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Short communication

Biosynthesis of (−)-*ent*-kaurenoic acid in *Smallanthus sonchifolius* and its effect against microbial biofilms

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ABSTRACT

The biosynthetic pathway of (−)-*ent*-kaurenoic acid (**1**) was investigated by incorporation of 1-D-^{13}C-glucose in *Smallanthus sonchifolius* (Asteraceae) plantlets. The ^{13}C-enrichment pattern indicated that methylerythritol-4-phosphate (MEP) pathway is the biosynthetic pathway involved in diterpenoid biosynthesis. Our studies in *S. sonchifolius* reinforce that the biosynthesis of different classes of terpenes should not be compartmentalized into cytosol and plastids. Additionally, (−)-*ent*-kaurenoic acid showed antimicrobial activity against *Staphylococcus aureus* biofilm.

1. Introduction

Smallanthus sonchifolius (Poepp & Endl.) H. Robinson (Asteraceae), popularly known as yacón, biosynthesizes *ent*-kaurenoic acid (**1**) (Valentová and Ulrichová, 2003), a biologically active diterpenoid that is a key intermediate in the biosynthesis of some plant secondary metabolites, including gibberellins (Kasahara et al., 2002; Bömke and Tudzynski, 2009). Plants can synthesize all terpenoids by the mevalonate (MVA) or methylerythritol-4 phosphate (MEP) pathways operating in the cytoplasm and plastids, respectively. Thereby, two independent pathways established in separate intracellular compartments are involved in the biosynthesis of isoprene units (DMAPP/IPP) (Vranová et al., 2013). For years, triterpenoids and sesquiterpenoids were considered biosynthesized by MVA pathway, while monoterpenoids, diterpenoids, tetraterpenoids were designed exclusively MEP pathway as proposed by some authors (Vranová et al., 2012; Kirby and Keasling, 2009), but this statement can no longer hold true (Hemmerlin et al., 2012). Precursors of germacrene D in *Tanacetum vulgare* L. (Asteraceae) are synthesized by MVA pathway (Umlauf et al., 2004), while the germacrene D in *Solidago canadensis* (Asteraceae) is biosynthesized by MEP pathway (Steliopoulos et al., 2002). We recently showed that *S. sonchifolius* biosynthesizes sesquiterpene lactones by MEP pathway (Lopes et al., 2013). Therefore, MVA and MEP metabolic pathways are compartmentalized into cytosol and plastids, but not the classes of terpenoids.

So far, there is only one report in the literature about gibberellins and *ent*-kaurene precursor's biosynthesis by incorporation of ^{14}C-mevalonic acid in *Phaseolus coccineus* (Faboideae) cell-free system (Turnbull et al., 1986). Years later, studies of steviol, an *ent*-kaurene derivative from *Stevia rebaudiana* Bertoni (Asteraceae), showed that the MEP pathway is involved in the diterpenoid biosynthesis (Totté et al., 2000). Gibberellin diterpenes are biosynthesized by contribution of the MEP pathway by which the intermediate *ent*-kaurene is produced from GGPP (trans-geranylgeranyl diphosphate) and its regulation occurs in proplastids (Hedden and Thomas, 2012).

Herein, we present the (−)-*ent*-kaurenoic acid biosynthesis by incorporation of 1-^{13}C-D-glucose precursor. To the best of our knowledge, (−)-*ent*-kaurenoic acid biosynthesis by ^{13}C-precursor is being described for the first time. Additionally, the effect of (−)-*ent*-kaurenoic acid on biofilm formation against *Staphylococcus aureus* has been evaluated.

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2. Results and discussion

With the aim to elucidate the biosynthetic origin of the isoprene units in the (−)-*ent*-kaurenoic acid, we used in vitro cultures of *Smallanthus sonchifolius* (Lopes et al., 2013). The pattern of isotopic incorporation was determined by quantitative ^{13}C NMR (Fig. 2). The ^{13}C-enrichment pattern of **1** showed that the positions C-2, C-6, C-11, C-14, C-15, C-17, C-19 and C-20 (Table 1) were highly labeled after ^{13}C-glucose metabolism (3.1% up to 6.3%). These data confirm that *ent*-kaurene was biosynthesized exclusively by isoprenoid units from MEP pathway once C-positions labeled were correspondent to C-1 and C-5 from IPP (Fig. 1). The metabolic pathway of glycolysis converts 1-^{13}C-D-glucose to 1-deoxy-D-xylulose-5-phosphate (DXP), the first intermediate from MEP pathway, from pyruvate and glyceraldehyde 3-phosphate by a thiamine diphosphate-dependent synthase (Dewick, 2009; Totté et al., 2000). The condensation of dimethylallyl diphosphate (DMAPP) with three IPP units generates the geranylgeranyl pyrophosphate (GGPP). The cyclization of GGPP to *ent*-kaurene scaffold occurs by the Wagner-Meerwein rearrangement in the chloroplasts and one specific oxidation at position C-18 occurs by microsomal mono-oxygenases at endoplasmic reticulum surface (Brandle and Telmer, 2007). Interestingly, diterpenes and sesquiterpene lactones biosynthesized by *S. sonchifolius* share the same MEP biosynthetic pathway.

