

Biosynthesis of Aphidicolin Proceeds via the Mevalonate Pathway in the Endophytic Fungus *Nigrospora sphaerica*

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Previamente o fungo endofítico *Nigrospora sphaerica* foi identificado como um profícuo produtor do diterpeno bioativo afidicolina. Neste artigo foi realizado um estudo a fim de se verificar as melhores condições para a produção do diterpeno em meio Czapek. A fonte de açúcar (glicose e sacarose) e o tempo de incubação (4-15 dias) foram otimizados, para posterior aplicação no estudo biossintético do diterpeno. Os níveis mais elevados da produção da afidicolina foram obtidos no 8º dia com 1% de glicose e no 12º dia com 3% de sacarose nos meios de cultura. A biossíntese da afidicolina foi estudada empregando [1-¹³C]-D-glicose como precursor, e o padrão de incorporação foi determinado através de espectroscopia de RMN de ¹³C quantitativo. A incorporação de [1-¹³C]-D-glicose mostrou que as unidades isoprênicas da afidicolina são derivadas da via do mevalonato.

We have previously identified the endophytic fungus *Nigrospora sphaerica* as a prolific producer of the bioactive diterpene aphidicolin. Herein we report a study to establish the best conditions for the production of aphidicolin by *N. sphaerica* in Czapek medium. The sugar source (glucose and sucrose) and the incubation time (4-15 days) were optimized for further application on biosynthetic studies of the diterpene. The highest levels of production of aphidicolin were found on the 8th day with 1%-glucose and on the 12th day with 3%-sucrose based media. The biosynthesis of aphidicolin was investigated using [1-¹³C]-D-glucose as a precursor, and showed that the isoprene units of aphidicolin are derived from the mevalonate pathway.

Keywords: *Nigrospora sphaerica*, aphidicolin, biosynthesis, [1-¹³C]-D-glucose, mevalonate pathway

Introduction

Terpenoids constitute one of the most diverse groups of natural products in nature. Dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) are the universal precursors in the biosynthesis of terpenoids. These precursors can be produced by two distinct biosynthetic pathways: the mevalonate pathway (MVA pathway) and methylerythritol pathway (MEP pathway).¹ The key regulatory step of the MVA pathway involves reduction of 3-hydroxy-3-methylglutaryl-CoA into mevalonic acid, catalyzed by the enzyme HMG-CoA reductase.² MVA pathway is of common occurrence among eukaryotes, including plants, fungi and yeasts, but is also present in some bacteria.³ The MEP pathway is nearly completely elucidated, and its distribution is now fairly well known.⁴ Many eubacteria, cyanobacteria,

green algae and higher plants are known to use the MEP pathway.^{5,6}

MVA and MEP pathways can be readily differentiated by the labeling pattern resulting from the incorporation of ¹³C after feeding with ¹³C-precursor into isoprenoids. Studies for the elucidation of the biosynthetic origin of terpenoid metabolites are usually based on [1-¹³C]-D-glucose as general precursor.^{7,8} Isoprenoids biosynthesized by the MVA pathway and by the MEP pathway show different enrichment pattern into isoprene units. MVA-derived terpenes will be labeled at C-2, C-4 and C-5, while MEP-derived terpenes will be labeled at C-1 and C-5.^{9,10}

The classical isotope tracer experiments still remain as an important tool to identify the origin of natural products and to infer which enzymes are involved in a particular natural product biosynthesis. A number of novel metabolic pathways have recently been identified based on the results of isotope tracer experiments.¹¹

