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Selective Production of Staplabin and SMTPs in Cultures of *Stachybotrys microspora* Fed with Precursor Amines

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Staplabin and SMTPs, a family of triprenyl phenol metabolites of *Stachybotrys microspora*, enhance fibrinolysis by modulating plasminogen conformation to increase its susceptibility to activation by plasminogen activators. We found that the production of these metabolites were markedly elevated by feeding the microbial culture with an amino acid or an amino alcohol that is a partial molecular constituent of the compound. Thus, the addition of 5-aminovaleric acid, 2-aminoethanol, Ser, Phe, Leu, Trp, Orn and Lys at 100 mg/ml resulted in 7- to 45-fold increases in the production of staplabin, SMTP-1, -3, -4, -5, -6, -7 and -8, respectively. Although the feeding at day 0 to 3 of culture supported the selective production, the supplementation after 5 days had little or no effect. When non-constituent amino acids were supplemented to cultures, production of hitherto uncharacterized congeners was observed.

The plasminogen/plasmin system is involved in a variety of physiological and pathological conditions including fibrinolysis, inflammation, tissue remodeling, ovulation, tumor metastasis and tissue invasion of pathogens[1~4]. In this system, the zymogen, plasminogen, is proteolytically activated to plasmin by plasminogen activators, such as urokinase-type and tissue-type plasminogen activators. The conformational status of plasminogen plays an important role in local propagation of plasminogen activation[5~9], and modulation of plasminogen conformation may be an attractive means to regulate physiological activity of the plasminogen/plasmin system.

Staplabin is a first low molecular weight compound to be discovered that enhances both plasminogen-fibrin binding and activation of plasminogen by relaxing plasminogen conformation[10,11]. The fungus *Stachybotrys microspora* produces a variety of staplabin analogs designated SMTPs[12~14], some of which are two to ten times more potent than staplabin. However, the level of production of such metabolites is relatively low, and the presence in culture of numerous minor congeners hampered the purification of a compound of interest. Staplabin and SMTPs have in their molecule in common a chromanlactam with 4,8-dimethyl-3,7-nonadienyl group. Each compound differs in an amino acid or an amino alcohol side chain attached to the nitrogen atom of the lactam moiety. In this study, we have attempted to produce selectively a compound of staplabin family by supplementing the microbial cultures with precursor amines, as these metabolites seems to be synthesized by incorporating amino acids or amino alcohol. We show that the production of a metabolite of interest is markedly elevated by this method. Moreover, this strategy provides a means to produce hitherto uncharacterized congeners.

Materials and Methods

Strains and Media

S. microspora IFO 30018 was obtained from the Institute for Fermentation, Osaka. The microorganism was subcultured on potato glucose agar slants. For the seed cultures, medium (pH 5.8) containing the followings was used: 30 g glucose, 10 g soybean meal, 3 g peptone, 3 g meat extract, 3 g yeast extract, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.1 g CB442 (an antifoam; Nippon Oil & Fat Co, Japan) in 1 liter of reverse osmotic water. The production medium (pH 5.5) contained 20 g glucose, 5 g

