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Full Length Research Paper

Feeding of L-phenylalanine as precursor enhances strobilurin A biosynthesis in the basidiomycte, *Strobilurus tenacellus*

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In this study, the effect of different concentrations of L-phenylalanine (0, 0.10, 0.25, 0.50, 1 and 2 mM) was used as a precursor to enhance antifungal natural product strobilurin A, which was investigated in the fungus *Strobilurus tenacellus*. The putative precursor was added to the fungal shake flask culture and the concentration of strobilurin A was quantified by liquid chromatography and mass spectroscopy (LCMS). The concentrations of the feeding flasks were compared with the control culture under similar conditions of fermentation. Highest yield (2.5 times higher than control) was observed in the flasks fed with 1 mM of L-phenylalanine. This supports the hypothesis that L-phenylalanine is the putative precursor of strobilurin A and during biosynthesis probably, the aromatic part of the compound is derived from this precursor.

Key words: Strobilurin A, *Strobilurus tenacellus*, phenylalanine precursor.

INTRODUCTION

Strobilurins are important fungal metabolites mainly produced by basidiomycetes (Sauter et al., 1999). They are strong antifungal compounds inhibiting mitochondrial electron transport chain at the site of bc1-complex of cytochromes of the respiratory chain (Becker et al., 1981). So far, more than 20 analogues of natural strobilurins have been isolated from various species of basidiomycetes including *Strobilurus tenacellus*, *Oudemans iellamucida*, *Pterula* sp., *Xerula longipes*, *Mycena crocota*, *Favolaschia calocera,* as well as Ascomycete, the *Bolinea lutea* (Malita, 2008). Strobilurin A1 is the parent member of the strobilurin family and it is hypothesized that other strobilurin homologues are its derivatives obtained during biosynthesis (Fredenhagen et al., 1990).

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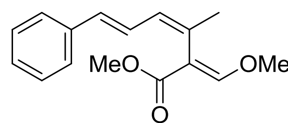

strobilurin A **1**

Previous classical labelling studies carried out by Nerud et al. (1982) on strobilurin A1 produced by *Oudemansiella mucida* suggested that the aromatic part in strobilurin A1 is derived from benzoic acid, while the aliphatic portion is acetate based and polyketide in nature. During the course of isotopic labelling studies, they also observed that all the three methyl groups are from methionine.

The aromatic metabolite benzoic acid is an important structural element in a number of natural products obtained from plants, fungi and bacteria. In eukaryotes, benzoate is a component of taxol 2 (Baloglu and Kingston, 1999), cocain 3 (Bjorklund and Leete, 1992),