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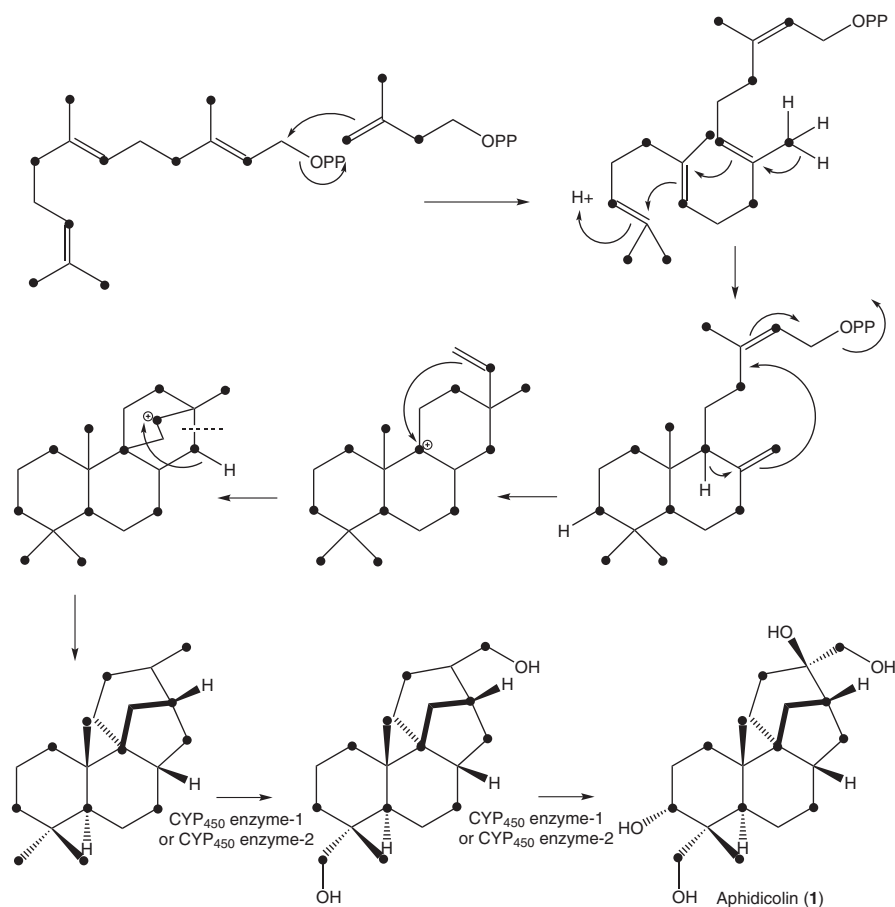


Figure 1. Biosynthesis of aphidicolin (**1**).

Recently, we reported the isolation and remarkable cytotoxic activity against cancer cell lines of the diterpene aphidicolin (**1**), produced in liquid and solid cultures by the endophytic fungus *Nigrospora sphaerica*.¹² Aphidicolin (**1**) was first isolated from *Cephalosporium aphidicola*¹³ as an antiviral agent against Herpes simplex type 1. The diterpene also showed a variety of biological activities such as antitumor, phytotoxic and specific inhibition of DNA polymerase α . This latter property makes aphidicolin a valuable tool for studies of eukaryotic DNA synthesis and cell cycles.^{14,15}

Considering the biological significance of aphidicolin and the absence of biosynthetic studies involving this diterpene, the aim of the present study was to investigate the biosynthetic pathway of aphidicolin (**1**) in the endophytic fungus *N. sphaerica*.

Experimental

Procedures

¹H and ¹³C NMR spectra were recorded in CD₃OD in a Varian INOVA 500 spectrometer. Chemical shift

values were recorded relative to the δ_{H} (4.80 ppm) and δ_{C} (49.0 ppm) signals of the predominantly deuterated solvent. The MS system used was a quadrupole time-of-flight instrument (UltrTOF-Q, Bruker Daltonics, Billerica, MA, U.S.A.), equipped with an ESI positive and negative ion source. The analyses were performed with the mass spectrometer in full scan mode. The following settings were applied for the analyses: capillary voltage 3,900 V; dry gas flow 4 L h⁻¹; nebulizer gas nitrogen. The instrumentations for HPLC analysis consisted of a Shimadzu (Kyoto, Japan) HPLC system, coupled of a LC-6AD model solvent pump, system controller SCL 10AVB, a column oven CTO-10ASVP, a Rheodyne model 7725 injector with a 20 μ L loop, a SPD-M10AVP diode array detector operating at 190 nm and a software Class VP for data acquisition.