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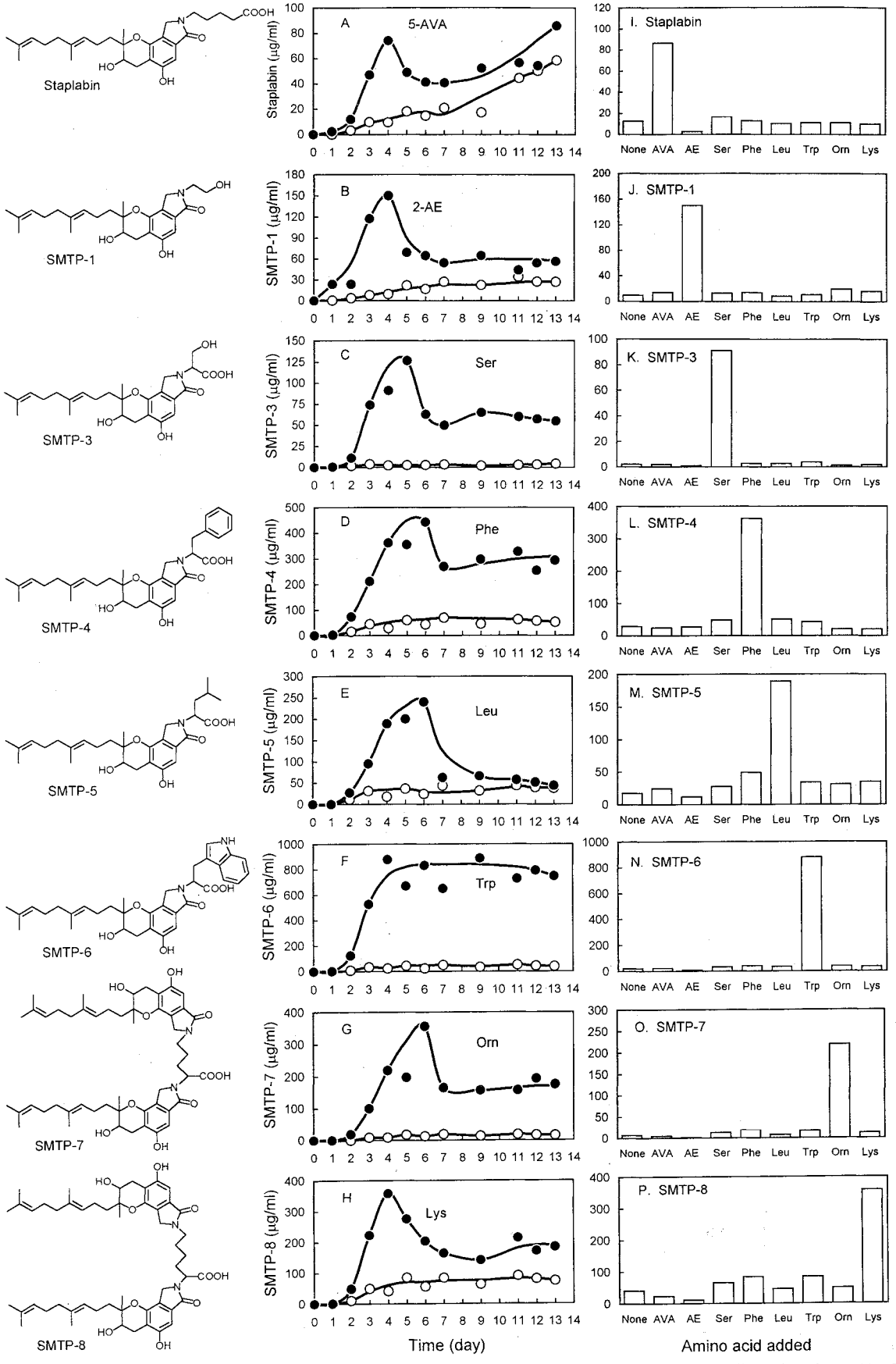
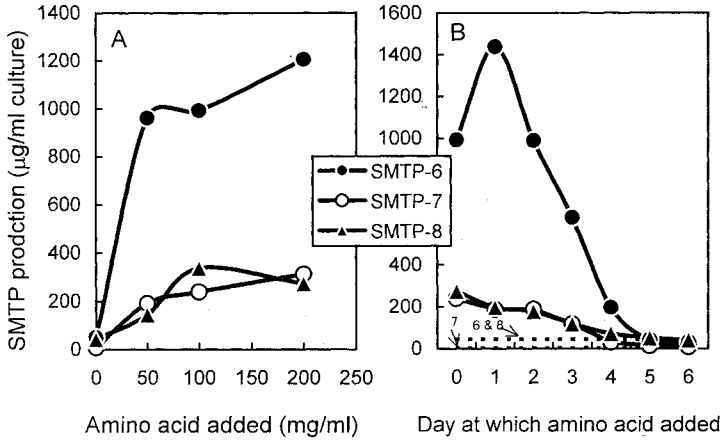
Fig. 1. Selective production of staplabin and SMTPs in *S. microspora* cultures supplemented with amino acid and amino alcohol.



