

Online Gas Chromatography Combustion/Pyrolysis–Isotope Ratio Mass Spectrometry (HRGC-C/P-IRMS) of (±)-Dihydroactinidiolide from Tea (*Camellia sinensis*) and Rooibos Tea (*Aspalathus linearis*)

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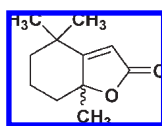
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Online capillary gas chromatography–isotope ratio mass spectrometry in both the combustion and the pyrolysis modes (HRGC-C/P-IRMS) was employed to perform authentication studies of the flavoring agent (±)-dihydroactinidiolide. Thus, the $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values of synthetic (ex synthetic β -ionone and natural β -carotene) as well as enzymatically (ex synthetic and natural β -carotene) produced references were studied in comparison with those of the natural substance isolated from black ($n = 17$) and green teas ($n = 6$) (*Camellia sinensis*) as well as Rooibos tea (*Aspalathus linearis*) ($n = 7$). The isotope values determined for both the synthetic and enzymatically produced samples of (±)-dihydroactinidiolide reflected the influence of the origin of their educts. Hence, in cases when synthetic educts were used, the $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values ranged from -27.0 to -28.4% and from -28 to -169% , respectively, whereas the use of natural educts led to ranges from -30.3 to -31.6% and from -154 to -228% , respectively. As to the tea samples, $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values ranging from -29.0 to -34.1% and from -153 to -274% , respectively, were recorded for (±)-dihydroactinidiolide from black and green teas, whereas that from Rooibos tea showed $^2\text{H}/^1\text{H}$ ratios ranging from -189 to -210% as well as slightly enriched values in the $^{13}\text{C}/^{12}\text{C}$ ratios ranging from -24.4 to -27.1% .

KEYWORDS: (±)-Dihydroactinidiolide; tea; *Camellia sinensis*; Rooibos; *Aspalathus linearis*; isotope ratio mass spectrometry; HRGC-C/P-IRMS; $^{13}\text{C}/^{12}\text{C}$ ratio; $^2\text{H}/^1\text{H}$ ratio; flavor authentication

INTRODUCTION

Dihydroactinidiolide is a C-11 terpenic lactone isolated for the first time from the essential oil of the leaves of *Actinidia polygama* by Sakan et al. (1). Since then, its presence has



also been described in several plant tissues such as black (2–4) and green teas (5, 6), Rooibos tea (7, 8), tobacco (9, 10), grapes (11), or apricots (12, 13). In the latter, dihydroactinidiolide has been found to occur as a racemate (14). Its natural occurrence in plant material is due to the degradation of carotenoids. Hence,

together with β -ionone and 5,6-epoxy- β -ionone, (±)-dihydroactinidiolide has been described as one of the main products of the thermal (15, 16) and oxidative degradation of β -carotene (17). In addition, the bicyclic lactone has been obtained from β -carotene under enzymatic co-oxidation conditions using xanthine oxidase (18, 19) and lipoxygenase (20, 21). Furthermore, microbial formation has been reported (22). Carotenoid degradation is also regarded to be responsible for the occurrence of (±)-dihydroactinidiolide in marine sediments (23) and as an off-flavor compound in freshwater (24). Due to its importance for the aroma of tea and tobacco, the synthesis of dihydroactinidiolide, in both racemic (25–28) and stereoselective forms (29–33), has found great attention in the past.

As in recent years the authenticity assessment of flavor substances has gained increased importance (34), it was of interest to include (±)-dihydroactinidiolide in the series of our recent studies dealing with carotenoids (35) and some of their common degradation products (36). Thus, we report here the $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ ratios of natural (±)-dihydroactinidiolide isolated from black and green teas (*Camellia sinensis*) and from Rooibos tea (*Aspalathus linearis*), as well as synthetic and enzymatically produced reference samples.

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MATERIALS AND METHODS

Samples and Chemicals. Black tea samples from India (Assam $n = 5$; Darjeeling $n = 5$), Egypt ($n = 1$), and Sri Lanka (Ceylon $n = 6$) and green teas from China ($n = 4$) and Japan ($n = 2$) as well as Rooibos teas from South Africa ($n = 7$) were purchased at local markets in Würzburg, Germany. Two synthetic samples of (\pm)-dihydroactinidiolide (with no information about their synthetic origin) were available from our laboratory collection of flavor compounds; two others were self-prepared, both from synthetic β -ionone (Aldrich, Steinheim, Germany) and natural β -carotene, respectively. β -Carotene (synthetic and natural) was also used for the enzymatic production of (\pm)-dihydroactinidiolide employing xanthine oxidase (grade III from buttermilk, Sigma Aldrich, Steinheim, Germany) (cf. below). The natural β -carotene was isolated and purified from palm oil as recently reported (35). All other chemicals were obtained from Sigma-Aldrich. Solvents were redistilled before use.

