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ACCUMULATION AND BIOSYNTHESIS OF BENZOFURANS IN ROOT CULTURES OF *EUPATORIUM CANNABINUM*

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Key Word Index—*Eupatorium cannabinum*; Asteraceae; root cultures; benzofuran derivatives; structure elucidation; biosynthesis.

Abstract—Root cultures of *Eupatorium cannabinum* were shown to accumulate a plethora of benzofuran derivatives. The benzofurans were qualitatively and quantitatively identical or similar to those found in roots of differentiated plants. Seven benzofurans including a new natural product were isolated and identified. The structure elucidation of the new compound is described. Studies on the biosynthesis of the benzofurans were carried out by feeding ^{14}C-labelled compounds and stable isotopes to the root cultures of *E. cannabinum*. The results obtained established that the aromatic ring, as well as the *C*-acetyl substituent linked to the aromatic ring, originate from the shikimic acid pathway via phenylalanine and cinnamic acid. The *C*-acetyl substituent was shown to arise directly from the phenylpropanoid side chain.

INTRODUCTION

2,2-Dimethylchromenes and 2-isopropenylbenzofurans are frequently accumulated by species of the Asteraceae belonging to the tribes Astereae, Eupatorieae, Heliantheae, Inuleae and Senecioneae [1, 2]. So far more than 200 different chromenes and benzofurans have been isolated from these tribes. Most of the chromenes and benzofurans have a *C*-acetyl group as a substituent of the aromatic ring. In spite of the frequent occurrence of these natural products their biosynthesis is still largely unknown. A previous report claimed that the aromatic ring and acetyl group (C_6–C_2 part of the molecule) of the benzofurans from *Eupatorium rugosum* (Asteraceae, tribe Eupatorieae) are biogenetically derived from the polyketide pathway [3]. In a recent study on the metabolism of chromene derivatives in seedlings of *Ageratina adenophora* (Asteraceae, tribe Eupatorieae), however, we obtained preliminary evidence for the shikimic acid pathway as the biogenetic origin of the C_6–C_2 part of the chromenes [4]. By using root cultures of *Eupatorium cannabinum* that accumulate the same benzofurans as those found in roots of differentiated plants we have now unambiguously shown, for the first time, that the aromatic ring as well as the acetyl substituent of the benzofurans are biogenetically derived from phenylalanine.

RESULTS AND DISCUSSION

Phytochemical analysis

Root cultures of *E. cannabinum* were established from aseptically grown seedlings and have been in continuous cultivation for more than two years. The root cultures accumulated a complex pattern of benzofuran derivatives all characterized by their intense yellow, orange or greenish fluorescence on TLC plates under $UV_{366\,nm}$. Seven benzofurans were isolated and identified (**1**–**7**, Fig. 1). Benzofurans **1**, **2** and **4**–**7** were known natural products. Compounds **2**, **3**, **6** and **7** are characterized by an acetyl substituent at C-6 rather than at C-5 as usually encountered. Whereas **2**, **6** and **7** were only recently described from roots of *Ageratum houstonianum* [5, 6], compound **3** proved to be a new natural product. The nature of an ester was evident from the mass spectrum of **3** exhibiting a characteristic fragment at *m/z* 216 that accounted for $[M - C_4H_7COOH]^+$ and the base peak at *m/z* 83 $[C_4H_7CO]^+$. The identity of the esterifying acid as angelic acid followed from the alkaline hydrolysis of **3**, GC-MS analysis of the trimethylsilylated acid and comparison with an authentic standard. The nature of the benzofuran, the position of the ester bond at C-3 as well as the relative stereochemistry at C-3 and C-2 followed from the ^1H NMR spectrum. The two singlets at δ 7.420 and 7.042 were indicative of H-7 and H-4 respectively. The signal of the acetyl group appeared at δ2.668. The signals of the heterocyclic protons H-3 and H-2 appeared as doublets at δ6.205 and 5.087 respectively. The *trans* disposition of H-3 relative to H-2 followed from the coupling constant ($J_{(3-2)} = 3.1$ Hz) that is characteristically smaller than in the corresponding *cis*-isomer [7]. The absolute configurations at C-3 and C-2, however, were not elucidated. The protons of the 2-isopropenyl side chain appeared at δ5.128 and 4.997 (H-11a, 11b) and 1.823 (H-12) respectively. Further signals in the ^1H NMR spectrum of **3** included those of the angelic acid residue at δ6.227 (H-3′) and at 1.998 (H-4′) and 1.908 (H-5′). The assignment of the acetyl group to C-6 rather than to C-5 was based on the ^1H NMR as well as on the UV spectrum of **3**. As both isomeric compounds **3** and **4** were available a distinction between them could be readily deduced from