Biological material

The endophytic fungus *N. sphaerica* SS67 was isolated from the roots of *Smallanthus sonchifolius* (Asteraceae).¹² The fungus has been maintained on potato dextrose agar slants, on mineral oil, on agar plugs in sterile water and on silica-gel (1-4 mm Synth, Brazil), stored at 4 °C at our

laboratory, under voucher number SS67. The [1-¹³C]-D-glucose (99% isotopic abundance) was purchased from Sigma-Aldrich.

Quantification of aphidicolin production

Liquid cultures were conducted in two steps. First, the slant stored fungi were inoculated in Petri dishes containing PDA medium and incubated at 30 °C for 7 days. After this period, three agar plugs (0.5 cm diameter) were cut from the *N. sphaerica* PDA cultures, inoculated in 20 mL of seed medium (2 falcon flasks of 10 mL each, supplemented with 5% of triptone, 10% of dextrose, 3% of yeast extract) and the resulting culture was incubated at 30 °C for 48 h with shaking at 120 °C rpm. After cultivation, the resulting mycelia were transferred to 180 mL of Czapek medium (flask of 500 mL) at pH 6.2, 30 °C, 120 rpm. Three different concentrations of sugar were used, 1% glucose, 3% glucose and 3% sucrose, and the cultures were incubated at the same conditions varying the time of incubation (4-16 days). Flasks with no fungal inoculation were incubated at the same conditions for control purposes. The culture broths were extracted by partition with ethyl acetate (3 × 70 mL) at the even days between the 4th and the 16th day. Crude extracts (from cultures and controls) were analyzed by HPLC. The quantification of aphidicolin was performed on a Shimadzu CLC-ODS (C-18) column 250 × 4.6 mm, 5 μ particle size protected with a 4-Pack endcapped guard column Agilent Technologies 12.5 × 4.6 mm; solvent, 35% acetonitrile-65% water (using an isocratic mobile phase; 1.0 mL min⁻¹ flow rate; detection at 190 nm). Aphidicolin was detected at 9.6 min, and the amount of aphidicolin was calculated from a calibration curve (prepared in acetonitrile at concentration 1, 2, 4, 6 and 8 mg mL⁻¹) of standard in respect of the peak area and amount.

Administration of [1-¹³C]-D-glucose to the fungal culture

N. sphaerica was inoculated onto PDA medium in Petri dishes and incubated at 30 °C for 7 days. After this period, three agar plugs (0.5 cm diameter) were cut and inoculated in 20 mL of seed medium (6 falcon flasks of 10 mL each, supplemented with 5% of triptone, 10% of dextrose, 3% of yeast extract) and the culture was incubated at 30 °C for 48 h with shaking at 120 °C rpm. The resulting mycelia (2 falcon flasks) were transferred to 180 mL of Czapek medium (total of 3 flasks of 500 mL with 180 mL of medium each) at pH 6.2, 30 °C, 120 rpm with 1% of labeled sugar ([1-¹³C]-D-glucose) and cultures were reincubated at 30 °C for 8 days at 120 rpm. Fungus in the 8th day of culture was extracted by the addition of 200 mL of ethanol 95% to the broth

(to cause the death of the microorganism), the resulting suspension was vacuum-filtered and the filtrate submitted to liquid-liquid partition with ethyl acetate (5 × 350 mL). The organic layer was evaporated under reduced pressure to produce the EtOAc crude extract (393 mg).

Extraction and isolation of ¹³C-labeled aphidicolin from N. sphaerica

The EtOAc crude extract (393 mg) was fractionated using column chromatography over silica gel (70-230 mesh; Merck, column size 8.5 × 1.5 cm) eluted with EtOAc (isocratic system) to yield five fractions. Fraction 5 (128 mg) was further purified by column chromatography over silica gel (70-230 mesh; Merck, column size 4.5 × 1.0 cm) eluted with EtOAc (isocratic system) to yield enriched aphidicolin (43 mg).

