BIOSYNTHESIS OF ASTROMICIN\textsuperscript{t} AND RELATED ANTIBIOTICS

II. BIOSYNTHETIC STUDIES WITH BLOCKED MUTANTS OF \textit{MICROMONOSPORA OLIVASTEROSPORA}

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An inosamine-idiotrophic mutant, KY11559, which produced no astromicin unless scyllo-inosamine was added to the fermentation medium, was isolated from \textit{Micromonospora olivasterospora}. Biotransformation studies were performed with resting cells of this mutant and compounds assumed to be precursors of 1,4-diaminocyclitol (fortamine). \textit{Scylo}-inosose, \textit{scylo}-inosamine and FU-10 were converted to astromicin.

A number of mutants blocked in the biosynthesis of astromicin were developed from \textit{M. olivasterospora}, and the intermediates accumulated by these mutants were isolated and identified. Twenty-five blocked mutants were classified into 10 groups, based on their complementation patterns by cosynthesis experiments. Further, utilizing these blocked mutants and the isolated compounds, biotransformation analyses were performed.

The results showed that the amination at position 4 in fortamine occurred after formation of the pseudodisaccharide. Subsequently, the aminosugar and aminocyclitol moieties were aminated, methylated, dehydroxylated, epimerized and acylated to produce astromicin. Thus it was demonstrated that the astromicin biosynthetic pathway has a unique feature which is not found in the biosynthesis of other aminoglycoside antibiotics.

Astromicin (ASTM) is an aminoglycoside antibiotic produced by \textit{Micromonospora olivasterospora}\textsuperscript{1-3} (Fig. 1). ASTM has a broad antibacterial spectrum covering Gram-negative and -positive bacteria and is effective against many aminoglycoside-resistant bacteria\textsuperscript{4}. Further, this antibiotic is characterized by markedly low ototoxicity\textsuperscript{5} and nephrotoxicity\textsuperscript{6} compared with other aminoglycosides.

In antibiotic production, many minor metabolites structurally related to the major one are generally co-produced. In the ASTM fermentation, the number of minor metabolites, the structures of which have so far been determined, amounts to about 50. The authors were able to assume a part of the biosynthetic pathway for ASTM on the basis of biotransformation experiments with the metabolites isolated from mutant strains, as described in the previous report\textsuperscript{7}.

\textsuperscript{t} Astromicin was initially designated as fortimicin A.
In the present study, the authors attempted to elucidate the biosynthetic pathway for ASTM in more detail. Complementation experiments revealed the blocked sites of many mutants. The intermediates produced by these mutants were isolated, and their structures were determined. Studies using these mutants and purified intermediates, especially biotransformation experiments using an inosamine idiotrophic mutant, provided more precise information about the biosynthesis of ASTM, including the steps for biosynthesis of fortamine, the aminocyclitol component. From the results, it is apparent that there exists a characteristic system in the biosynthesis of ASTM which is not found in the biosynthesis of other aminoglycoside antibiotics.

Materials and Methods

Microorganisms

*M. olivasterospora* KY11520 was used as the parent strain for isolating mutants blocked in the biosynthesis of ASTM. *Bacillus subtilis* ATCC 6633 was used as the test strain in cosynthesis, bioassay and bioautography.

Media

ATCC No. 5 agar medium containing glucose 0.6%, Stabilose K (soluble starch) 0.4%, Tryptose 0.2%, yeast extract 0.1%, beef extract 0.1%, agar 2.0% and FeSO₄·7H₂O 100 mg/liter was used for isolation of *M. olivasterospora* mutants, or for sporulation. SK₁ medium was used as the seed medium. It was composed of Stabilose K 2.0%, glucose 0.5%, yeast extract 0.5%, peptone 0.5%, meat extract 0.3%, KH₂PO₄ 0.02%, MgSO₄·7H₂O 0.06% and CaCO₃ 0.1%, and its pH value was 7.6 (before autoclaving). The composition of the fermentation medium was Stabilose K 6%, soybean meal 2%, Ebios (dried yeast) 5%, peptone 0.5%, KH₂PO₄ 0.1%, K₂HPO₄ 0.3%, MgSO₄·7H₂O 0.05%, NaCl 0.2% and Ca-pantothenate 10 mg/liter. For cosynthesis and the agar piece screening method¹⁴, the fermentation medium was diluted two-fold, and 2% agar was added.