Isolation of (\pm)-Dihydroactinidiolide from Tea Samples. Tea (100 g) was brewed using 1300 mL of distilled boiling water for 30 min. The tea leaves were separated, and the solution was submitted to liquid–liquid extraction (LLE, 48 h), using a pentane/dichloromethane (2:1, v/v) mixture. The extract was dried over anhydrous sodium sulfate and fractionated by liquid chromatography (LC) on a silica gel glass column (2 cm \times 30 cm), filled with silica gel 60, 0.2–0.5 mm (Merck, Darmstadt, Germany) using a pentane/diethyl ether mixture (from 0 to 100% diethyl ether in 10% steps, each 150 mL; flow rate = 3 mL/min). (\pm)-Dihydroactinidiolide was collected in the 40:60 pentane/diethyl ether fraction. The solvent was removed by rotary evaporation, and the residue was dissolved in approximately 1 mL of diethyl ether and subjected to HRGC-MS and HRGC-IRMS analyses. The influence of sample preparation on the $^2\text{H}/^1\text{H}$ and $^{13}\text{C}/^{12}\text{C}$ isotope ratios was checked by model LLE and LC separation. No significant isotope discrimination effects were observed by the applied workup procedures.

Synthesis of (\pm)-Dihydroactinidiolide. (i) *From β -Ionone.* The preparation was performed from β -ionone (synthetic) via formation of 5,6-epoxy- β -ionone and its thermal degradation at 80 °C over 24 h as described by Bosser et al. (19). Purification by LC on silica gel (cf. sample preparation for tea) yielded the product (12 mg) with 97% purity (HRGC).

(ii) *From β -Carotene.* Thermal degradation of β -carotene (natural) performed over 4 h at 90 °C according to the method described by Kanasawud and Cruzet (16) delivered a product mixture from which (\pm)-dihydroactinidiolide was separated by LC on silica gel as described above [yield, 2 mg; purity (HRGC), 95%].

(iii) *Enzymatic Production from β -Carotene.* Co-oxidation of β -carotene (synthetic and natural) (4 h, 37 °C) with xanthine oxidase was performed as described by Bosser and Belin (18). Subsequent LC purification on silica gel (as described above) yielded products (6 mg each) with 97% purity each (HRGC).

All samples of dihydroactinidiolide under study were subjected to enantioselective multidimensional gas chromatography–mass spectrometry (enantio-MDGC-MS). In all cases, the racemic form was found (data not shown).

Gas Chromatography (HRGC). An Agilent 6890 series gas chromatograph (GC) with split injector (220 °C, split ratio 1:20) and flame ionization detector (FID, 240 °C) was used. The GC was equipped with a J&W DB-1 (Agilent J&W, Waldbronn, Germany) fused silica capillary column (30 m \times 0.25 mm i.d., $d_f = 0.25 \mu\text{m}$). The temperature program was from 60 to 250 at 4 °C/min. Nitrogen was used as the carrier gas, 1.5 mL/min being the flow rate. The injected volume was 1 μL ; external standard (1-decanol) was used for product purity control.

Gas Chromatography–Mass Spectrometry (HRGC-MS). An Agilent 6890 series GC with split injector (220 °C, split ratio 1:20) was directly coupled to a HP Agilent 5973 Network mass spectrometer (Agilent Technologies Inc., Palo Alto, CA). The GC was equipped with a J&W DB-Wax (Agilent J&W, Waldbronn, Germany) fused silica capillary column (30 m \times 0.25 mm i.d., $d_f = 0.25 \mu\text{m}$). The temperature program was 3 min isothermal at 50 °C, then raised at 4 °C/min to 220 °C. Helium was used as the carrier gas, 1 mL/min being the flow rate. The temperatures of the ion source and the transfer line were 220 and 200 °C, respectively. The electron energy for the EI mass spectra was 70 eV, and the injected volume was 1 μL . Identification was performed by comparison of

linear retention indices and mass spectrometric data (recorded by full scan in the EI mode) with those of authentic reference.

