Hausen and Helmke, 1995) were also reported for 6-hydroxytremetone.

While some ambiguities still persist, some of the benzofuran derivatives are considered as the causative agents for trembles in cattle when the feed is contaminated with benzofuran producing plants (especially with *Eupatorium rugosum*, *Aplopappus heterophyllus* and *A. fruticosus*). In humans, the so-called milk sickness is associated with the consumption of milk or milk products containing benzofuran derivatives (Christiensen, 1965; Sharma et al., 1998).

Several reports have been published on the biosynthetic origin of benzofurans in plants. Most of these studies were performed with radioisotopes, and the precise topology of the label distribution could not be diagnosed within the limitations of that method. Bohlmann and Grenz (1970) proposed dehydrotremetone (**6**) to be biosynthesised in Asteraceae by isoprenylation of *para*-hydroxyacetophenone (**15**) (Fig. 2). Compound **15** has been reported to originate from acetyl-CoA units (**13**) via polyketide-type intermediates in *Eupatorium rugosum* (route A in Fig. 2) (Lin et al., 1974; Lin and Heinstein, 1974). On the other hand, **15** has been claimed to be derived from phenylalanine (**14**) in *Eupatorium*

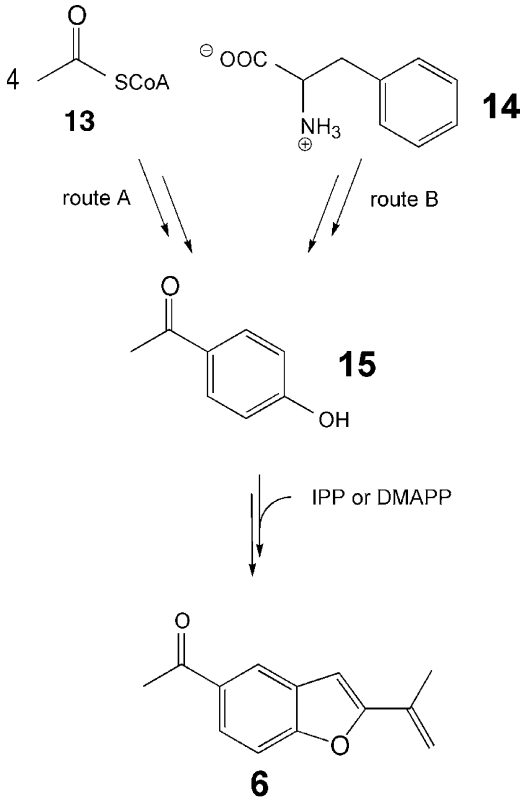

Fig. 2. Published biosynthetic routes of dehydrotremetone (**6**) by isoprenylation of acetophenone (**15**) (Bohlmann and Grenz, 1970). The acetophenone moiety has been proposed to be obtained via a polyketide-type pathway (route A) (Lin et al., 1974; Lin and Heinstein, 1974) or via a phenylpropanoid pathway (route B) (Siebertz et al., 1989; Monir and Proksch, 1989).

cannabinum and *Ageratina adenophora* (route B in Fig. 2) (Siebertz et al., 1989; Monir and Proksch, 1989). Dimethylallyl diphosphate (DMAPP) (Bohlmann and Grenz, 1970) or isopentenyl diphosphate (IPP) (Lin and Heinstein, 1974) have been reported to serve as putative electrophiles in the assembly of **6**.

The DMAPP (**18**) and IPP (**17**) precursors of meroterpenoids from plants are known to be derived either from mevalonate (**16**) or 1-deoxyxylulose 5-phosphate (**21**) (Fig. 3). For example, the biosynthesis of shikonin in cell cultures of *Lithospermum erythrorhizon* is dependent on mevalonate (Li et al., 1998), whereas the prenyl moieties of humulone, hyperforin and anthraquinones have been shown to be formed predominantly via the deoxyxylulose phosphate pathway (Eichinger et al., 1999; Goese et al., 1999; Adam et al., 2002).

This dichotomy is well in line with the fact that both isoprenoid pathways (Fig. 3) operate simultaneously in higher plants (for reviews see, Eisenreich et al., 2001, 2004). The enzymes of the mevalonate pathway are located in the cytoplasm and the enzymes of the deoxyxylulose phosphate pathway are operative in the plastids of plant cells. In a simple model, the compartment in which the biosynthesis of the target compound occurs (i.e., cytoplasm or plastid) determines the trunk pathway of terpenoid biosynthesis (i.e., mevalonate or 1-deoxyxylulose phosphate pathway, respectively). However, it has also been shown that this compartmental separation of the two pathways is not absolute (Hemmerlin et al., 2003; Bick and Lange, 2003; Schuhr et al., 2003).

A powerful method for the elucidation of the biosynthetic origin of plant metabolites is based on ^{13}C-labeled glucose as a general precursor (Eisenreich et al., 1996; Werner et al., 1997; Glawischnig et al., 2001). Since glucose is closely connected to the central metabolism in plants, the ^{13}C-label from the proffered precursor is diverted to virtually all metabolic compartments and metabolites of the plant cell. In conjunction with the retrobiosynthetic analysis of amino acids, the specific label distribution in the target molecule yields quantitative information about its biosynthetic history (for review, see Bacher et al., 1999).

This methodology is now used for the analysis of isoeuparin (**1**) and (−)-4-hydroxytremetone (**2**) biosynthesis in root cultures of *T. patula*. Evidence is provided that phenylalanine (**14**) and DMAPP (**18**) obtained via the deoxyxylulose phosphate pathway serve as basic building blocks of **1** and **2**.

2. Results

NMR spectroscopy and GC/MS analysis showed that root cultures of *T. patula* produce isoeuparin (**1**) and 4-hydroxytremetone (**2**) at levels of 1.7 and 4.5 mg per g of dry cell mass, respectively. The ^{1}H NMR and ^{13}C NMR signals of **1** and **2** could be unequivocally assigned on the basis of ^{1}H^{1}H homocorrelation experiments (COSY, NOESY), ^{1}H^{13}C heterocorrelation experiments (HMQC, HMBC) and INADEQUATE experiments with ^{13}C-enriched samples from the experiment with [U-$^{13}C_6$]glucose (Tables 1 and 2).

The optical rotation of **2** was determined as $[\alpha]_D^{20} = -107.8°$ (c = 0.256 g/ml of $CHCl_3$). On the basis of the (*R*)-configuration of (−)-tremetone (**11**) and (−)-6-hydroxytremetone (**3**), isolated from Asteraceae species (Bonner et al., 1964; Harada et al., 1968; Zalkow et al., 1979; Yamaguchi et al., 2003), it appears safe to assume that (−)-4-hydroxytremetone (**2**) has (*R*)-configuration.

Preliminary experiments with root cell cultures of *T. patula* using [1,2-$^{13}C_2$]acetate as precursor showed that the incorporation of acetate into isoeuparin (**1**) and (−)-4-hydroxytremetone (**2**) was well below 1% (data not shown). The low enrichments may reflect poor uptake rates of the proffered acetate, poor utilization of exogenous acetate in the root cells, or an acetate-independent biosynthetic origin of the metabolites under study.

