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Absolute configuration of the α-methylbutyryl residue in longipinene derivatives from *Stevia pilosa*

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Abstract

The absolute configuration of the α-methylbutyryl residue in (4*R*,5*S*,7*S*,8*S*,9*S*,10*R*,11*R*,2″*S*)-7-angeloyloxy-9-hydroxy-8-(α-methylbutyryloxy)-longipin-2-en-l-one and (4*R*,5*S*,7*S*,8*R*,10*R*,11*R*,2″*S*)-7-angeloyloxy-8-(α-methylbutyryloxy)- longipin-2-en-l-one was determined by chemical correlation with (*S*)-(+)-benzyl α-methylbutyrate prepared from authentic (*S*)-(+)-α-methylbutyric acid. Both compounds were isolated from the hexane extracts of roots of *Stevia pilosa* Lag. together with four other longipinene derivatives. The developed correlation method is useful to ascertain the chirality of natural α-methylbutyryl esters found in nature and to reinforce the hypotheses on the biogenetic origin of these residues.

Keywords: *Stevia pilosa*; Compositae; Absolute configuration; (*S*)-(+)-α-Methylbutyric acid; (*S*)-(+)-2-Methylbutanoic acid; Longipinene derivatives

1. Introduction

Chiral ester moieties derived from short-chain-length fatty acids are commonly found in terpenoids (Torres-Valencia et al., 1999). In most cases, reports on the structure of these substances focusses on the stereochemical assignment of the main framework, but lacks information about the absolute configuration of ester residues. In previous work, we have carried out the preparation, studied the absolute configuration, and measured the optical activity properties of the four stereoisomers of 2,3-epoxy-2-methylbutanoic acid named (+)- and (−)-epoxyangelic (Torres-Valencia et al., 1998; Torres-Valencia et al., 2002) and (+)- and (−)-epoxytiglic acids (Torres-Valencia et al., 1995), As a continuation of this research, we describe herein the chirality of the α-methylbutyryl residues found in two longipinene derivatives (**1** and **2**) isolated from the roots of *Stevia pilosa*, and four other longipinene (**3**–**6**) derivaties. The absolute configuration of the α-methylbutyryl fragment is assigned as *S* by means of chemical correlation with (*S*)-(+)-benzyl α-methylbutyrate (**7**) prepared from commercially available (*S*)-(+)-methylbutyric acid. The α-methylbutyryl moiety has been found attached to three sesquiterpene lactones isolated from *S. sanguinea* (De Hernández et al., 1997) and two longipinene derivatives obtained from *S. serrata* (Román et al., 1985). This is the first time that the absolute configuration of the α-methylbutyryl residues of natural products is elu-

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cidated by chemical correlation, thus providing a methodology of general usage.

In addition to compounds **1** and **2**, the present study allowed the isolation of four polyoxygenated longipinene derivatives (**3–6**) which contained angeloyl ester residues [(*Z*)-2-methyl-2-butenoyl]. Previous investigation of the aerial parts of *S. pilosa* yielded a guaianolide-type sesquiterpene lactone named leukodine (Martınez et al., 1988).

2. Results and discussion

The hexane extracts from the roots of *S. pilosa* showed, by ^{1}H and ^{13}C NMR spectroscopic analyses, high concentrations of longipinene derivatives containing angelate and α-methylbutyrate ester residues. Column chromatography separation of this extract, using hexane and hexane–EtOAc mixtures as eluents, afforded a fraction containing the longipinene derivatives, which was subjected to alkaline hydrolysis, using KOH in MeOH/H_2O. A mixture of angelic and α-methylbutyric acids was obtained from the aqueous phase after removal of the sesquiterpenes by extraction with EtOAc, neutralisation with HCl, and extraction with CH_2Cl_2. Esterification of the acid mixture with benzyl alcohol, followed by HPLC separation, gave benzyl α-methylbutyrate (**7**) and benzyl angelate (**8**), which were characterised from their physical and spectroscopic data. The identity and absolute configuration of the benzyl α-methylbutyrate (**7**) determined as *S*, after comparison of its optical activity $[\alpha]_D^{20}$ + 11.6 ($CHC1_3$, *c* 0.20) and ^{1}H and ^{13}C NMR spectroscopic data with that of an authentic sample of **7** $[\alpha]_D^{20}$ + 11.6 ($CHC1_3$, *c* 1.87) prepared by esterification of commercial benzyl alcohol and (*S*)-(+)-α-methylbutyric acid.