S. microspora was grown in medium supplemented at day 0 with 100 mg/ml of the indicated amines. (A~H) After cultivation for indicated time, the levels of staplabin and SMTPs were determined with control culture (○) and amine-fed cultures (●). (I~P) Each culture at day 4 was analyzed for the levels of staplabin and SMTPs. The structures of staplabin and SMTPs are shown on the left.

Fig. 2. Effects of dose and timing of amino acid feeding on the production of SMTPs.



(A) *S. microspora* was grown in medium supplemented at day 0 with the indicated concentrations of Trp, Orn or Lys, and the levels of SMTP-6, -7 and -8, respectively, were determined at day 4.

(B) Trp, Orn or Lys (100 mg/ml) was added to cultures at the indicated time, and the levels of SMTP-6, -7 and -8, respectively, were determined at day 13. Dashed lines show the levels of the three metabolites in control cultures.

peptone, 3 g yeast extract, 3 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$ and 0.1 g CB442 in 1 liter of reverse osmotic water.

Production of Triprenyl Phenols

A loopful of a slant culture of *S. microspora* was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium. The flask was incubated at 25°C for 4 days on a rotary shaker at 180 rpm. A 1 ml portion of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of production medium, and the flask was incubated as above for up to 14 days. Where indicated, amino acid or amino alcohol was added to cultures at the day shown. Unless otherwise noted, the concentration of an amine was 100 mg/ml.

HPLC Analysis

One milliliter of microbial culture was extracted thrice with 2.5 ml of methyethlyketone, and the organic extracts were combined, dried over sodium sulfate and concentrated to dryness. The resulting residue was dissolved in 100 μl of MeOH. A portion of the extract was analyzed for the contents of staplabin and SMTPs as follows using authentic samples as standards. An Inertsil PREP-ODS column (6×250 mm; GL Sciences, Tokyo, Japan) was developed at a rate of 1 ml/minute at 40°C with a gradient of 60% MeOH in 50 mM ammonium acetate, aq as follows: MeOH concentration was kept at 60% for initial 55 minutes, followed by linearly increasing MeOH concentration to 88% for next 15 minutes, and finally increasing MeOH concentration to 100% for further 13 minutes. For determination of SMTP-7 and -8, the column was also developed with isocratic 80% MeOH in 50 mM ammonium acetate, aq. The elution was monitored at 200~350 nm using a Hitachi 7450 photodiode array detector. Calculation of the content of metabolite was made by comparing peak area or peak height of interest with that of authentic compounds. The identification of metabolites was performed by co-chromatography with authentic samples and, for most metabolites, by mass and NMR spectrometry.

Results and Discussion

The accumulation of staplabin and SMTPs in the *S. microspora* culture with normal medium gradually increased after 2~3 days of cultivation and reached 3~80 μg/ml at day 5 to 13 (Fig. 1A~H). The feeding of 5-aminovaleric acid (5-AVA) at day 0 resulted in a transient increase in the production of staplabin (Fig. 1A). Similarly, the supplementation with 2-aminoethanol (2-AE), Ser, Phe, Leu, Trp, Orn and Lys gave rise to marked augmentation of the production of SMTP-1, -3, -4, -5, -6, -7 and -8, respectively (Fig. 2B~H). The peak of accumulation of these metabolites was observed at day 3~6, and the amount

Table 1. Prominent peaks in the HPLC analyses of *S. microspora* cultures supplemented with various amines.