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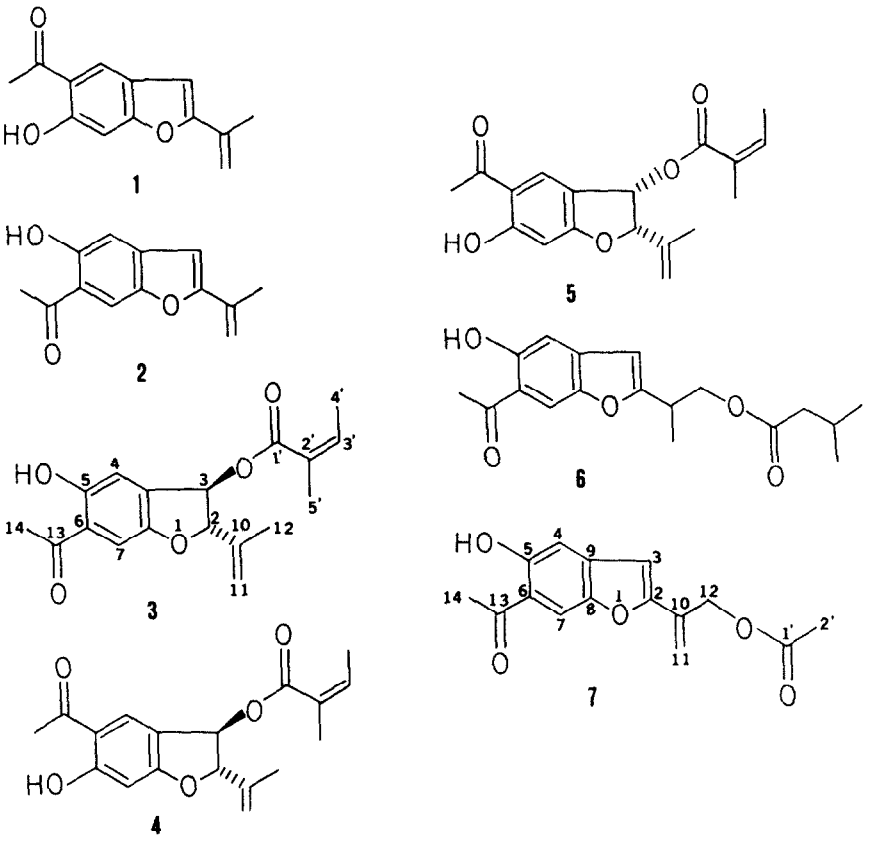

Table 1. Abundance of major benzofurans in root cultures and roots from differentiated plants of *E. cannabinum*

	Amount (μmol/g fr. wt)						
Abundance of benzofurans	1	2	3	4	5	6	7
Root cultures	1.1	+	+	0.9	1.0	+	1.4
Roots from differentiated plants	2.0	+	+	0.7	0.7	+	0.5

+: ≦ 0.1 μmol/g fr. wt.

a consideration of the substituent effects on the chemical shifts of H-4 and H-7 [8]. The UV spectrum of **3** recorded in MeOH exhibited maxima at λ366 and 256 nm and was thus similar to that of 2,5-OH-acetophenone whereas the UV spectra of the benzofurans **4** and **5** showed maxima at 318 and 274 nm and were similar to the UV spectrum of 2,4-OH-acetophenone. The benzofurans were present only in the roots and not in the liquid growth media.