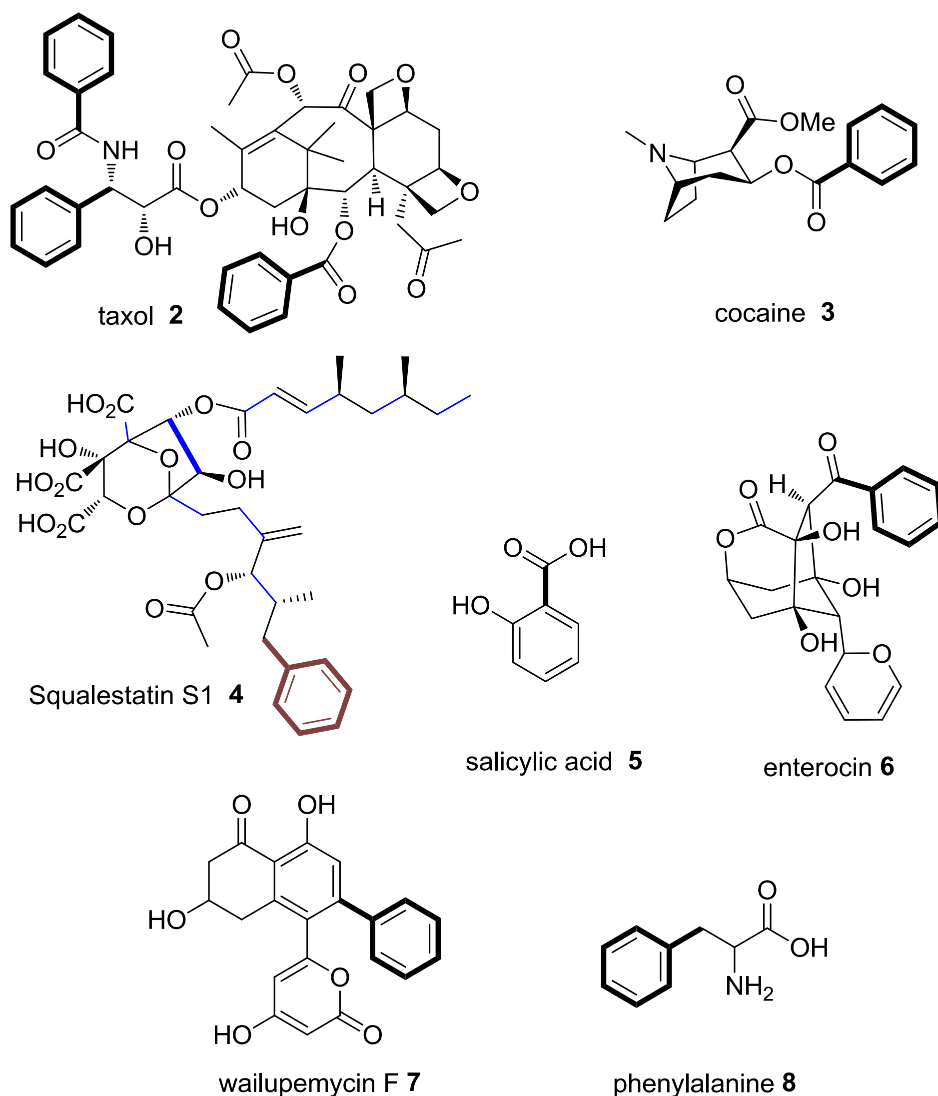

Figure 1. Various natural products with benzoate as important part of the structure.

squalestatin 4 (Cox et al., 2005) and salicylic acid 5, (Ribnicky et al., 1998). Benzoic acid is also an important part of bacterial metabolites such as enterocin 6, (Hertweck and Moore, 2000) and Wailupemycin 7 (Kalaitzis et al., 2009). It is hypothesized that the benzoic acid originates from various organisms from shikimate pathway *via* phenylalanine 8 (Stocker-Worgotter, 2008), all the natural products mentioned above are highlighted in Figure 1.

We assumed that the strobilurin A benzoate might also be from phenylalanine 8. To get insight into this, the present study was planned to assess the effect of adding phenylalanine 8 into the shake flask culture of strobilurin A1 produced by *S. tenacellus*. Enhancement of the yield with feeding of phenylalanine 8 is anticipated, which support the idea that the benzoate part of 1 strobilurin A is derived from phenylalanine 8.

MATERIALS AND METHODS

S. tenacellus culture used in this study was purchased from CBS-KNAW, Fungal Diversity Centre, Netherland. It was received on malt extract agar (MEA) medium in a glass ampule. For maintenance, it was transferred to pre-sterilized agar plate on MEA medium (50 gL^{-1} water). For the production of mycelial biomass of *S. tenacellus*, the modified method of Anke et al. (1977) was followed*.* The medium was autoclaved prior to inoculation of the culture. The agar plate cultures were prepared and good growth of the strain on the agar plates was observed. From the agar plate culture, the mycelia was transferred to a flask containing M2 medium (10 g malt extract, 4 g yeast extract, 4 g glucose L^{-1}) as