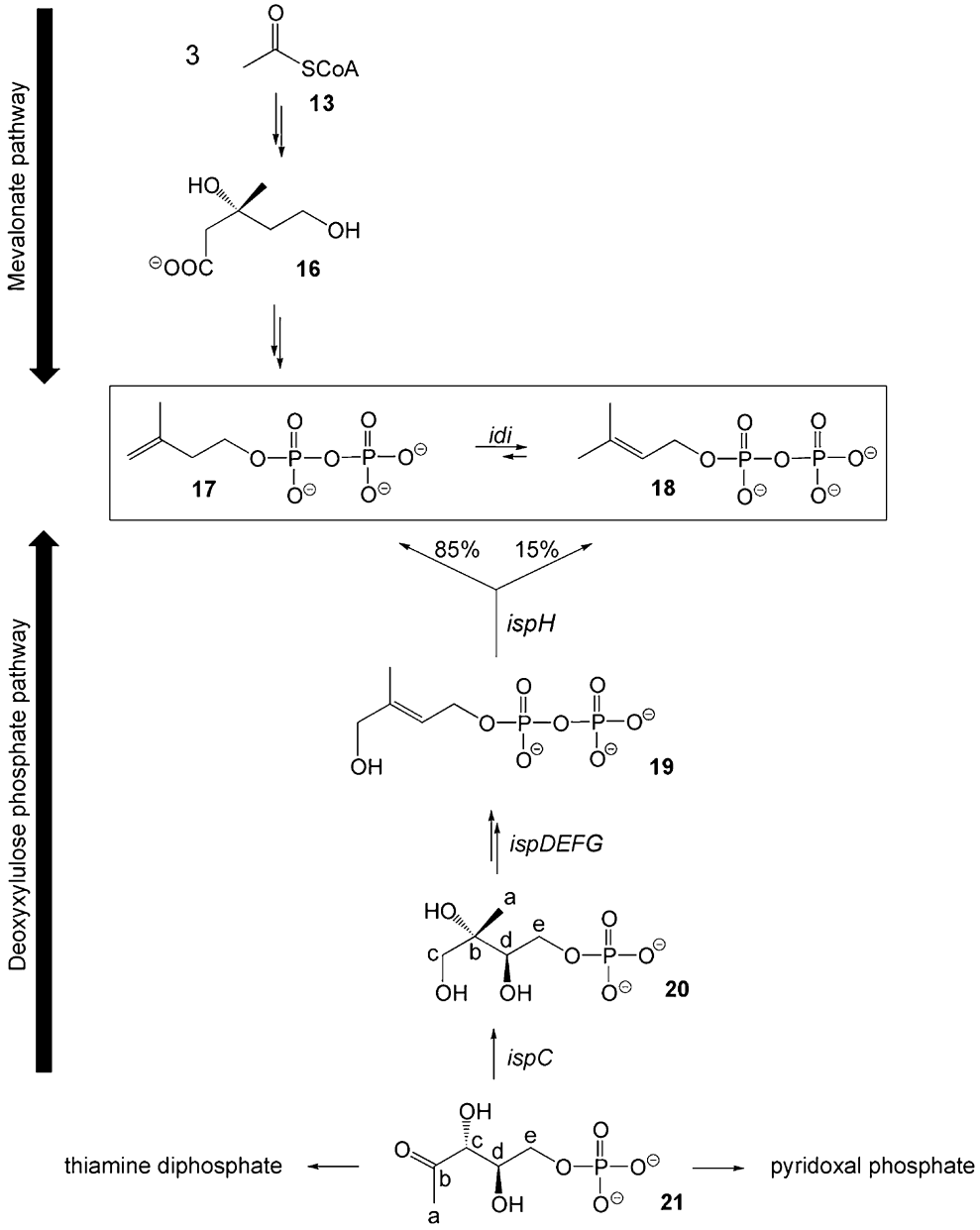

Fig. 3. Biosynthesis of isopentenyl diphosphate (**17**) and dimethylallyl diphosphate (**18**) via the mevalonate pathway or the 1-deoxyxylulose 5-phosphate (DOXP) pathway. Biosynthetically equivalent atoms in **20** and **21** are indicated by small characters.

In order to quantitatively determine the carbon flux from a general carbon source into **1** and **2**, we exploited the experimental approach used in a previous biosynthetic study with root cultures of *T. patula* (Margl et al., 2001), where we showed that exogenous ^{13}C-labelled glucose is efficiently utilised.

Following the earlier experimental setup (Margl et al., 2001), root cultures of *T. patula* were grown in a medium containing a mixture of natural abundance glucose and [U-$^{13}C_6$]glucose or [1-^{13}C]glucose at ratios of 10:1 and 2:1 (w/w), respectively. After 13 days, the biomass was harvested, and the benzofuran derivatives **1** and **2** were isolated by solvent extraction. The compounds were purified chromatographically and analysed by quantitative NMR spectroscopy. Hydrolysis of the residual biomass afforded amino acids that were isolated chromatographically and analysed by quantitative NMR spectroscopy.

^{13}C NMR signals of **2** from the experiments with [U-$^{13}C_6$]glucose are shown in Fig. 4. All signals in that spectrum have satellites due to $^{13}C^{13}C$ coupling, mostly of relatively high intensity, which indicate the presence of multiply ^{13}C-labeled molecular species. Similarly, the spectrum of **1** from the experiment with [U-$^{13}C_6$]glucose displayed a complex pattern of satellite signals.

All satellite signals were assigned by the analysis of coupling constants delineated from the one-dimensional spectrum and from the correlation patterns observed in

Table 1
NMR data of isoeuparin (**1**)

Position	Chemical shift, ppm[a]		Coupling constants, Hz[b]			Coupling patterns			
	$\delta^{13}C$	$\delta^{1}H$	J_{CC}	J_{HH}	J_{CH}	INAD[c]	COSY	NOESY	HMBC
OH		13.21(*s*)							
10	204.08		42.9(11)						6, 11
8	159.51		69.0(7), 58.6(9), 5.5(4), 2.0(6)						7, 3
4	158.53		70.6(9), 63.2(5), 5.5(8), 2.2(7)						6, 7, 3
2	156.75		74.3(3), 6.4(14)						3, 13, 13′, 14
12	132.38		71.9(13), 43.6(14)			13			14, 13′, 3
6	127.37	7.62(*d*)	60.8(5,7) 2.0(8)	8.8(7)	159.2(6), 12.3(8)	7, 5	7	7, 11	11
$13_{(E)}$	113.46	5.17(*s*)	71.9(12)	1.5(13′, 14)	160.2(13)	12	13′, 14	13′, 14	14, 3
$13'_{(Z)}$		5.74(*s*)			159.4(13)		13, 14	13	
7	103.40	6.97(*d*)	69.2(8), 61.5(6), 6.2(9) 2.2(4)	9.0(6)	167.7(7)	6	6	6	
3	100.86	6.82(*s*)	74.1(2)		178.8(3), 9.5(2)			14	13′
11	26.87	2.63(*s*)	43.1(10)		128.0(11), 5.8(10)			6	
14	19.24	2.10(*s*)	43.6(12), 6.2(2), 1.1(3)		127.6(14)			3, 13	13, 13′
9	114.36		65.2(4), 60.6(8)						3, 7
5	119.01		60.6(4, 6)						11, 7

[a] Referenced to external tetramethylsilane.
[b] Coupling partners are given in parentheses. Carbon–proton (J_{CH}) and carbon–carbon coupling constants (J_{CC}) are determined with ^{13}C-enriched samples.
[c] Two-dimensional INADEQUATE experiment with the sample from the experiment with [U-$^{13}C_6$]glucose.