All analysed benzofurans were conveniently studied even in crude root culture extracts by GC-MS except esters **4** and **5** which proved to be labile. Upon GC-MS analysis of a mixture of **4** and **5** only euparin was detected when the sample was injected into the hot injection port and consequently the peaks of both esters were missing from the GC-MS trace of the root culture extract. Satisfactory GC-MS spectra of both compounds could only be achieved by on-column injection. In contrast, the isomeric ester **3** was not labile and could easily be analysed by GC-MS employing a hot injection port as normal.

The major benzofurans present in the root cultures (**1**, **4**, **5** and **7**) were quantified by HPLC (Table 1). Compound **7** was the dominating benzofuran amounting up to 1.4 μmol/g fr. wt followed by euparin(**1**) (1.1 μmol/g fr. wt) and esters **4** and **5** (0.9 and 1.0 μmol/g fr. wt respectively). All other benzofurans analysed were present as minor components equalling or below 0.1 μmol/g fr. wt. The benzofuran pattern of root cultures from *E. cannabinum* was similar to that of roots from differentiated plants (Table 1). All benzofurans detected in root cultures were also present in roots from flowering plants whereas they were absent in all aerial parts of *E. cannabinum* analysed including stems, leaves and flowering heads. Euparin (**1**) was the major benzofuran in roots from plants amounting to 2.0 μmol/g fr. wt, followed by esters **4** and **5** (both 0.7 μmol/g fr. wt) and ester **7** (0.5 μmol/g fr. wt). From the benzofurans identified in this study only compounds **1** and **5** had previously been reported for *E. cannabinum* [9, 10].

Table 2. Incorporation of ^{14}C-labelled precursors into major benzofurans (**1**, **4**, **5**, **7**) by root cultures of *E. cannabinum*

^{14}C-Tracers (dose: 1 μCi = 37 kBq each)	Assay conc. (mM)	Uptake by root cultures (% of applied dose)	Incorporation into benzofurans (% of applied dose)			
			1	**4**	**5**	**7**
L-[U-^{14}C]Phenylalanine	0.5	97	1.2	1.1	1.2	3.7
trans-[*side chain*-3-^{14}C] Cinnamic acid	0.1	97	1.5	1.9	1.3	4.4
Sodium [U-^{14}C]acetate	0.5	99	n.d.	n.d.	n.d.	1.9
L-[U-^{14}C]Tyrosine-hydrochloride	0.1, 0.5, or 1.0	92–98	n.d.	n.d.	n.d.	n.d.
[*ring*-U-^{14}C]Benzoic acid	0.1, 0.5, or 1.0	90–95	n.d.	n.d.	n.d.	n.d.
[*ring*-U-^{14}C]Acetophenone	0.1, 0.5, or 1.0	40–50	n.d.	n.d.	n.d.	n.d.

Incubation conditions: growing roots (0.8 g fr. wt) in 5 ml culture medium plus tracer, 48 hr at 25°, 120 rpm.; n.d., no measurable incorporation detectable.