Additionally, column chromatography followed by HPLC separation of the longipinene fraction allowed isolation of the naturally occurring longipinene derivatives containing the α-metylbutyryl residues **1** (Román et al., 1985) and **2** (Román et al., 1985), together with angelate esters **3** (Sánchez-Arreola et al., 1995), **4** (Amaro et al., 1988), **5** (Román et al., 1985), and **6** (Sánchez-Arreola et al., 1995, 1999).

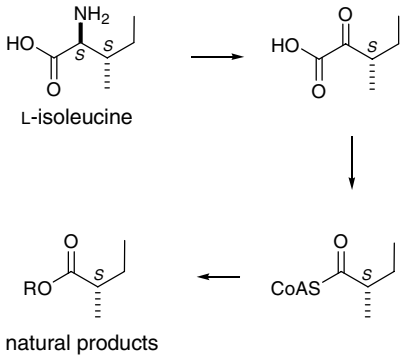
These results, together with previous reports (Torres-Valencia et al., 2002, 1999) for the stereochemical assignment of functionalized α-methylbutyric residues in natural products, contribute to the knowledge of the absolute configuration of the branched short-chain-length fatty acids, which is relevant to reinforce the proposed biogenetic origin of these moieties from amino acids (Manitto, 1981). Biosynthetic studies indicated that L-isoleucine is converted to α-methylbutyryl CoA which produced the α-methylbutyrate derivatives in many species (Rawlings, 1998). This pathway has also been considered for the biosynthesis of secondary metabolites produced by bacteria including cyclic hexadepsipetides (Umezawa et al., 2002) and polyketides (Denoya et al., 1995). If the biogenesis of the α-methylbutyric residues starts from L-isoleucine, which possesses the 2*S*,3*S* configuration (Khawas, 1970), these residues will always have the *S* configuration. However, there are some examples where the *R* isomer is the predominant, as is the case for medicinal plants of the genus *Rheum* (Dregus et al., 2003) and *Veratrum* (Weisenborn et al., 1954).

The present study supports the presence of the L-isoleucine pathway in the biosynthesis of the secondary metabolites of *Stevia pilosa* as shown in Scheme 1. The chiral center at C-2 in the α-methylbutyryl residue is *S* in agreement with the chirality of the carbon atom of L-isoleucine which originates C-2 in the α-methylbutyryl residues. On the other hand, an alternate pathway starting from L-threonine has been proposed (van der Hoeven and Steffens, 2000), which involves conversion of this aminoacid into α-ketobutyric acid by threonine dehydratase, followed by a multienzyme conversion into α-keto-β-methylvaleric acid and subsequent decarboxylation. The coexistence of both biogenetic routes would explain those cases when a mixture of (*S*)-(+)- and (*R*)-(−)-α-methylbutyrates is present.

3. Experimental

3.1. General

IR spectra were measured in $CHCl_3$ on a Perkin–Elmer 2000 FT-IR spectrophotometer. Optical rotations were determined in $CHCl_3$ on a Perkin–Elmer 341 polarimeter. NMR measurements were performed at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Jeol Eclipse 400 spectrometer or at 300 MHz for ^{1}H and 75.4 MHz for ^{13}C on a Varian Mercury 300 spectrometer from $CDCl_3$ solutions containing TMS as internal standard. Mass spectra were recorded at 70 eV on a Hewlett Pack-



Scheme 1. Stereochemical analysis for the biosynthesis of α-methylbutyryl residues in natural products of *Stevia* starting from L-isoleucine.

ard 5890 Series II spectrometer. CC was carried out on Merck silica gel 60 (Aldrich, 230–400 mesh ASTM). HPLC separations were done on a chromatographic system composed by a Varian Prostar 215 pump, a Varian Prostar 320 UV detector operating at 260 nm and a ChromSpher 5 Si column of 250 mm length × 4.6 mm i.d. or on a Perkin–Elmer Series 200 chromatograph using a Dynamax C18 reverse phase column of 10 mm i.d. × 250 mm length + 100 mm (pre-column) employing UV detection at 254 nm.