Table 2
NMR data of 4-hydroxytremetone (**2**)

Position	Chemical shift, ppm[a]		Coupling constants, Hz[b]			Coupling patterns			
	$\delta^{13}C$	$\delta^{1}H$	J_{CC}	J_{HH}	J_{CH}	INAD[c]	COSY	NOESY	HMBC
OH		12.75(*s*)							
10	202.65		43.1(11)						6
8	166.85		68.3(7), 60.8(9), 6.0(4), 1.9(6)						3, 3′, 6, 7
4	160.32		69.6(9), 61.0(5), 5.1(8), 4.1(7)						3, 3′, 6
2	89.97	5.29(*dd*)	33.8(3), 6.1(14)	8.0(3), 9.6(3′)	151.6(2)	3	3, 3′	13′	14, 3, 3′, 13, 13′
12	143.27		73.0(13), 43.1(14)			13			14, 3, 3′, 2,
6	133.25	7.58(*d*)	61.1(5,7), 1.8(8), 3.1(9),	8.5(7)	158.1(6)	7, 5	7	11, 7	11
13_E	112.93	4.91(*d*)	73.0(12)		160.2(13)	12	13′, 14	13′, 14	14
$13'_{(Z)}$		5.06(*s*)			159.4(13′)		13, 14	2, 13	
7	101.81	6.38(*d*)	69.2(8), 61.5(6), 6.2(9), 4.1(4)	8.5(6)	166.6(7)	6	6	6	3, 3′
3	30.95	2.98(*dd*)	33.6(2)	15.6(3′), 9.9(2)	134.3(3)	2	2, 3′	14	2
3′		3.32(*dd*)		15.6(3), 7.7(2)	136.0(3′)		2, 3		
11	26.30	2.53(*s*)	42.9(10)		127.8(11), 5.8(10)			6	
14	17.00	1.74(*s*)	43.1(12), 6.2(2), 1.1(3)		127.0(14)		13, 13′	13, 3	2, 13, 13′
9	113.46		61.0(4), 60.6(8)						3, 3′, 7
5	114.7		61.1(4,6)						11, 7

[a] Referenced to external tetramethylsilane.
[b] Coupling partners are given in parentheses. Carbon–proton (J_{CH}) and carbon–carbon coupling constants (J_{CC}) are determined with ^{13}C-enriched samples.
[c] Two-dimensional INADEQUATE experiment with the sample from the experiment with [U-$^{13}C_6$]glucose.

the INADEQUATE spectra (Tables 1 and 2). On the basis of this analysis, isotopolog groups comprising more than one ^{13}C atom were assigned (Table 3, cf. also Fig. 4). From the relative fractions of the satellite groups in the overall signal of a certain carbon atom, the molar contributions of each respective isotopolog group were calculated (Table 3).

Two isotopologs with four contiguous ^{13}C atoms, two isotopologs with three ^{13}C atoms and four groups with two contiguous ^{13}C atoms were observed in the benzenoid ring of **1** and **2** (Fig. 5). The formation of $^{13}C_3$ and $^{13}C_4$ isotopologs cannot be explained by the involvement of a polyketide pathway where only contiguous $^{13}C_2$ units can be transferred from the acetyl-CoA (**13**) precursor to the downstream product. However, the observed labelling pattern can be explained by the involvement of a shikimate-derived precursor (for details see below). It is also worth noting that the labeling