Evaluation of the isotopic abundance

The ¹³C NMR spectra of samples of **1** obtained from ¹³C at natural abundance and **1**-¹³C-labeled obtained from incorporation experiments involving [1-¹³C]-D-glucose as precursor, respectively, were measured under identical experimental conditions. ¹³C NMR spectra were acquired using NS = 65536. The Spin Works 3.0 version 3.1.2.0 was the software used to process the spectra obtained by measuring the integral of the carbons. The relative ¹³C-isotopic abundances of individual carbon atoms in **1** and **1**-¹³C-labeled were calculated from the integrals obtained from the labeled sample in comparison with those of the natural abundance sample (Table 1). The values were normalised with reference to an abundance value of 1.1% for the carbon with the lowest possible ¹³C enrichment.

Results and Discussion

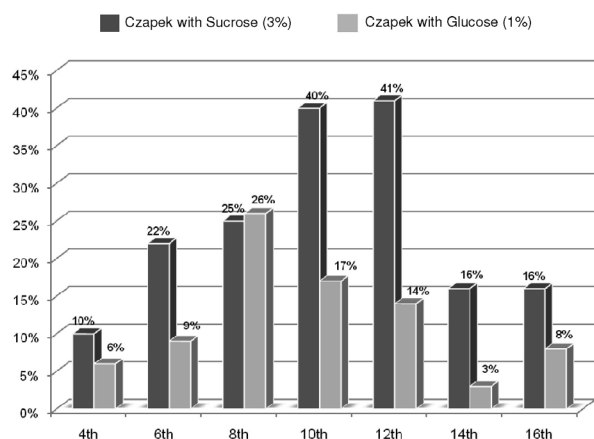
Two different pathways are known for the formation of IPP: the well-known acetyl-CoA/MVA route and MEP pathway starting from glyceraldehyde 3-phosphate and pyruvate. They can be clearly distinguished by incorporation of a ¹³C-labeled carbon source such as glucose (especially when they are utilized as single carbon and energy source) and examination of the ¹³C NMR spectra of the analyzed isoprenoids.

In order to elucidate the biosynthetic origin of the isoprene units in the diterpene aphidicolin, we performed preliminary studies to verify the best conditions for the production of **1** in liquid medium (Czapek) at pH 6.3, 30 °C, 120 rpm with unlabeled sugars. The Czapek medium was supplemented with 3% of sucrose in the commercial composition. Three

Table 1. ^{13}C NMR data (125 MHz, CD_3OD) and isotopic abundance (%) of aphidicolin $1\text{-}^{13}\text{C}$ -labeled

Carbons	δ (ppm) ^a	Relative enrichment (%) ^b
C-1	27.9	9.37
C-2	27.5	-
C-3	77.3	7.72
C-4	41.4	-
C-5	34.8	8.95
C-6	24.2	0.15
C-7	28.8	10.25
C-8	41.3	-
C-9	50.3	6.45
C-10	40.8	-
C-11	33.7	0.53
C-12	42.3	7.43
C-13	32.4	7.84
C-14	25.7	0.32
C-15	27.7	8.62
C-16	75.6	-
C-17	68.8	6.58
C-18	72.1	7.43
C-19	18.0	8.48
C-20	15.6	8.05

^a Referenced to CD_3OD ; ^b Calculated by comparison of integrals of signals in the ^{13}C NMR spectrum of $1\text{-}^{13}\text{C}$ -labeled, in comparison with integrals of signals in the ^{13}C NMR spectrum of **1** with ^{13}C at natural abundance.

**Figure 2.** The optimization of production of **1** on the 12th day with sucrose medium (3%), and 8th day with glucose medium (1%).

different experiments have been carried out, using 3% sucrose, 3% glucose and 1% glucose in the Czapek medium. The highest level of production of **1** (Figure 2) was found on the 12th day with 3% sucrose based medium exhibiting a yield of 41% (m/m), followed by the 8th day with 1% glucose based medium exhibiting a yield of 26% (m/m). The production

of **1** was not observed in glucose based medium (3%). It is well known that high concentrations of glucose may inhibit the fungal secondary metabolism.¹⁶ Once determined the best conditions for the production of **1** with 1% glucose based medium, *N. sphaerica* was cultivated with $[1\text{-}^{13}\text{C}]\text{-D-glucose}$, a sugar source of ^{13}C -enrichment.