Gas Chromatography–Isotope Ratio Mass Spectrometry (HRGC-IRMS). A Finnigan Delta plus XL isotope ratio mass spectrometer was coupled by an open-split, via a combustion/pyrolysis (C/P) interface, to an HP 6890 gas chromatograph (GC). The GC was equipped with a J&W DB-Wax fused silica capillary column (60 m \times 0.32 mm i.d.; $d_f = 0.25 \mu\text{m}$). The following conditions were employed: 1 μL splitless injection (250 °C); temperature program, from 50 to 200 °C at 5 °C/min; helium flow, 2 mL/min.

Interfaces. For combustion to CO₂ an oxidation reactor (Al₂O₃; 0.5 mm i.d., 1.5 mm, o.d., 320 mm) with Cu/Ni/Pt metal (each 240 mm \times 0.125 mm) at 960 °C was used. Water separation was performed with a Nafion membrane. For pyrolysis to H₂ a ceramic tube (Al₂O₃, 0.5 mm i.d., 320 mm) kept at 1440 °C was employed.

Daily system stability checks were performed by measuring reference samples with known $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ ratios. Stability check of the used reference gases was carried out by measuring International Atomic Energy Agency (IAEA, Vienna, Austria) standards with defined $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ ratios (for $^{13}\text{C}/^{12}\text{C}$, IAEA-CH-7; and for $^2\text{H}/^1\text{H}$, IAEA-CH-7, NBS 22 oil, and V-SMOW).

The isotope ratios are expressed in per mil (‰) deviation relative to the V-PDB and V-SMOW international standards. For $^{13}\text{C}/^{12}\text{C}$ determinations, the mass spectrometer was calibrated against reference CO₂ gas (Messer Griesheim, Frankfurt, Germany) with a defined $\delta^{13}\text{C}_{\text{V-PDB}}$ of -24.9‰ . Results are expressed in $\delta^{13}\text{C}_{\text{V-PDB}}$ values as

$$\delta^{13}\text{C}_{\text{V-PDB}} (\text{‰}) = \left(\frac{R_{\text{sample}} - R_{\text{V-PDB}}}{R_{\text{V-PDB}}} \right) \times 1000$$

where R is the isotope ratio $^{13}\text{C}/^{12}\text{C}$.

For $^2\text{H}/^1\text{H}$ measurements the mass spectrometer was calibrated against reference H₂ gas (Messer Griesheim) with a defined $\delta^2\text{H}_{\text{V-SMOW}}$ of -255‰ . Results are expressed in $\delta^2\text{H}_{\text{V-SMOW}}$ values as

$$\delta^2\text{H}_{\text{V-SMOW}} (\text{‰}) = \left(\frac{R_{\text{sample}} - R_{\text{V-SMOW}}}{R_{\text{V-SMOW}}} \right) \times 1000$$

where R is the isotope ratio $^2\text{H}/^1\text{H}$.

In general, 6-fold determinations were carried out and standard deviations were calculated. The latter were ± 0.1 and $\pm 5\text{‰}$ for $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$, respectively. The area of linearity for the $\delta^2\text{H}_{\text{V-SMOW}}$ determinations was 1–5 μg (on column) for (\pm)-dihydroactinidiolide. Finally, the influence of sample preparation on the $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ isotope ratios checked by model LLE and LC separations was found to be within the range of standard deviation and thus negligible (data not shown).

RESULTS AND DISCUSSION

The $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values of (\pm)-dihydroactinidiolide of various origins measured by HRGC-C/P-IRMS are compiled in **Table 1**. Five categories were taken into account: (i) black tea; (ii) green tea; (iii) Rooibos tea; and (iv) synthetic and (v) enzymatically produced reference samples. The $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ ratios are correlated in **Figure 1**.

As can be seen from the data, the isotope values recorded for (\pm)-dihydroactinidiolide from black and green tea samples comprised ranges from -29.0 to -34.1‰ and from -153 to -274‰ for $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$, respectively. These ranges did not allow analytical differentiation of the various tea groups/origins. With a range from -24.4 to -27.1‰ for the $\delta^{13}\text{C}_{\text{V-PDB}}$ values of (\pm)-dihydroactinidiolide isolated from Rooibos tea, however, clear-cut distinction from the group of black and green teas was given. As to Rooibos tea, the corresponding $\delta^2\text{H}_{\text{V-SMOW}}$ values were found to be in the narrow range from -189 to -210‰ .

The differences in the $\delta^{13}\text{C}_{\text{V-PDB}}$ values of (\pm)-dihydroactinidiolide observed for tea samples and Rooibos tea are more probably due to the botany – Theaceae and Fabaceae families, respectively (both C3 plants) – than processing technology.