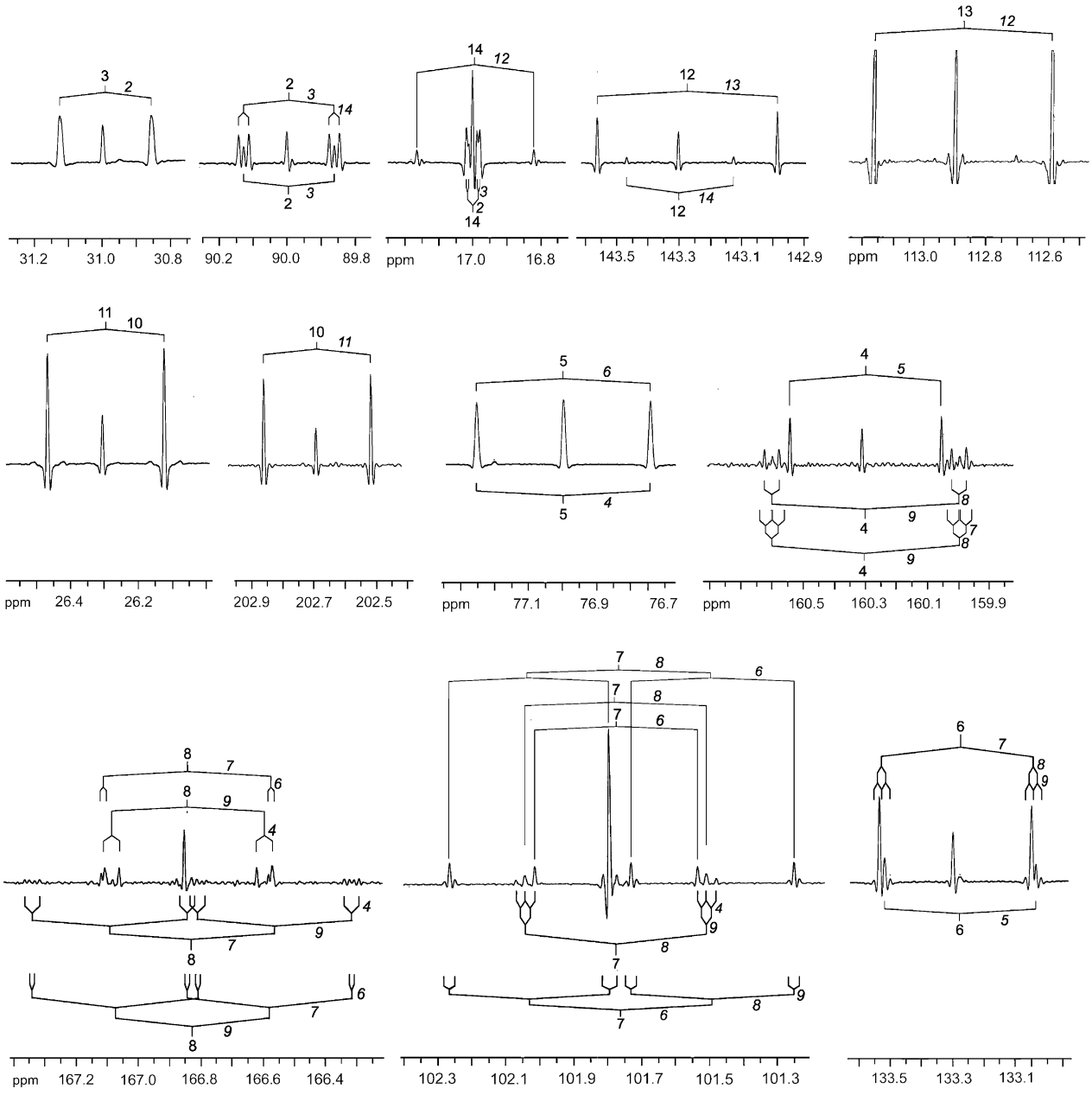

Fig. 4. High-resolution ^{13}C NMR signals of 4-hydroxytremetone (**2**) isolated from *Tagetes patula* in the experiment with [U-$^{13}C_6$]glucose. ^{13}C–^{13}C coupling patterns are indicated. Prior to Fourier transformation, the free induction decay was multiplied with Gaussian functions.

pattern of the benzenoid ring was symmetric. This documents the involvement of an inherently symmetrical intermediate such as acetophenone or 4-hydroxyacetophenone (**15**).

The isotopolog composition of the furan or dihydrofuran moiety in **1** and **2**, respectively, was characterized by two $^{13}C_2$ units (Fig. 5). The terminal carbon atom 14 shows no intense coupling to the direct bonding partner (C-12) but does show long-range coupling via two or three bonds to carbon atoms 2 and 3 (cf. Fig. 4). The presence of a [2,3,14-$^{13}C_3$]-isotopolog is also confirmed by the long-range coupling between C-14 and C-2 as detected in the signal of C-2 ($^2J_{CC}$ = 6.2 Hz) and the broadened satellite signal of C-3 (Fig. 4). The triple-labelled isotopolog indicates that carbon atom C-14, C-2 and C-3 can be incorporated jointly from a single [U-$^{13}C_6$]glucose molecule. The fact that these jointly introduced carbon atoms are not directly connected in **1** and **2** indicates that a rearrangement had occurred in the biosynthetic process. As discussed in detail below, this labelling pattern is in line with the contribution of an isoprenoid moiety, more specifically dimethylallyl diphosphate (DMAPP) (**18**), via the non-mevalonate isoprenoid biosynthetic pathway. This conclusion is also

Table 3
^{13}C labeling data of 4-hydroxytremetone (**2**) and isoeuparin (**1**) from the experiments with [1-$^{13}C_1$]glucose and [U-$^{13}C_6$]glucose

Position	[1-^{13}C]glucose	[U-$^{13}C_6$]glucose					
	% ^{13}C	% ^{13}C		% ^{13}C^{13}C[b]		mol%[c]	
	2	**2**	**1**	**2**	**1**	**2**	**1**
2	1.4	11.0	11.0	19.1 (2, 3)	18.3 (2, 3)	2.1 (2, 3)	2.0 (2, 3)
				55.4 (2, 3, 14)	50.0 (2, 3, 14)	6.1 (2, 3, 14)	5.6 (2, 3, 14)
3	13.2	10.8	10.8	18.2 (3, 2)	19.1 (3, 2)	2.0 (3, 2)	2.1 (3, 2)
4	13.0	11.0	10.9	38.2 (4, 5)		4. 2 (4, 5)	
				13.6 (4, 9)	40.4 (4, 5)	1.5 (4, 9)	4. 4 (4, 5)
				32.7 (4, 9, 8)	11.9 (4, 9)	3.6 (4, 9, 8)	1.3 (4, 9)
				9.1 (4, 9, 8, 7)	32.1 (4, 9, 8)	1.0 (4, 9, 8, 7)	3.5 (4, 9, 8)
5	1.1	10.7	10.7	39.1 (5, 6)	33.9 (5, 6)	4.3 (5, 6)	3.7 (5, 6)
				40.0 (5, 4)	39.4 (5, 4)	4.4 (5, 4)	4.3 (5, 4)
6	13.4	11.6	10.7	40.0 (6, 5)	40.4 (6, 5)	4.4 (6, 5)	4.4 (6, 5)
				30.0 (6, 7, 8,)	30.2 (6, 7, 8)	3.3 (6, 7, 8)	3.3 (6, 7, 8)
7	1.9	11.3	11.6	14.5 (7, 6)		1.6 (7, 6)	
				11.8 (7, 8)	12.8 (7, 6)	1.3 (7, 8)	1.4 (7, 6)
				23.4 (7, 8, 6)	10.9 (7, 8)	2.9 (7, 8, 6)	1.2 (7, 8)
				11.0 (7, 6, 8, 9)	26.6 (7, 8, 6)	1.2 (7, 6, 8, 9)	2.9 (7, 8, 6)
				10.3 (7, 8, 9, 4)		1.1 (7, 8, 9, 4)	
8	2.1	12.3	12.2	12.7 (8, 7)		1.4 (8, 7)	
				13.6 (8, 9)	11.7 (8, 7)	1.5 (8, 9)	1.3 (8, 7)
				28.0 (8, 9, 4)	11.8 (8, 9)	3.1 (8, 9, 4)	1.3 (8, 9)
				28.2 (8, 7, 6)	30.3 (8, 9, 4)	3.2 (8, 7, 6)	3.3 (8, 9, 4)
				12.0 (8, 7, 9, 4)	29.4 (8, 7, 6)	1.1 (8, 7, 9, 4)	3.2 (8, 7, 6)
				12.0 (8, 9, 7, 6)		1.1 (8, 9, 7, 6)	
9	1.1	11.8	10.8	13.6 (9, 4)	13.8 (9, 4)	1.5 (9, 4)	1.5 (9, 4)
				12.7 (9, 8)	10.8 (9, 8)	1.4 (9, 8)	1.2 (9, 8)
				34.5 (9, 8, 4)	32.1 (9, 8, 4)	3.8 (9, 8, 4)	3.5 (9, 8, 4)
10	15.8	11.6	11.8	72.7 (10, 11)	75.2 (10, 11)	8.0 (10, 11)	8.2 (10, 11)9
11	1.3[a]	9.8[a]	10.3[a]	76.4 (11, 10)	76.1 (11, 10)	8.4 (11, 10)	8.3 (11, 10)
12	1.9	10.6	9.8	69.1 (12, 13)	66.1 (12, 13)	7.6 (12, 13)	7.2 (12, 13)
				12.6 (12, 14)	10.6 (12, 14)	1.4 (12, 14)	1.1 (12, 14)
13	12.5	10.6	10.6	72.7 (13, 12)	68.8 (13, 12)	8.0 (13, 12)	7.5 (13, 12)
14	2.3	9.2	9.7	11.2 (14, 12)	11.8 (14, 12)	1.3 (14, 12)	1.3(14, 12)
				61.0 (14, 2, 3)	54.1 (14, 2, 3)	6.7 (14, 2, 3)	5.9 (14, 2, 3)

[a] Absolute ^{13}C abundance determined from the ^{13}C-satellites in ^{1}H NMR spectra. These values were taken to normalize relative ^{13}C-abundances.
[b] Fraction of satellite signals corresponding to certain multiple ^{13}C labeled isotopologs (coupling partner in parentheses) in the overall ^{13}C signal of the index carbon atom.
[c] Absolute abundance of multiple ^{13}C labelled isotopologs (coupling partners are given in parentheses).

confirmed by the diagnosis of the labelling pattern observed in the experiment with [1-$^{13}C_1$]glucose.