The aphidicolin labeling pattern was determined by quantitative ^{13}C NMR spectroscopy analysis (Figure 3). Unequivocal ^1H and ^{13}C NMR chemical shifts assignments for aphidicolin were based on the previous data¹²⁻¹³ and obtained HMQC and HMBC experiments. The ^{13}C NMR spectrum obtained for $1\text{-}^{13}\text{C}$ -labeled showed that significant intensity increase occurred for C-1, C-3, C-5, C-7, C-9, C-12, C-13, C-15, C-17, C-18, C-19 and C-20 signals (Table 1). Analysis of integrals showed relative enrichment higher than 6% for all the enriched carbon signals. A retrobiosynthetic analysis allowed us to establish that C-2, C-4 and C-5 of the isoprene precursor were labeled. This labeling pattern is consistent with the operation of the MVA pathway in the biosynthesis of aphidicolin by *N. sphaerica*.

It had already been proposed in the literature that the aphidicolane skeleton of aphidicolin is the result of further cyclization of a $9\beta\text{-labdadienyl}$ system with additional rearrangement as shown in Figure 1. The pathway also involves sequential hydroxylation of aphidicolin-16 β -ol at C-18, C-3 α and C-17, and this oxidation sequence has been established earlier.^{17,18} However, the number of cytochrome P_{450} enzymes is inconsistent with the three necessary hydroxylation reactions in the biosynthesis of aphidicolin. It is possible that one of the two cytochrome P_{450} enzymes may catalyze more than one hydroxylation reaction, as previously found for the gibberellin biosynthesis.¹⁹ Mass spectrometric based methods have been successfully used as powerful metabolite profiling tools for evaluating metabolic pathways.²⁰ The positive ion, high resolution electron spray ionization mass spectra, (HRESIMS) of $1\text{-}^{13}\text{C}$ -labeled in comparison with the mass spectrum of **1** showed amounts of $[\text{M} + \text{Na} + 1]^+$ ions m/z 362.2326, $[\text{M} + \text{Na} + 2]^+$ ions m/z 363.2358, $[\text{M} + \text{Na} + 3]^+$ ions m/z 364.2386 and $[\text{M} + \text{Na} + 4]^+$ ions m/z 365.2413 that were higher than those of the natural abundance $[\text{M} + \text{Na}]^+$ ions m/z 361.2349 (Figure S1), indicating that $[1\text{-}^{13}\text{C}]\text{-D-glucose}$ was incorporated. This result confirms that the endophytic fungus *N. sphaerica* biosynthesized aphidicolin by the mevalonate pathway.

Conclusions

Our results using ^{13}C -labeled glucose confirmed that *N. sphaerica* produced aphidicolin via the MVA pathway. These results are in agreement with the literature that suggests that