Feeding of ^{14}C-labelled compounds to root cultures

The biosynthesis of the benzofurans from *E. cannabinum* was studied *in vivo* by feeding ^{14}C-labelled compounds to the root cultures as they provided a convenient experimental tool for biosynthetic studies. The ^{14}C-labelled compounds employed in this study were L-phenylalanine, L-tyrosine, *trans*-cinnamic acid, benzoic acid, acetophenone and acetic acid. The incorporation of radioactivity from the tracers into the benzofurans was separately monitored for the major compounds **1**, **4**, **5** and **7** (Table 2). Uptake of the ^{14}C-labelled compounds by the roots was calculated from the radioactivity remaining in the liquid growth media 48 hr after feeding when the roots were harvested. Uptake of the various ^{14}C-labelled compounds by the root cultures was almost equal ranging from 90 to 99% of the applied dose (always 1 μCi) except for acetophenone where an uptake of only 40–50% was observed. Of the ^{14}C-labelled compounds studied only phenylalanine and cinnamic acid yielded efficient incorporations into the benzofurans. ^{14}C-Cinnamic acid was the most efficient precursor with an incorporation of 4.4% (relative to the applied dose of 1 μCi) into the major compound **7**, followed by 1.9% for **4**, 1.5% for **1** and 1.3% for **5**. ^{14}C-Phenylalanine was in comparison slightly less efficient: 3.7% incorporation for **7**, 1.2% for **1**, 1.1% for **4** and 1.2% for **5**. Feeding of ^{14}C-acetate resulted only in an incorporation into compound **7** (1.9%) whereas no radioactivity was found in the other benzofurans analysed. When **7** was subjected to alkaline hydrolysis, the radioactivity following feeding of ^{14}C-acetate was lost and did not appear in the benzofuran alcohol indicating that ^{14}C-acetate had been incorporated only as an esterifying acid. Feeding of the remaining ^{14}C-labelled compounds (tyrosine, benzoic acid, acetophenone) yielded no measurable incorporation into the benzofurans analysed. Other unknown metabolites of the root cultures that were not identical with the benzofurans, however, were labelled indicating metabolism of the latter ^{14}C-compounds after uptake by the root cultures.

Feeding of stable isotopes to root cultures

The feeding experiments with ^{14}C-labelled compounds indicated phenylalanine and cinnamic acid as biogenetic precursors of the benzofurans from root cultures of *E. cannabinum* (Table 2). Based on the structural comparison between the two aromatic precursors and the benzofurans it was obvious to assume that the precursors were incorporated into the C_6–C_2 part of the benzofurans. To obtain unequivocal proof for the precise site of incorporation into the benzofuran molecule several stable isotopes including L-[2-^{13}C]-phenylalanine, L-[*ring*-$^2H_5$]phenylalanine and [2-^{13}C]acetate were fed to the root cultures. Following feeding the benzofurans **1** and/or **7** were isolated and the incorporation determined by ^{13}C NMR or GC-MS.

The ^{13}C spectra of **1** and **7**, reported here for the first time, were assigned from considerations of the DEPT spectra and from a comparison of the two sets of data. By using substituent chemical shift increments for the acetyl and hydroxyl groups [11], the benzofuran ring carbons were readily distinguished and assigned. Comparison of the ^{13}C spectra of **1** and **7** with and without enrichment with L-[2-^{13}C]phenylalanine showed significantly increased signal intensities for C-14 in the spectra of both compounds (enrichment 10.7% for **1** and 24.0% for **7**). Enrichment with [2-^{13}C]acetate in comparison showed only an increased signal intensity for C-2′ of compound **7** (enrichment 6.8%) and none for the other carbons which agreed with the data obtained by alkaline hydrolysis of **7** after feeding of ^{14}C-acetate.

The incorporation of L-[*ring*-$^2H_5$]phenylalanine into benzofurans **1** and **7** was determined by GC-MS and direct comparison of the mass spectra with those of non-deuterated compounds. The deuterated benzofurans clearly showed higher $[M+2]^+$ signals as well as $[M+2-15]^+$ fragments compatible with the expected incorporation of two deuterium atoms into the molecule. The intensities of the $[M+2]^+$ and the $[M+2-15]^+$ signals of deuterated **1** were both 4.2% (relative to the $[M]^+$ and $[M-15]^+$ signals) compared to 1.2% for the $[M+2]^+$ and $[M+2-15]^+$ signals (relative to the $[M]^+$ and $[M-15]^+$ signals) of the non-deuterated compound. For deuterated **7** the intensities of the $[M+2]^+$ and $[M+2-15]^+$ signals were 6.9 and 7.1% respectively (relative to the $[M]^+$ and $[M-15]^+$ signals) compared to 1.6% for the $[M+2]^+$ and $[M+2-15]^+$ signals (relative to the $[M]^+$ and $[M-15]^+$ signals) of the non-deuterated compound. The intensities of the $[M+1]^+$ and $[M+1$

$-15]^+$ fragments in the mass spectra of deuterated and non-deuterated **1** or **7** were of almost equal intensity. Smaller fragments in the mass spectra of **1** and **7** could not be evaluated for the incorporation of deuterium due to their low relative intensities [5].