A part of the ^{13}C NMR spectrum of (−)-4-hydroxytremetone (**2**) obtained from cultures with [1-$^{13}C_1$]glucose is shown in Fig. 6B. The corresponding section of a ^{13}C NMR spectrum of **2** with natural ^{13}C-abundance is displayed in Fig. 6A. From the modulation of the signal amplitudes it is immediately obvious that some carbon atoms had acquired significant ^{13}C label from [1-^{13}C]glucose. Absolute ^{13}C abundances for selected carbon atoms were gleaned from the $^{1}H^{13}C$ coupling satellites in the ^{1}H NMR spectrum of the ^{13}C-enriched sample. With these values at hand, the relative abundances of the other carbon atoms in **2** were converted into absolute ones (Table 3). These data are also summarised graphically in Fig. 6B.

C-4, C-6 and C-10 of the benzoid ring and its acetoxy side chain, C-3 of the dihydrofuran ring and C-13 of its isopropylidene side chain acquired significant ^{13}C-enrichment from [1-^{13}C]glucose. The averaged ^{13}C-abundance was $13.6 \pm 1.3\%$ for these carbon atoms. The abundance of the other carbon atoms was $1.6 \pm 0.6\%$. The slight ^{13}C excess above the natural abundance contribution (i.e., 1.1% ^{13}C) could reflect some reshuffling of the ^{13}C label in the biosynthetic network of the plant cell.

The specific labelling pattern in **1** and **2** from [1-^{13}C]glucose or [U-$^{13}C_6$]glucose can be interpreted by a retrodictive/predictive approach (see Section 3). For the purpose of this reconstruction, leucine (**22**), phenylalanine (**14**) and valine (**23**) were isolated from hydrolysates of the residual cell mass obtained after extraction of **1** and **2** in the labelling experiments. The isotopolog compositions were determined by quantitative NMR experiments as described above for **1** and **2**. The data are summarised in Figs. 7 and 8, respectively.

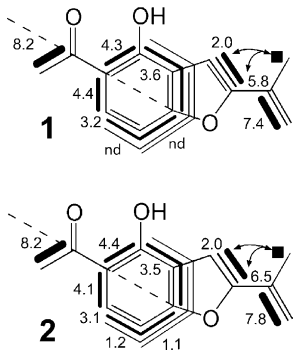

Fig. 5. Labelling patterns of isoeuparin (**1**) and 4-hydroxytremetone (**2**) from the experiment with [U-$^{13}C_6$]glucose. Bold lines indicate ^{13}C-labelled isotopolog groups with directly adjacent ^{13}C atoms; numbers represent the molar abundances of multiply ^{13}C-labelled isotopologs; arrows indicate ^{13}C isotopolog groups with two directly adjacent ^{13}C atoms and one ^{13}C atom in the same molecule detected by long range coupling in the ^{13}C NMR spectra. The symmetry axis in the benzyl ring system of the putative precursor is indicated by a dashed line. nd, not determined due to signal overlap.

3. Discussion

Isoeuparin (**1**) and (−)-4-hydroxytremetone (**2**) were isolated as major metabolites from root cell extracts of *T. patula*. To the best of our knowledge, this is the first documentation of **2** as a natural product. A compound isolated earlier from *Lasiolanea morii* (Asteraceae) was also claimed to be 4-hydroxytremetone (Bohlmann et al., 1982). However, the compound was reassigned later by Yamaguchi et al. (1986) as 4-acetyl-2-isopropenyl-2,3-dihydrobenzofuran-6-ol (**12**, Fig. 1).

As previously shown in biosynthetic studies with ^{13}C-labelled glucose as precursor, the ^{13}C labelling patterns of amino acids can serve as the basis for the reconstruction of the labelling patterns of certain compounds with central positions in intermediary metabolism (Werner et al., 1997; Eisenreich and Bacher, 2000; Sola et al., 2004). The labelling patterns of these central intermediates cannot be determined directly with an acceptable effort because they are present in low amounts despite their enormous metabolic significance. Specifically, the labelling patterns of phosphoenol pyruvate and the biosynthetically closely related glyceraldehyde 3-phosphate (**25**) can be deduced from that of the side chain of phenylalanine (**14**) which is generated by the condensation of phosphoenol pyruvate and phosphoshikimate affording enolpyruvylshikimate phosphate and finally chorismate, the universal aromatic amino acid precursor (Figs. 7 and 8). The labeling patterns of the hydroxyethyl moiety of hydroxyethyl-TTP (**26**) can be deduced from carbons 3 and 4 of valine (**23**), and the labeling patterns of the acetyl moiety of acetyl-CoA (**13**) can be deduced from carbons 1 and 2 of leucine (**22**), respectively (Figs. 7 and 8). The arguments for these deductions have been described elsewhere in more detail (Eisenreich and Bacher, 2000).

With the deduced labeling patterns of putative precursors of benzofuran derivatives at hand, the labeling patterns of **2** or **1** were now predicted via hypothetical biosynthetic pathways. More specifically, the isotopolog compositions of *para*-hydroxyacetophenone (**15**) can be easily predicted from the observed isotopolog patterns in the phenyl ring and the β- and γ-carbon atoms of phenylalanine (**14**) (prediction C in Figs. 7 and 8). On the other hand, the isotopolog composition of **15** obtained via a polyketide type intermediate (**24**) can be predicted from the labelling patterns of acetyl-CoA (**13**) (prediction A in Figs. 7 and 8). The isotope composition of the hypothetical terpenoid precursor can be predicted via the mevalonate pathway (prediction B in