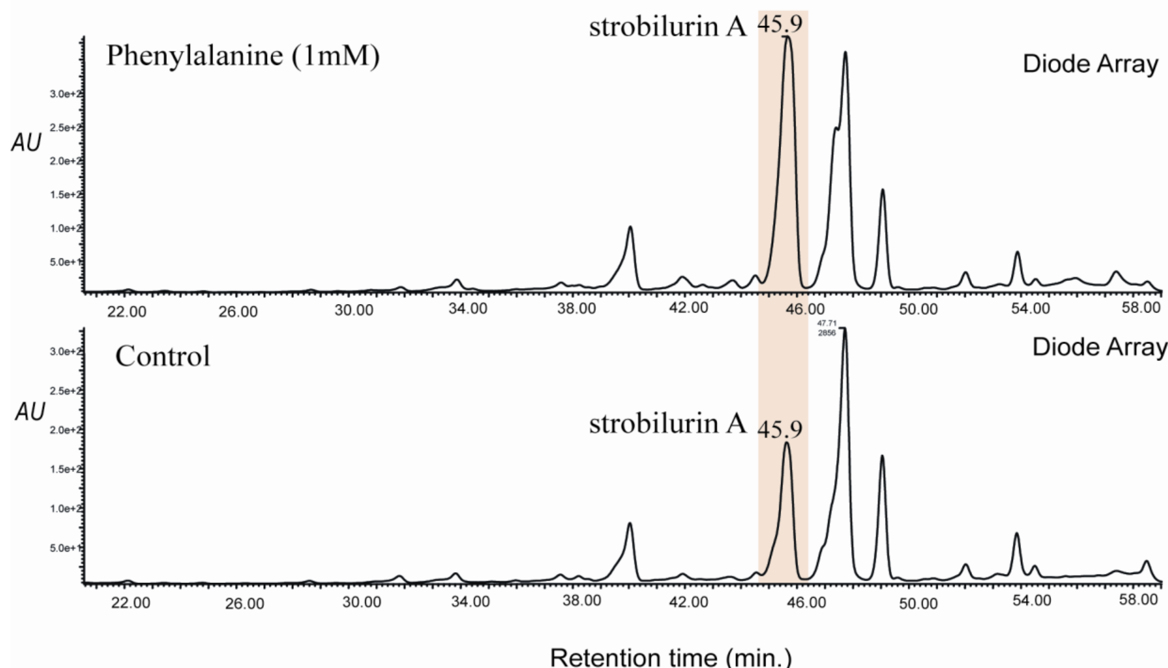

Figure 2. 2.5 times higher yield enhancement of strobilurin A observed with 1 mM of phenylalanine administered to the shake flask culture of *S. tenacellus.*

seed culture and incubated for 5 days at 25°C and 150 rpm. Production culture in the MEA medium (150 ml) in 500 ml conical flask was inoculated with the seed culture (10 ml) and grown at 25°C and 150 rpm. After 8 days of incubation and shaking, the flask containing the liquid culture was collected, filtered under vacuum and washed several times with distilled water. The wet cells were extracted with MeOH/acetone (2:1) and then with MeOH. The combined extracts were evaporated to remove most of the acetone and the metabolites were extracted with 100 ml $CHCl_3$ from the residue. The organic extract was dried over anhydrous $MgSO_4$, filtered and evaporated in vacuum, to produce dark brown oil. The crude extract solution (10 mg mL^{-1} in MeOH) was first centrifuged to remove solids and then subjected to LCMS analysis.

The proposed precursor, phenylalanine 8 was supplied as a pulse feed on days 2, 3 and 4 of cultivation as DMSO solution. Controls in parallel were run with each experiment for systematic comparison. After fermentation for 8 days, the flasks were collected for extraction of strobilurin A1.

The crude extract method was used to quantify strobilurin A1 using HPLC integration method. The elution time was determined by using the standard sample of strobilurin A1. For quantification, a series of known concentrations of strobilurin A1 (0.03 to 1 mg mL^{-1} in MeOH) were detected with diode array detector (200to 400 nm) by LC. Calibration curve was plotted with area under HPLC peaks *vs*. a series of standard of known strobilurin A 1 concentrations (Figure 1).