Ent-kaurenoic acid (**1**) has previously shown biological potential in different assays: it displayed cytotoxic and embryotoxic effects (Costa-Lotufo et al., 2002; Cavalcanti et al., 2009; Dutra et al., 2014), induced genotoxicity (Cavalcanti et al., 2006), inhibited vascular smooth muscle contractility (Ambrosio et al., 2006), demonstrated promising antinociceptive activity in inflammatory pain models (Mizokami et al., 2012), and leishmanicidal activity (Miranda et al., 2015). In addition, there are evidences that **1** exhibits antibacterial activities against *Staphylococcus aureus* (Velikova et al., 2000; Padla et al., 2012), *Bacillus cereus* (Wilkens et al., 2002), *Staphylococcus epidermidis* (Padla et al., 2012) and *Staphylococcus aureus* ATCC 6538 (Pereira et al., 2012). *Ent*-kaurenoic acid also displayed antimicrobial activity against oral pathogens (Ambrosio et al., 2008; Porto et al., 2009; Carvalho et al., 2011; Andrade et al., 2011; Moreira et al., 2016). Recently, *ent*-kaurenoic acid isolated from *Aralia continentalis* (Araliaceae) inhibited biofilm formation of *Streptococcus mutans* (Jeong et al., 2013). Considering this previous antibacterial potential, we decided to investigate the activity of **1** against Gram-positive *Staphylococcus aureus* biofilm, once it is necessary to understand the relation between bacterial biofilms and human diseases (Archer et al., 2011).

Table 1
^{13}C NMR data of (−)-*ent*-kaurenoic acid (**1**) isolated from *S. sonchifolius* after incorporation of 1-^{13}C-D-glucose into cultures ($CDCl_3$, 25 °C).

C	δ	1-^{13}C-D-glucose		
		Relative intensity of signal		ΔC
		U	L	
1	***40.7***	0.9	1.0	1.2
2	***19.1***	0.8	3.1	**4.3**
3	***37.8***	0.8	0.9	1.2
4	***43.7***	2.2	2.0	1
5	***57.1***	1.2	1.1	1
6	***21.8***	0.9	2.8	**3.4**
7	***33.1***	0.8	0.9	1.2
8	***44.2***	2.1	1.4	0.7
9	***55.1***	1.1	1.0	1
10	***39.7***[b]	2.1	2.6	1.4
11	***18.4***	0.8	2.6	**3.6**
12	***41.3***	1.0	1.1	1.2
13	***43.8***	1.6	1.4	1
14	***39.7***[b]	2.1	2.6	**1.4**
15	***48.9***	0.9	2.7	**3.3**
16[a]	***155.9***	1.4	1.4	1.1
17	***103.0***	1.0	3.3	**3.6**
18	***28.9***	1.0	1.1	1.2
19	***184.3***	0.4	2.0	**5.5**
20	***15.6***	1.1	3.2	**3.2**

L: labeling experiment with ^{13}C precursor; U: control experiments with unlabeled precursor; ΔC = 1.1% × L/U: increase in the relative intensity (significant increases in bold for enriched carbons).
[a] Used as reference.
[b] Overlapping resonance signals.

The minimum biofilm inhibitory concentration (MBIC) assay showed that the minimal concentration of 0.206 mM seems to be sufficient to inhibit 100% of *S. aureus* biofilm, whereas vancomycin at 2.760×10^{-3} mM inhibited 100% and high concentration of gentamicin at 62.814 mM inhibited 85% of *S. aureus* biofilm (Table 2). The minimum biofilm eradication concentration (MBEC) assays showed that the concentrations 0.412 mM and 0.206 mM inhibited 56% and 52% of strip biomass, respectively. Interestingly, (−)-*ent*-kaurenoic acid showed the best biomass eradication when compared to gentamicin that showed 13% biofilm eradication at 62.814 mM (Table 3), and this antibiotic is used to treat bovine subclinical mastitis (Nader et al., 2014). Regarding the eradication of bacterial population, the concentrations 0.412 mM and 0.206 mM decreased about five logarithm cycles of standard strain. These data were similar when compared to vancomycin at the concentration of 22.080×10^{-3} mM, and gentamicin at concentration higher of 62.814 mM after approximately three logarithmic cycles of the of *S. aureus* population (Table 3). In fact, our data showed that **1** exhibited a better antimicrobial action when compared to gentamicin. Biofilm represents the complex association of bacteria attached to several surfaces. The biofilm formation can increase the pathogenicity of the bacteria and protects the bacteria from external treatment (Gupta et al., 2016). Thus, searching for molecules with anti-biofilm properties can be useful to inhibit biofilm development and bacterial infectivity.

Here we report, for the first time, the biosynthesis of (−)-*ent*-kaurenoic acid by MEP pathway as well as we had already observed for sesquiterpene lactones in *S. sochifolius* (Lopes et al., 2013). Therefore, diterpenes e sesquiterpene lactones share the same terpenoid biosynthetic route in this plant. Additionally, (−)-*ent*-kaurenoic acid exhibited a better antibacterial action when compared to gentamicin.

3. Experimental

3.1. Chemicals

The labeled precursor 1-^{13}C-D-glucose, vancomycin and gentamicin were purchased from Sigma-Aldrich®.

3.2. Culture condition

The source of *S. sonchifolius* was described previously (Lopes et al., 2013). The plantlets were grown in glass tubes (8.5 cm × 2.5 cm) containing 5 mL of Murashige & Skoog culture medium, supplemented with 3% of D-glucose (w/v) and solidified with 0.2% Phytagel® (pH 6.0). Cultures were stored at 25 ± 2 °C (55–60% relative humidity with a 16-h photoperiod of 40 μmol m^{-2} s^{-1} intensity, provided by 85 W cool-white GE fluorescent lamps) and subcultured at intervals of 8 weeks until feeding experiments.

3.3. Labeled kaurenoic acid from plantlets fed with 1-^{13}C-D-glucose

Nodal segments of *S. sonchifolius* plantlets were inoculated in liquid medium supplemented with 1-^{13}C-D-glucose (3% m/v) and transferred into glass tubes (8.5 cm × 2.5 cm) containing 2.5 mL of