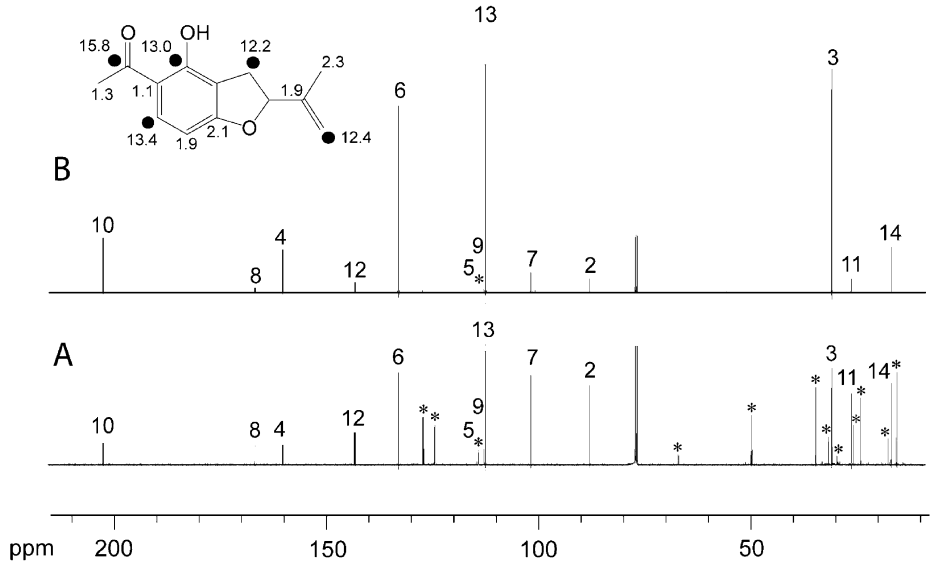

Fig. 6. Sections of ^{13}C NMR spectra of 4-hydroxytremetone at natural ^{13}C-abundance (**A**) and from the experiment with [1-$^{13}C_1$]glucose (**B**). Filled circles indicate significantly enriched ^{13}C atoms and numbers represent absolute ^{13}C abundances. Signals from impurities are indicated by asteriks.

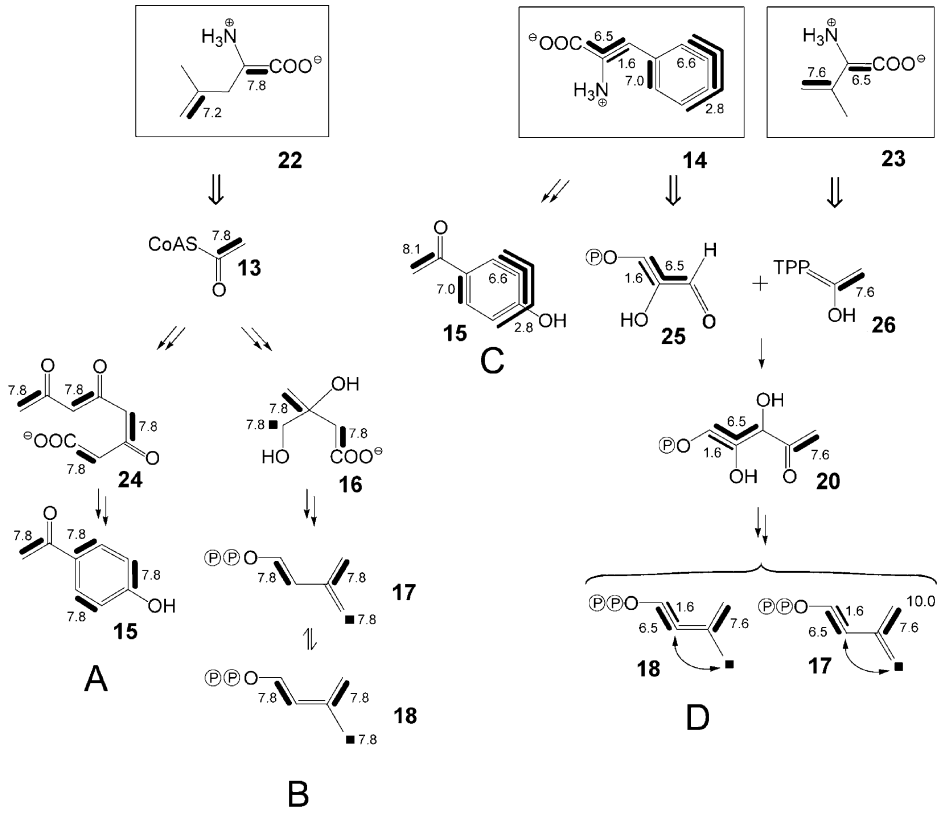

Fig. 7. Predicted labelling patterns for the putative precursors of benzofuran derivatives from the experiment with [U-$^{13}C_6$]glucose. The labelling patterns of 4-hydroxyacetophenon (**15**), IPP (**17**) and DMAPP (**18**) are based on the labelling patterns of acetyl-CoA (**13**), glyceraldehyde 3-phosphate (**25**) and hydroxyethyl-TTP (**26**) which were reconstructed by retrobiosynthesis of leucine (**22**), phenylalanine (**14**) and valine (**23**), respectively. Bold lines indicate ^{13}C-labelled isotopolog groups with directly adjacent ^{13}C atoms; numbers represent the molar abundances of multiply ^{13}C-labelled isotopologs; arrows indicate ^{13}C isotopologs with two directly adjacent ^{13}C atoms and one ^{13}C atom in the same molecule. A, predicted labelling pattern of 4-hydroxyacetophenon (**15**) via a polyketide-type biosynthesis; B, predicted labelling pattern of DMAPP (**18**) via the mevalonate pathway; C, predicted labelling pattern of 4-hydroxyacetophenone (**15**) via phenylalanine; D, predicted labelling pattern of DMAPP (**18**) via the 1-deoxyxylulose phosphate pathway.

Figs. 7 and 8) or the non-mevalonate pathway (prediction D in Figs. 7 and 8) from the respective precursors, i.e., acetyl-CoA (**13**) in the mevalonate pathway or glyceraldehyde 3-phosphate (**25**) and hydroxyethyl-TPP (**26**) in the non-mevalonate pathway. Notably, the non-mevalonate pathway involves a rearrangement reaction which cleaves the adjacent 3-carbon unit in **20** introduced from glyceraldehyde 3-phosphate (**25**).

All hypothetical combinations for the assembly of **2** from **15** and **18** using the patterns from the different predictions are shown in Fig. 9. In each of the two labelling experiments, specific isotopolog compositions are generated using the different isotopolog patterns in the building blocks **15** and **18** (predictions A–D). Only the prediction via the non-mevalonate pathway (prediction D) and via phenylalanine (prediction C) is in agreement with the spectroscopically determined patterns (Fig. 9). It follows that phenylalanine (including the β- and γ-carbon atoms of the side chain) and DMAPP formed via the 1-deoxyxylulose phosphate pathway serve as basic precursors in the biosynthesis of the benzofuran derivatives in *T. patula*.

A hypothetical pathway for the assembly of **1** and **2** is shown in Fig. 10. It is based on the finding that the benzenoid ring is derived from the phenyl ring of phenylalanine, the acetoxy side chain is derived from the three-carbon side chain of that amino acid by loss of the carboxylic group, and two carbon atoms of the furan ring and the isopropyl side chain of **2** are contributed en bloc from DMAPP (**18**) biosynthesised via the non-mevalonate pathway.