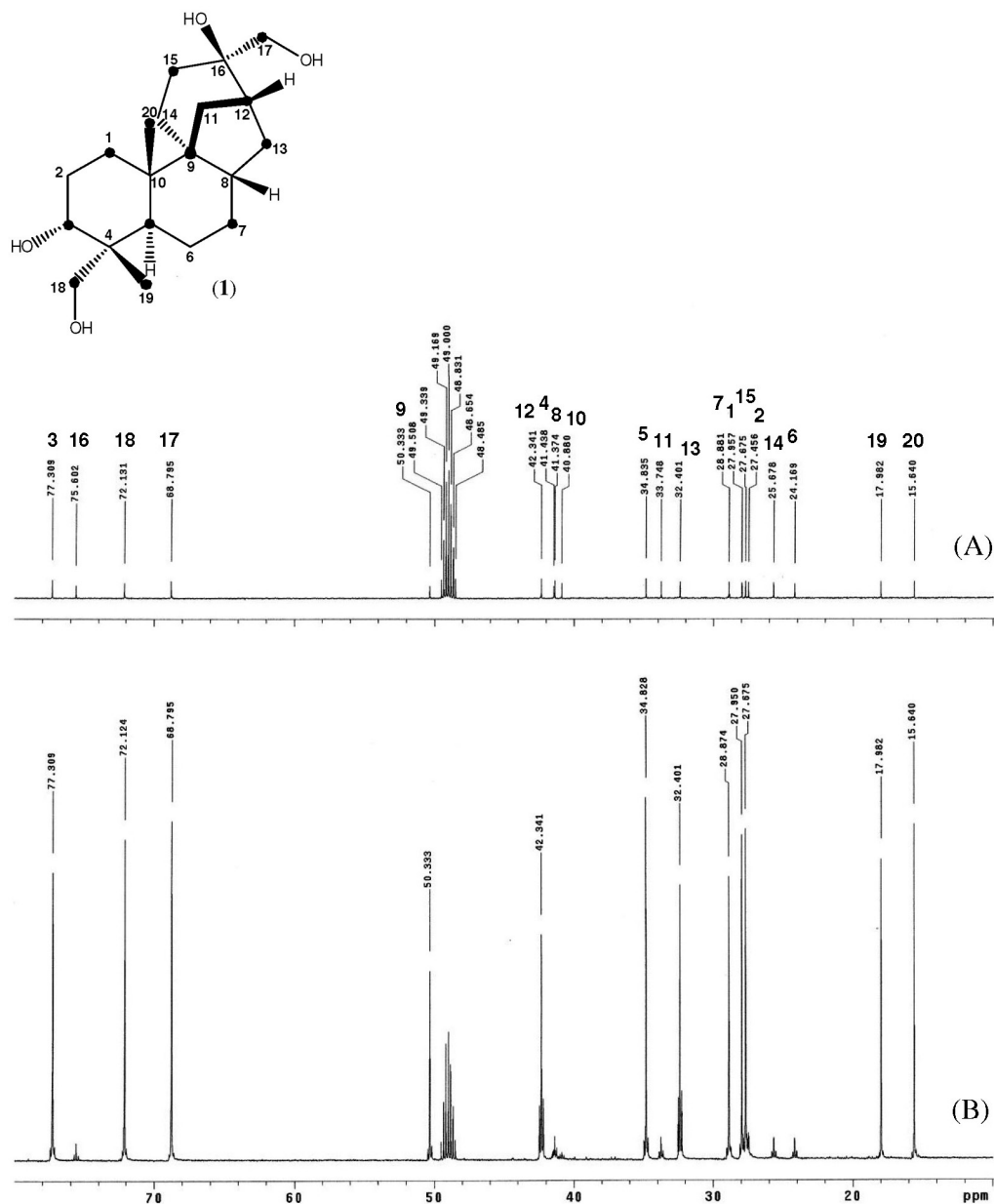


Figure 3. ¹³C NMR (125 MHz, CD₃OD) spectra of 1 with natural ¹³C-isotopic abundance (A); and 1 after *N. sphaerica* was fed with [1-¹³C]-D-glucose (B).

majority of fungi and yeasts biosynthesized metabolites through MVA pathway from a single acetyl-CoA,²¹ and therefore most probably do not present compartmentation of two different routes (MVA and MEP pathways) to IPP as observed in higher plants. There are few described examples of natural product biosynthesis experiments based on isotopic tracer experiments with endophytic fungi. Recently, biosynthetic studies with a Brazilian endophytic strain showed incorporation of two units of amino acids on brasiliamide structures.²² In addition, to the best of our knowledge this is the first report of an experimental evidence for the biosynthetic pathway involved in the formation of the bioactive diterpene aphidicolin.

Supplementary Information

Supplementary data are available free of charge at <http://jbcs.sbq.org.br>, as PDF file.

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