3.2. Plant material

Specimens of *Stevia pilosa* Lag. were collected at Mineral del Monte Municipality, in the state of Hidalgo, Mexico, during October 2001. A voucher specimen (M. Torres Valencia 10 (IEB)) is deposited at the Herbarium of Institute de Ecología, A.C., Pátzcuaro, Michoacán, Mexico, where Professor Jerzy Rzedowski identified the plant material.

3.3. Extraction and isolation

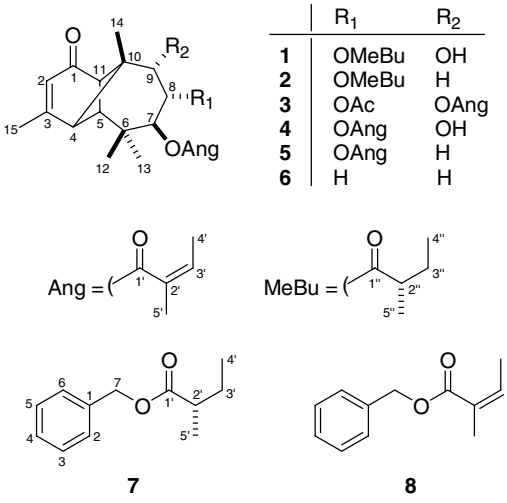
Air-dried grounded roots (1.1 kg) of *S. pilosa* were extracted (×3) with hexane under reflux for 6 h (×3). Filtration and evaporation of the extract afforded a yellow viscous oil (65 g), which was dissolved in MeOH at 50 °C, kept at 0 °C for 12 h and filtered to remove fatty materials. The filtrate was evaporated under vacuum and a portion of the pale yellow oily residue (5 g) was subjected to silica gel CC(120 g). Fractions (20 ml) were collected using hexane (1 l), mixtures of hexane–EtOAc (9:1, 600 ml), hexane–EtOAc (4:1, 1.0 l), hexane–EtOAc (1:1, 500 ml) and pure EtOAc (100 ml). Combined fractions 99–137 (1.25 g) were applied to a column using hexane–EtOAc 19:1 and collecting fractions of 10 ml. The mixture of compounds from fractions 165–211 was purified by TLC (hexane–EtOAc 9:1, 18 developments) affording **6** (22 mg, R_f 0.74) and **5** (17 mg, R_f 0.64). The mixture contained in fractions 238–280 (142 mg) was purified by HPLC (MeOH–H_2O, 7:3, flow 4.5 ml min^{-1}, injecting 2.5 mg) to afford **2** (1 mg) with R_t of 82 min. Compound **3** (126 mg) was isolated from fractions 324–329. Finally, fractions 146–148 afforded longipinene derivatives **1** and **4** (109 mg), which were identified by comparison of ^{1}H NMR and ^{13}C NMR spectra with the literature data (Amaro et al., 1988; Román et al., 1985; Sánchez-Arreola et al., 1995).

3.4. Alkaline hydrolysis of the mixture of longipinene derivatives

CC of the hexane extract (10 g) from the roots of *S. pilosa*, using hexane (1 l), hexane–EtOAc (9:1) and hexane–EtOAc (4:1) as eluents, gave a mixture of longipinene derivatives (7.2 g from the latter fractions). The mixture was dissolved in MeOH (14 ml), treated with KOH (4.29 g) in H_2O (19 ml) and stirred at room temperature during 20 min and extracted with EtOAc. The aqueous layer was acidified with concentrated HCl until pH 3.0, saturated with NaCl and extracted with CH_2Cl_2 (300 ml) (×2). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to provide 687 mg of the mixture of angelic and α-methylbutyric acids.