Electrophilic substitution of the aromatic nucleus of the 4-hydroxyacetophenone (**15**) with DMAPP (**18**) could afford **27** as an intermediate. Notably, a prenyl substituted acetophenone derivative has been isolated earlier from *Eupatorium rugosum* by Lin and Heinstein (1974). The formation of a dihydrofuran ring system in **29** could be triggered by the intermediary formation of the epoxide **28** (cf. also Bohlmann and Grenz, 1970) which is then attacked by the phenolic hydroxyl function. Subsequent dehydration of **29** could then afford **11** or **2** bearing the isopropylidene side chain. A marked feature of the labelling pattern from the experiment with [U-$^{13}C_6$]glucose is a $^{13}C_2$ isotopolog with ^{13}C atoms at

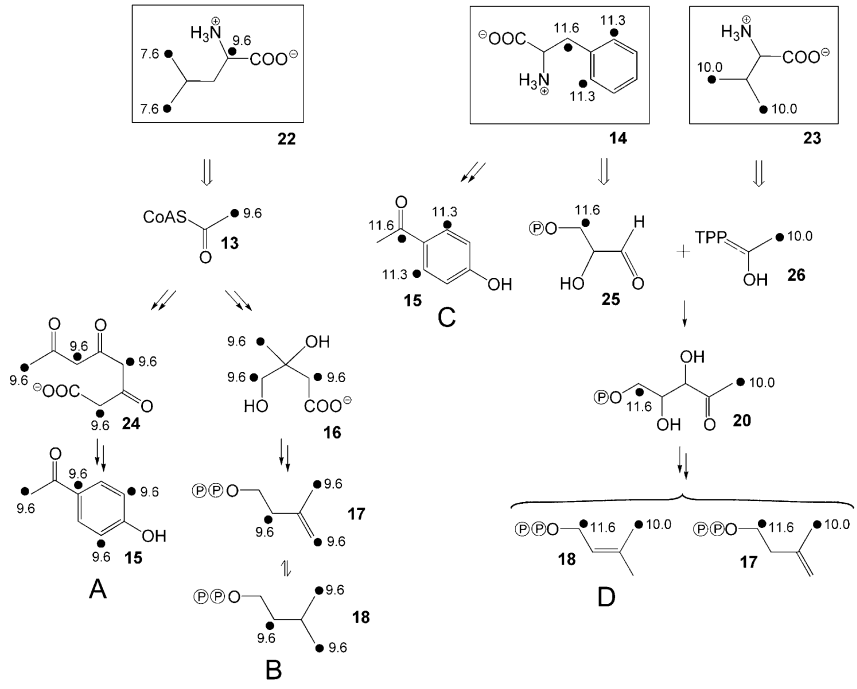

Fig. 8. Predicted labelling patterns for the putative precursors of benzofuran derivatives from the experiment with [1-$^{13}C_1$]glucose. Filled circles indicate significantly enriched ^{13}C atoms and numbers represent the corresponding ^{13}C abundances. For details, see legend to Fig. 7.

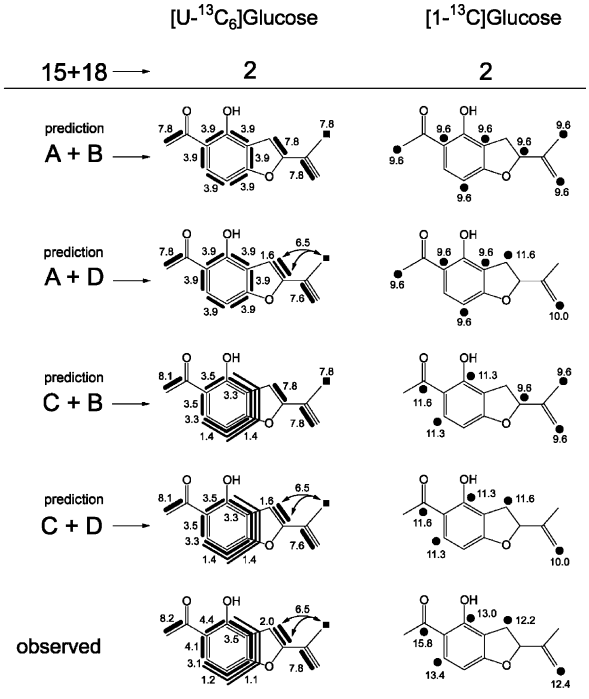

Fig. 9. Comparison of predicted labelling patterns in 4-hydroxytremetone (**2**) with the spectroscopically determined labelling patterns in **2** from root cultures of *Tagetes patula*. Bold lines indicate ^{13}C-labelled isotopolog groups with directly adjacent ^{13}C atoms. Arrows indicate ^{13}C isotopolog groups with two directly adjacent ^{13}C atoms and one ^{13}C atom in the same molecule. Filled circles indicate significantly enriched ^{13}C atoms. Numbers represent the corresponding ^{13}C abundances of the indexed isotopolog. The hypothetical labelling patterns of **2** are based on the predicted labelling patterns in 4-hydroxytremetone (**15**) and DMAPP (**18**) elaborated in Figs. 7 and 8.

position 12 and the exomethylene position in **1** and **2** (indicated by a black bar in Fig. 10). An equivalent isotopolog can be found in the (*Z*)-methyl group and C-2 of DMAPP (**18**). Therefore, the methyl group derived from the (*Z*)-methyl group of DMAPP (**18**) must have been deprotonated in this process. The formation of the furan ring in **6** could proceed by dehydration of a putative hydroxyl intermediate **30**. It remains unknown when the hydroxy function at position 4 in **1** or **2** is introduced.

4. Experimental

4.1. Materials

[1-^{13}C]glucose (99% ^{13}C) was obtained from Omicron (South Bend, Indiana, USA). [U-$^{13}C_6$]glucose (99% ^{13}C) and [1,2-$^{13}C_2$]acetate (sodium salt) were purchased from Isotec (Miamisburg, Ohio, USA).

4.2. Root cultures

Seeds of *T. patula* (L.) cv. Carmen were treated with 70% (v/v) ethanol and 10% (v/v) household bleach (HYPO), rinsed twice with sterile water, placed on hormone-free Nitsch medium (Nitsch and Nitsch, 1969) and incubated for 1 week in darkness at 23 °C. Roots of seedlings with an approximate length of 1 cm were cut and transferred into 10 ml of MS medium (Murashige and Skoog, 1962), pH 6, containing 100