Amine added	Retention time* (minute)	Peak area** (V x s)	Area of corresponding peak in control** (V x s)	Fold increase as compared with control	Identification
5-AVA	48.2	1.45	0.21	7	Staplabin
2-AE	54.9	3.36	0.21	16	SMTP-1
Gly	35.9	13.93	0.25	56	
L-Ala	38.3	4.94	0.15	33	
L-Val	47.0	3.80	0.06	63	
D-Val	62.7	9.86	< 0.05	-	
L-Leu	58.0	6.43	0.59	11	SMTP-5
D-Leu	70.1	18.36	< 0.05	-	
L-Ile	59.5	15.13	0.16	95	
L-Phe	58.1	18.13	1.42	13	SMTP-4
D-Phe	66.5	21.52	< 0.05	-	
L-Trp	50.3	37.89	0.84	45	SMTP-6
D-Trp	56.7	31.05	< 0.05	-	
L-Tyr	31.2	4.74	0.06	79	
D-Tyr	34.6	5.47	< 0.05	-	
L-Ser	25.0	2.71	0.06	45	SMTP-3
D-Ser	29.5	4.83	< 0.05	-	
L-Thr	28.2	2.75	0.11	26	
L-His	24.9	1.83	< 0.05	-	
	75.3	3.20	0.36	9	
	76.1	2.38	< 0.05	-	
	77.1	4.25	0.34	13	
	81.5	12.33	0.29	42	
L-Asp	17.0	1.17	0.09	13	
L-Arg	26.3	6.37	< 0.05	-	
D-Arg	26.6	5.21	< 0.05	-	
L-Orn	77.8	29.52	0.90	33	SMTP-7
D-Orn	78.3	15.99	< 0.05	-	
L-Lys	79.0	23.66	2.65	9	SMTP-8
D-Lys	79.8	24.14	< 0.05	-	

* Retention times shown are from analyses using a gradient elution program as described in *Materials and Methods*.
** Extracts from 4-day cultures were analyzed for the level of accumulation of staplabin congeners. The peak area is calculated by multiplying signal intensity (volt) by time (second).

was reduced afterward. In contrast, the maximum accumulation of triprenyl phenols in control culture required 5~13 days. Fig. 1I~P shows the amount of the 8 metabolites in control and precursor amine-fed cultures at day 4. From these results, it was evident that the supplementation of an amino alcohol or amino acid resulted in specific accumulation in culture of a particular metabolite that has an added precursor in respective molecule. A marked enrichment of SMTP-6, for example, was achieved by Trp-fed culture. The levels of the 8 metabolites in respective precursor amine-fed culture were 7- to 45-fold higher than those in control culture, while the production of unrelated metabolites unchanged (Fig. 1).

Next, effects of dose and timing of precursor amine feeding on the product accumulation were investigated. As shown in Fig. 2A, specific accumulation of a metabolite of interest was achieved at a dose of 50 mg/ml, while the level was slightly increased at higher doses. A specific accumulation of SMTP-6, -7 and -8 was observed when Trp, Orn and Lys, respectively, were supplemented at day 0~3 of culture, whereas the levels were comparable to those in control culture when addition was made after

day 5 (Fig. 2B). These results suggest that microbial accumulation of another precursor, possibly an acceptor of amine, is a limiting factor for the production of SMTPs.

When amino acids that are not occurring in the structure of previously isolated SMTPs were added to the *S. microspora* culture, we observed significant increases in the accumulation of unidentified metabolites with UV spectra similar to SMTPs (Table 1). For example, the supplementation with Val, Ile or Tyr resulted in >60-fold increase in the product appearing at a retention time of 47.0, 59.5 or 31.2 minutes, respectively, under the analytical conditions employed. In addition, the addition of D enantiomers of Ser, Phe, Leu, Trp, Orn and Lys gave rise to the production of compounds that were distinct from metabolites enriched in respective L enantiomer-fed cultures.

In the present study, we found that supplementing *S. microspora* cultures with amino acids and amino alcohols markedly elevated the production of staplabin and SMTPs. The production was selective with regard to amine added. Thus, the major product had an added amino acid or amino alcohol as a structural moiety. The production of unrelated metabolites were unchanged. These results suggest that the formation of staplabin and SMTPs in such conditions involves direct incorporation of an amine added to culture. This notion is supported by the fact that a large amount of hitherto unknown metabolites were accumulated when D-amino acids such as D-Trp, D-Orn and D-Lys were supplemented to a culture. Similarly, when amino acids that are not occurring in the structure of the previously isolated SMTPs were added, significant accumulation of uncharacterized congeners was observed.

In conclusion, the precursor amine-fed culture should make it possible to selectively produce a variety of staplabin analogs including new congeners. Specific accumulation of a metabolite of interest may aid the isolation of such a compound.

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