There was a discrepancy between the degree of enrichment of benzofurans **1** and **7** following feeding of L-[2-^{13}C]phenylalanine and L-[*ring*-$^2H_5$]phenylalanine. Thus the enrichment of the two benzofurans recorded following feeding the of former tracer was larger than following feeding of the latter. This discrepancy cannot be explained satisfactorily at present. It must be assumed that some of the deuterium label at C-4 and C-7 of the benzofurans is lost during substitution of the aromatic ring of phenylalanine (hydroxylation and attachment of the heterocycle).

CONCLUSIONS

The organ specific phytochemical analysis of differentiated plants of *E. cannabinum* and of root cultures as well as the feeding experiments with ^{14}C-labelled compounds and stable isotopes demonstrate that the benzofurans are not only stored but are also synthesized in the roots of this species. The biogenetic experiments with ^{14}C-labelled compounds and with stable isotopes further prove that the C_6–C_2 part of the benzofurans from *E. cannabinum* originates in the shikimic acid pathway via phenylalanine and cinnamic acid as precursors. The acetyl group of the benzofurans is directly derived from the C_3-side chain of the phenylpropanoid precursor by loss of the carboxylic group. As tyrosine, by comparison with phenylalanine, is not incorporated into the benzofurans the oxygen substituent at C-8 of the benzofuran molecule can be assumed to be introduced not at the amino acid level but at some later step in the biosynthesis of the benzofurans. Acetate is only incorporated as the esterifying acid in compound **7** and not into the C_6–C_2 part of the benzofurans analysed.

The biosynthesis of the benzofurans from *E. cannabinum* as elucidated in our study shows similarities to that of acetophenone derivatives (e.g. pungenine) in needles of *Picea pungens* [12]. Feeding of various ^{14}C-precursors to needles of *P. pungens* indicated that L-^{14}C-phenylalanine, ^{14}C-cinnamic acid and ^{14}C-caffeic acid were effectively incorporated into pungenine (3,4-dihydroxyacetophenone) whereas by comparison ^{14}C-acetate was only a poor precursor. Our data on the biosynthesis of benzofurans, however, are in contrast to a previous report on the biosynthesis of benzofurans from *Eupatorium rugosum* [3]. The authors studied the biosynthesis of dehydrotremetone [6-desoxyeuparin; euparin (**1**)] by feeding various ^{14}C-precursors to differentiated plants of *E. rugosum* employing the wick technique followed by chemical degradation of dehydrotremetone to determine the site of incorporation of the tracers into the benzofuran. ^{14}C-Acetate was reported as the best precursor for the C_6–C_2 part of dehydrotremetone. The incorporation of ^{14}C-acetate into dehydrotremetone, however, amounted only up to 0.06% (compared to 0.0006% with phenylalanine) and is thus almost two orders of magnitude smaller than the incorporation of ^{14}C-phenylalanine into benzofuran **7** obtained here using root cultures of *E. cannabinum*. These highly divergent results on the biosynthesis of benzofurans are hard to bring into line at the moment if we consider the close structural similarity of the compounds studied and the close systematic relationship of both species (*E. cannabinum* and *E. rugosum*.). A reanalysis of the biosynthesis of dehydrotremetone in *E. rugosum* is thus desirable.

EXPERIMENTAL

Plant material. Flowering plants and achenes of *E. cannabinum* were collected in the vicinity of Braunschweig. A voucher specimen is on file in the Institut für Pharmazeutische Biologie. Root cultures were established from aseptically grown seedlings. Roots (length 1–2 cm) from 4–5 seedlings were excised and transferred into 10 ml MS-medium [13], pH 5.8, containing 4% sucrose and no phytohormones [14]. As soon as the culture was established it was subcultured in 100 ml flasks containing 50 ml medium. The cultures were transferred every four to six weeks and kept in the dark on a gyratory shaker (120 rpm at 25°). At the beginning of the feeding experiments the root cultures had been in continuous cultivation for more than two years.