Control DMSO was administered on the same days to monitor any effect shown by DMSO on the fungal cells and strobilurin A1 production. Whole cell culture flasks without feeding with phenylalanine 8 were also kept for comparison. Control flasks (DMSO fed and only culture) and phenylalanine 8 fed flasks were collected at the eighth day of fermentation on shaker at room temperature. The cultures were extracted following the above standard protocol. Wet filtered mycelia and crude extract were weighed for comparison. Crude extracts were then dissolved in 5 ml HPLC grade methanol and 20 µl of the solution was subjected to LCMS analysis to quantify the strobilurin A1 concentration to evaluate metabolite profile of the feeding flasks and the control.

RESULTS AND DISCUSSION

In this project, we investigated the effect of different concentration of L-phenylalanine 8 on production of strobilurin A1. The feeding experiments were performed in triplicate. Strobilurin A1 produced by *S. tenacellus* was analyzed by LCMS and quantified in comparison with the standard calibration curve of Strobilurin A1. It was observed that with the increasing concentration of phenylalanine 8, the yield of strobilurin A1 increased reciprocally up to 1 mM. The highest yield of strobilurin A, (2.5 times higher than control) was obtained when the feeding substrate concentration was 1 mM (Figure 2). Phenylalanine 8, fed in higher concentration (2 mM), had some toxic effects on the cells growth and consequently strobilurin A1 production.

This study shows that *S. tenacellus* enzymatic machinery is capable of incorporating phenylalanine in strobilurin A1 biosynthesis. Pulse feeding of the precursor significantly increased the production of strobilurin A1. DMSO as control had no variation in cell growth and strobilurin A yield. Phenylalanine effect on yield was

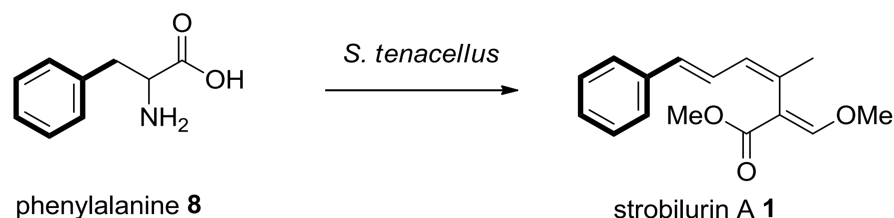

Figure 3. Incorporation of phenylalanine 8 in the structure of strobilurin A1.

interestingly very high when cell culture was extracted on day 8, in the case of strobilurin.

Various studies conducted previously have suggested that phenylalanine is a precursor during biosynthesis of benzoic acid in plants, fungi and bacteria (Herrmann and Weaver, 1999). It is proposed that phenylalanine is first converted to phenylalanine ammonia lyase (PAL), a pathway enzyme ubiquitous in plants and certain fungi (Camm and Towers, 1969). Cinnamic acid is believed to be converted to cinnamoyl CoA by a hypothetical enzyme, acyl CoA ligase and then the cinnamoyl CoA is broken down to benzoyl CoA in plant like β-oxidation pathways (Hertweck and Moore, 2000).

We opined that during strobilurin A1 biosynthesis, the benzoate part is likely a starter unit for the specific enzymes to accomplish biosynthesis as shown in Figure 3.

The yield enhancement of strobilurin A1 as depicted in Figure 2 by feeding phenylalanine to the culture of *S. tenacellus* suggested that benzoate in strobilurin A1 originated from shikimate pathway and is gotten from the aromatic amino acid, phenylalanine 8. It is assumed that phenylalanine may follow the PAL route in strobilurin A biosynthesis and *via* cinnamate, which is broken down to benzoate. Further feeding studies of labelled precursors are needed to prove this hypothesis.

Conclusion

It is concluded that the benzoate part of the important antifungal metabolite, strobilurin A1 during biosynthesis most likely originated from the shikimate pathway. Phenylalanine is the precursor during biosynthesis of strobilurin A and probably it forms benzoate *via* cinnamate followed by β-oxidative pathway. Phenylalanine administration to the culture of *S. tenacellus* enhances the yield by 2.5 times, so the substrate can be utilized for higher production of strobilurin A1 in shake flask cultures.

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