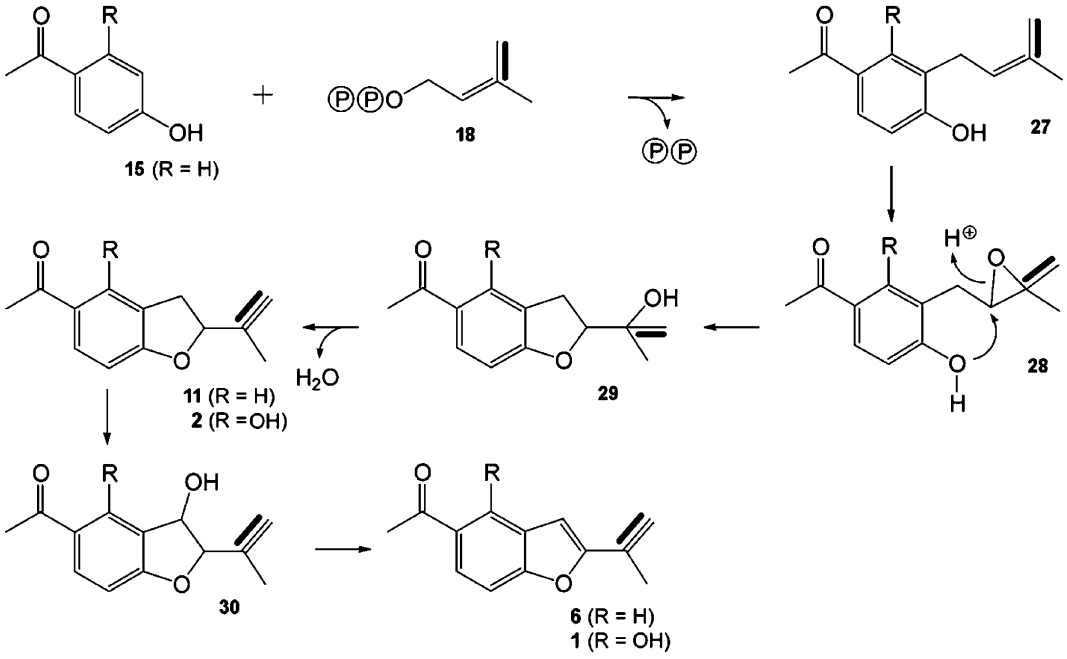

Fig. 10. Hypothetical mechanism of isoeuparin (**1**) and 4-hydroytremetone (**2**) formation in *Tagetes patula*. Bold lines indicate $^{13}C_2$-labelled isotopologs from [3,4-$^{13}C_2$]DMAPP (**18**) in the experiment with [U-$^{13}C_6$]glucose.

mg of myo-inositol, 100 mg of NZ-amine and 30 g of sucrose per litre (medium A). After 10 days, 10 ml of medium A were added. After 3 weeks, the root cultures were transferred into 300 ml shake flasks containing 100 ml of medium A and transferred to fresh medium every two weeks.

4.3. Labelling experiments

Roots of *T. patula* (35 g of wet cells) grown on medium A were transferred into a 300-ml Erlenmeyer flask with 100 ml of medium A containing 27 g of unlabelled glucose and 3 g of [U-$^{13}C_6$]glucose or 20 g of unlabelled glucose and 10 g of [1-$^{13}C_1$]glucose per litre as sole carbon source. The cultures were grown in darkness at 23 °C with shaking (100 rpm) for 8 days. A sterile solution of 100 mM methyljasmonate (50 µl) was added, and the cultures were incubated for 5 days. The biomass was harvested by suction and washed with a small amount of water. The yield was 63 g (wet weight).

4.4. Isolation of isoeuparin and 4-hydroxytremetone

The plant material was extracted three times with 200 ml of 70% (v/v) methanol. The extract was filtered. The solution was extracted twice with 600 ml of a mixture of hexane/*tert*-butylmethyether (1:1, v/v) (Croes et al., 1989). The organic phase was concentrated to dryness under reduced pressure. The residue was dissolved in 3 ml of hexane. Aliquots of 600 µl were spotted onto TLC plates (Polygram, SIL/UV 254, Macherey-Nagel) which were then developed with a mixture of hexane/dioxane/butanol (75:25:1, v/v) and dried. Zones containing benzofurans were detected on 365 nm as orange fluorescent bands by $R_f = 0.60$; they were scraped off and eluted with freshly distilled diethylether. The solution was concentrated to dryness under reduced pressure. The residue was dissolved in 300 µl of hexane and placed on top of a column of silica gel 60 (0.032–0.063 mm, Merck, 6×0.5 cm) which had been equilibrated with hexane. The column was developed sequentially with 4 ml portions of hexane containing 0%, 1%, 3%, 5% and 10% (v/v) diethylether, respectively. The last two fractions containing the benzofuran derivatives **2** and **1** were concentrated to dryness under a stream of nitrogen. All procedures were carried out under dim light. The isolated compounds were identified by NMR spectroscopy and GC/MS analysis (Bigi et al., 1983; Sütfeld et al., 1985; Burke et al., 1986; Yamaguchi et al., 1986).

4.5. Isolation of amino acids

Subsequent to extraction, biomass was hydrolysed and amino acids were isolated as described elsewhere (Eisenreich et al., 1991).

4.6. NMR spectrometry

^{1}H and ^{13}C NMR spectra were recorded at 25 °C using a DRX 500 spectrometer (Bruker Instruments, Karlsruhe, Germany) at transmitter frequencies of

500.1 and 125.6 MHz, respectively. Samples were dissolved in $CDCl_3$. Two-dimensional COSY, NOESY, HMQC, HMBC and INADEQUATE experiments were performed according to standard Bruker software (XWINNMR).

4.7. Mass spectrometry

The purity of isoeuparin (**1**) and 4-hydroxytremetone (**2**) was >95% as estimated by GC/MS in the electron ionization (EI, 70 eV) mode using a Finnigan MAT SSQ 4500 mass spectrometer coupled with a Perkin–Elmer 8500 gas chromatograph. Separation of the benzofuran derivatives was achieved using a PERMABOND fused silica capillary column (OV-1 DF, Macherey-Nagel; 25 m × 0.25 mm) with helium as carrier gas. Analysis were carried out with increasing oven temperature from 120 °C (held for 2 min) to 300 °C, at 5 °C min^{-1}; $p = 1$ bar. The injector temperature was kept at 300 °C. The retention times of **2** and **1** were 10.76 and 11.51 min, respectively.

4.8. Determination of ^{13}C enrichments

^{13}C-Enrichments were determined by quantitative NMR spectroscopy (Eisenreich and Bacher, 2000). For this purpose, ^{13}C NMR spectra of the biolabelled specimens and of the compounds with natural ^{13}C abundance (i.e., with 1.1% ^{13}C abundance) were measured under the same experimental conditions. The ratios of the signal integrals of the biolabelled compounds and of the compound at natural abundance were then calculated for each respective carbon atom. Absolute ^{13}C abundances for certain carbon atoms (i.e., for carbon atoms with at least one attached hydrogen atom displaying a ^1H NMR signal in a non-crowded region of the spectrum) were determined from the ^{13}C coupling satellites in the ^1H NMR spectra. As an example, the signal for the methyl hydrogen H-11 showed a pair of satellites characterised by one-bond $^1H^{13}C$ coupling ($^1J_{CH}$ = 128 Hz). The signal contribution of the satellite pair in the overall ^1H NMR intensity for H-11 accounted for 1.3% indicating that C-11 had a ^{13}C-abundance of 1.3%. The relative ^{13}C abundances determined for all other positions in **2** were then referenced to this value, thus affording absolute ^{13}C abundances for every single carbon atom (% ^{13}C in Table 3).

In the ^{13}C NMR spectrum of **2** from the experiment with [U-$^{13}C_6$]glucose, each signal was integrated separately. The relative fractions of each respective satellite pair (corresponding to a given coupling pattern, Table 3) in the total signal integral of a given carbon atom were calculated. These values were then referenced to the global absolute ^{13}C abundance for each carbon atom affording concentrations of multiple ^{13}C-labelled isotopolog groups (mol%).

Acknowledgements

This research was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and the Hans-Fischer-Gesellschaft. We thank Fritz Wendling and Angela Grygier for expert technical assistance.

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