Extraction, isolation and chromatographic analysis. All plant material was extracted exhaustively with Me_2CO. Extracts were concentrated for TLC analysis which was carried out on premade silica gel plates with CH_2Cl_2–MeOH (99:1) as eluent. The benzofurans were detected by their yellow, green or orange fluorescence on TLC plates under $UV_{366\,nm}$.

For isolation of the benzofurans root culture extracts were taken to dryness, redissolved in CH_2Cl_2 and chromatographed on a column filled with silica gel employing the same solvent. Fractions (10 ml) were taken and monitored by TLC. Similar fractions were combined and subjected to CC on a Sephadex LH-20 column with MeOH as solvent. Final purification was achieved by prep. TLC on premade silica gel plates with CH_2Cl_2 or mixtures of CH_2Cl_2 and MeOH as solvent.

For HPLC analysis the extracts were diluted with H_2O (1:1), centrifuged (10 000 *g* for 5 min) and the supernatant injected into a HPLC-system (Pharmacia, LKB, Sweden). The separation was achieved by applying a linear gradient from 100% A (30% MeCN, 70% H_2O, 1% H_3PO_4) to 100% B (80% MeCN, 20% H_2O, 1% H_3PO_4) in 20 min followed by 5 min isocratic at 100% B. The flow rate was 1.0 ml/min. Detection was at 300 nm (**1**, **2**, **4**–**7**) or 360 nm (**3**). The separation column (125 × 4 mm i.d.) was prefilled with Novapak C-18 (5 μ) (Waters Millipore GmbH, Eschborn, F.R.G.). Quantification was achieved by the external standard method using previously isolated and purified compounds.

Spectroscopic analysis. ^{1}H and ^{13}C NMR: CD_3OD or DMSO-d_6, ambient temperature, 300 or 400 MHz. All 1D or 2D spectra were obtained using the standard Bruker software. ^{13}C NMR spectra, used to determine the percentage enrichments in **1** and **7**, were taken in the inversegated mode to nullify the NOE and the time between pulses was 6.0 sec (relaxation delay + acquisition time). GC-MS: Carlo Erba 5160 GC (equipped with a 30 m × 0.32 mm i.d. fused silica quartz capillary column coated with DB-1 (J & W Scientific), temp program 100–300° at 6°/min, quadrupole mass spectrometer (Finnigan MAT 4515). EIMS: 70 eV in combination with the Incos data system. 2,4-Dihydroxy Acetophenone and 2,5-dihydroxy acetophenone were purchased from Aldrich (Steinheim, F.R.G.).

^{14}C-Precursors and stable isotopes. L-[U-^{14}C] Phenylalanine, L-[U-^{14}C]tyrosine HCl, [U-^{14}C]acetate (as Na salt), [*ring*-U-^{14}C]benzoic acid and *trans*-[*side chain*-3-^{14}C] cinnamic acid were obtained from Amersham Buchler (Braunschweig, F.R.G.). [U-^{14}C] Acetophenone was available from Sigma Chemie GmbH (Deisenhofen, F.R.G.).

L-[2-^{13}C, 99%]Phenylalanine and L-[*ring*-$^{2}H_5$, 98%]-phenylalanine were obtained from ICN Biomedicals (Eschwege, F.R.G.), [2-^{13}C, 93%]acetate (as Na salt) was purchased from Amersham Buchler (Braunschweig, F.R.G.).

Feeding experiments. For experiments with ^{14}C-tracers, growing roots from root cultures (*ca* 800 mg wet wt) were incubated in small Erlenmeyer flasks with 5 ml culture medium containing 1 μCi of the ^{14}C-tracer at various assay concentrations. Incubation was under continuous shaking (120 rpm) at 25° for 48 hr in the dark followed by extraction. All feeding experiments were repeated at least three times, those employing ^{14}C-phenylalanine six times.