Table 1. $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ Values of (\pm)-Dihydroactinidiolide Obtained from Tea and Rooibos Tea Samples as well as Synthetic and Enzymatic Origin^a

	$\delta^{13}\text{C}_{\text{V-PDB}}$ (‰)	$\delta^2\text{H}_{\text{V-SMOW}}$ (‰)
black tea		
Assam, India	-31.9	-246
Assam, India	-32.3	-229
Assam, India	-31.5	-216
Assam, India	-33.0	-185
Assam, India	-33.0	-207
Darjeeling, India	-32.5	-245
Darjeeling, India	-30.8	-208
Darjeeling, India	-30.5	-181
Darjeeling, India	-31.7	-223
Darjeeling, India	-30.8	-153
Ceylon, Sri Lanka	-31.4	-187
Ceylon, Sri Lanka	-29.0	-185
Ceylon, Sri Lanka	-30.5	-206
Ceylon, Sri Lanka	-32.5	-187
Ceylon, Sri Lanka	-32.9	-194
Ceylon, Sri Lanka	-31.7	-178
Egypt	-30.1	-231
green tea		
China	-32.1	-195
China	-30.9	-204
China	-31.8	-222
China	-30.2	-188
Japan	-34.1	-274
Japan	-31.7	-259
Rooibos tea		
South Africa	-26.9	-203
South Africa	-24.4	-189
South Africa	-26.4	-210
South Africa	-27.1	-200
South Africa	-26.5	-204
South Africa	-27.1	-203
South Africa	-26.6	-210
synthetic		
from β -ionone (synthetic)	-28.1	-32
from β -carotene (natural)	-30.3	-154
laboratory sample	-27.8	-28
laboratory sample	-28.4	-30
enzymatically produced		
from β -carotene (synthetic)	-27.0	-169
from β -carotene (natural)	-31.6	-228

^a Standard deviations: ± 0.1 and $\pm 5\%$ for $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$, respectively.

Nonetheless, it would be interesting to clarify in further studies whether technological factors – traditional Rooibos as used in our study undergoes “fermentation” (37) – or the interaction of carbon and nitrogen metabolism as known from legumes (38) is also a significant factor influencing the isotope values.

The $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values of both synthetic and enzymatically produced samples of (\pm)-dihydroactinidiolide nicely reflected the influence of the origin of their educts. The data determined for the sample synthesized ex β -ionone (synthetic) were in the range of those found previously for the synthetic educt (36). According to the values measured in our study, the unknown laboratory samples have obviously been synthesized from synthetic β -ionone. As to β -carotene as educt, both $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values of (\pm)-dihydroactinidiolide formed thereof also corresponded with those recently described for the educt (35).

Despite the still limited number of samples, the C/P-IRMS application of this study shows again the power (and limits) of the technique in flavoring authentication (34, 39).

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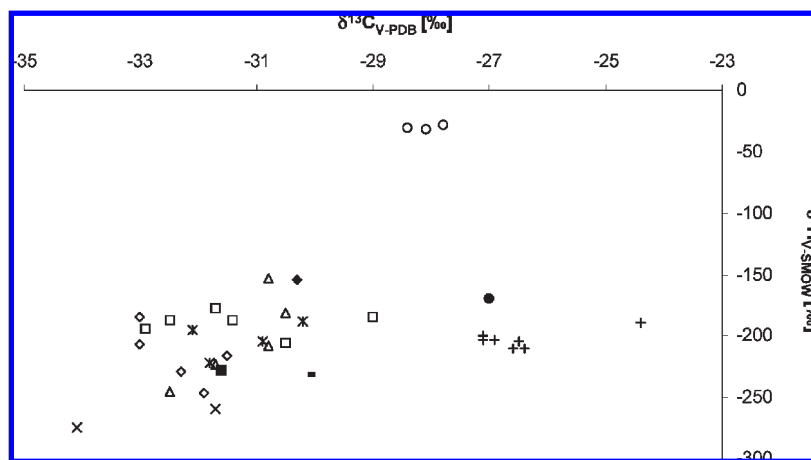


Figure 1. Correlation of $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values (‰) of (\pm)-dihydroactinidiolide after synthesis from natural (\blacklozenge) and synthetic educt (\circ), enzymatic production from synthetic (\bullet) and natural β -carotene (\blacksquare), as well as isolated from black tea (*Camellia sinensis*) [Ceylon, Sri Lanka (\square); Assam, India (\diamond); Darjeeling, India (Δ); and Egypt (-)], green tea (*C. sinensis*) [China (*) and Japan (\times)], and Rooibos tea (*Aspalathus linearis*) [South Africa (+)]. Standard deviations were ± 0.1 and $\pm 5\%$ for $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ determinations, respectively.

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