3.5. Esterification of the mixture of the carboxylic acids with benzyl alcohol

A solution of benzyl alcohol (1.46 g) in CH_2Cl_2 (10 ml) was treated with dicyclohexylcarbodiimide (2.78 g), 4-*N,N*-dimethylaminopyridine (5 mg) and the mixture of angelic and α-methylbutyric acids, obtained above. The mixture was stirred for 16 h at room temperature, filtered and evaporated. The crude product was subjected to silica gel CC(45 g) eluting with hexane (600 ml), followed by hexane–EtOAc (19:1, 250 ml), from which fractions 150–158 (483 mg) afforded a mixture of benzyl esters. A portion of this mixture (87 mg) was re-applied to normal phase HPLC, injecting 2.9 mg of sample in 30 μl of hexane and using hexane–EtOAc (99:1) as the mobile phase at flow rate of 3 ml min^{-1} to afford benzyl angelate (**8**) (4 mg, 5%) and benzyl α-methylbutyrate (**7**) (10 mg, 11%).



3.6. (S)-(+)-Benzyl α-methylbutyrate (7)

A solution of benzyl alcohol (159 mg, Aldrich) in CH_2Cl_2 (10 ml) was treated with dicyclohexylcarbodiimide (303 mg), 4-*N,N*-dimethylaminopyridine (5 mg) and (*S*)-(+)-α-methylbutyric acid 98% ee (100 mg, Aldrich), stirred for 5 h at room temperature, filtered and evaporated. The residue was purified on silica gel (18 g). Elution with hexane yielded **7** as a colourless

oil (94 mg, 50%), EIMS-*m*/*z* (rel. int.): 192 $[M]^+$ (16), 108 (22), 91 (100), 77 (8), 65 (16), 57 (34); IR ν_{max} ($CHCl_3$): 2968, 1735, 1456, 1182, 1147 cm^{-1}.

$$[\alpha] = \frac{\begin{array}{ccccc} 589 & 578 & 546 & 436 & 365 \\ +11.6 & +12.1 & +13.8 & +23.5 & +37.4 \end{array}}{\times (CHCl_3, c1.87)}$$

^1H NMR (400 MHz): δ 7.40–7.30 (5H, *m*, H-2–H-6), 5.12 (2H, *s*, H-7), 2.43 (1H, sext, $J = 7.0$, H-2′), 1.71 (1H, *m*, H-3′a), 1.49 (1H, *m*, H-3′b), 1.17 (3H, *d*, $J = 7.0$, Me-5′) 0.90 (3H, *t*, $J = 7.3$, Me-4′) ^{13}C NMR (100 MHz): δ 176.5 (C-1′), 136.2 (C-1), 128.5 (C-2, C-6), 128.1 (C-3, C-4, C-5), 66.0 (C-7), 41.1 (C-2′), 26.8 (C-3′), 16.6 (C-5′), 11.6 (C-4′).

3.7. Benzyl angelate (**8**)

Colourless oil; EIMS *m*/*z* (rel. int.): 190 $[M]^+$ (3), 172 (26), 145 (13), 91 (100), 83 (52), 55 (36); IR ν_{max} ($CHCl_3$): 2956, 1710, 1650, 1226, and 1206 cm^{-1}; λ_{max} (EtOH): 217 nm (logε 3.80); ^1H NMR (300 MHz): δ 7.40–7.30 (5H, *m*, H-2–H-6), 6.08 (1H, *qq*, $J = 7.1$ and 1.5 Hz, H-3′); 5.19 (2H, *s*, H-7), 1.98 (3H, *dq*, $J = 7.1$ and 1.5, Me-5′) 1.91 (3H, *quint*, $J = 1.5$, Me-4′). ^{13}C NMR (75.4 MHz): δ 167.8 (C-l′), 138.4 (C-3′), 136.3 (C-1), 128:5 (C-2, C-6), 128.0 (C-3′, C-4, C-5), 127.7 (C-2′), 65.8 (C-7), 20.6 (C-5′), 15.8 (C-4′).

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