Feeding experiments with stable isotopes were carried out on a larger scale employing Erlenmeyer flasks containing 100 ml medium and *ca* 3 g (wet wt) roots. The stable isotopes were fed to the cultures at an assay concentration of 0.5 mM each. For feeding of L-[^{13}C]phenylalanine 12 flasks, for L-[*ring*-$^{2}H_5$]phenylalanine 15 flasks and for [2-^{13}C]acetate 25 flasks were employed. After 48 hr, the roots from each experiment were combined, harvested and extracted followed by isolation of the benzofurans **1** and **7**.

Compound **1**. ^{13}C NMR (DMSO-d_6): δ204.33 (*s*, C-13), 160.27 (*s*, C-8), 158.59 (*s*, C-6), 156.75 (*s*, C-2), 132.03 (*s*, C-5), 124.79 (*d*, C-4), 121.30 (*s*, C-9), 117.15 (*s*, C-10), 113.12 (*t*, C-11), 103.35 (*d*, C-3), 98.48 (*d*, C-7), 27.23 (*q*, C-14), 18.69 (*q*, C-12); GC-MS (*m/z*, rel. int.) (only characteristic fragments shown): 218 (1), 217 (11.8), 216 (86) [M]$^+$, 203 (1.1), 202 (12.4), 201 (100); following feeding of L-[*ring*-$^{2}H_5$]phenylalanine: 218 (3.6), 217 (12.3), 216 (86.4) [M]$^+$, 203 (4.1), 202 (12.7), 201 (100).

Compound **3**. UV λ_{max}^{MeOH} nm: 256, 366; ^{1}H NMR (CD_3OD): δ7.420 (H-7, *s*), 7.042 (H-4, *s*), 6.227 (H-3', *dd*, *J* (3'-4') = 7.3 Hz, *J*(3'-5') = 1.5 Hz), 6.205 (H-3, *d*, *J* (3-2) = 3.1 Hz), 5.128 (H-11a, *br s*), 5.087 (H-2, *d*, *J* (2–3) = 3.1 Hz), 4.997 (H-11b, *br s*), 2.668 (H-14, *s*), 1.998 (H-4', *dd*, *J* (4'-3') = 7.3 Hz, *J*(4'-5') = 1.5 Hz), 1.908 (H-5', *dd*, *J* (5'-3') = 1.5, *J* (5'-4') = 1.5 Hz), 1.823 (H-12, *s*); GC-MS (*m/z*, rel. int.): 316 (4) [M]$^+$, 216 (38), 201 (26), 83 (100), 55 (64).

2,5-*Dihydroxy acetophenone*. UV λ_{max}^{MeOH} nm: 258, 372.

Compounds **4**, **5**. UV λ_{max}^{MeOH} nm: 274, 318.

2,4-*Dihydroxyacetophenone*. UV λ_{max}^{MeOH} nm: 276, 315.

Compound **7**. ^{13}C NMR (DMSO-d_6): δ204.19 (*s*, C-13), 169.95 (*s*, C-1'), 158.02 (*s*, C-5), 157.16 (*s*, C-2), 147.14 (*s*, C-8), 135.60 (*s*, C-9), 132.35 (*s*, C-6), 118.12 (*t*, C-11), 117.34 (*s*, C-10), 112.83 (*d*, C-7), 107.31 (*d*, C-4), 103.77 (*d*, C-3), 62.94 (*t*, C-12), 27.41 (*q*, C-14), 20.51 (*q*, C-2'); GC-MS (*m/z*, rel. int.) (only characteristic fragments shown): 276 (0.7), 275 (7.0), 274 (46.3) [M]$^+$, 261 (1.5), 260 (13.0), 259 (89.7); following feeding of L-[*ring*-$^{2}H_5$]phenylalanine: 276 (3.4), 275 (7.8), 274 (50.0) [M]$^+$, 261 (6.9), 260 (14.4), 259 (96.6).

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