Mutagenesis

The spore suspension was prepared as follows: *M. olivasterospora* KY11520 was incubated on the ATCC No. 5 agar plate for 3 weeks. After full development of spores was confirmed, spores were scraped off and suspended in 0.1 M Tris-HCl buffer (pH 8.0), 1/15 M phosphate buffer (pH 7.4) or 0.2 M acetate buffer (pH 4.0). After they were washed 4 or 5 times by centrifugation, the suspension was made to contain 1~2 x 10⁶ spores/ml and treated with mutagens. The mycelial suspension was prepared by the method reported previously⁹. Mutagens used were N-methyl-N'‐nitro-N-nitrosoguanidine (NTG), methanesulfonic acid ethyl ester (EMS), methanesulfonic acid methyl ester (MMS), 2-amino purine (2AP), 8-methoxypsoralen (8-MOP) and sodium nitrite (NaNO₂).

Mutagenesis was performed as follows: Treatment with NTG was carried out using the conditions recommended by DELIC et al.⁹. Treatment with EMS and MMS was performed according to the methods described by ENGEL¹⁰ and THOMA¹¹; spores suspended in 1/15 M phosphate buffer were mixed with EMS or MMS to a final concentration of 1~3% or 0.1~0.3% and incubated at 35°C for 8 hours under gentle shaking. For treatment with 2AP, spores were suspended in the synthetic medium containing 1,300 μg of 2AP/ml and incubated with shaking at 30°C for 4 days. Treatment with NaNO₂ was performed according to KLANOVA¹¹; the spore suspension prepared in 0.2 M acetate buffer (pH 4.0) was mixed with NaNO₂ at a final concentration of 1 M and shaken at 35°C for 60 minutes. The reaction was stopped by adding several volumes of 0.1 M Tris-HCl buffer. Irradiation with near ultraviolet light (N-UV) was performed by the methods described by SCOTT et al.¹² and TOWNSEND et al.¹³. Namely, 8-MOP was added to the spore suspension to the final concentration of 100 μg/ml, 5 ml of the suspension was transferred to a Petri dish and irradiated with a black light lamp (Toshiba) from a distance of 20 cm at a dose allowing 1.0~0.1% of the spores to survive. Irradiation with ultraviolet light was performed according to THOMA's report¹⁴. Irradiation from ⁶⁰Co was carried
out with spores suspended in 1/15 M phosphate buffer.

Spores and mycelia treated with mutagens were spread on ATCC No. 5 agar plates after diluting adequately and incubated at 30°C for about 3 weeks. Each colony developed was picked and examined for production of the antibiotic by the agar piece method\(^{15}\).

**Cosynthesis**

Cosynthesis was performed according to the methods described by McCormick et al.\(^{16}\) and Delic et al.\(^{17}\) Blocked mutants were incubated in SK\(_1\) medium at 30°C for 3 days with shaking. One milliliter of the culture was transferred into 10 ml of fresh SK\(_1\) medium and incubated at 30°C overnight. Mutant cultures obtained were used for cosynthesis. The schema for cosynthesis tests is illustrated in Fig. 2.

**Biotransformation with Resting Cells**\(^{8,10}\)

Mutants of *M. olivasterospora* were incubated in the fermentation medium for 3 days. Mycelia which grew in the medium were harvested, washed 3 times with 0.1 M Tris-HCl buffer (pH 7.5), suspended in the same buffer and used for biotransformation experiments. The reaction mixture containing 150 mg (dry weight) of mycelia and 500–1,000 µg of substrate in 10 ml of Tris-HCl buffer (pH 7.5) was incubated at 30°C for about 20 hours. Biotransformation products in the reaction mixture were detected by thin-layer chromatography (TLC) and paper chromatography (PPC). TLC was performed by using Merck Silica Gel 60 plates and the lower phase of chloroform - methanol - 17% ammonium hydroxide (1:1:1) as the solvent system. ASTM and its precursors were visualized by the Rydon-Smith reaction\(^{20}\). The amount of each product was determined fluorometrically with a Shimadzu CS-910 double-beam densitometer\(^{21}\). PPC was performed with Toyo No. 51 paper and the lower phase of chloroform - methanol - 17% ammonium hydroxide (1:1:1) as the solvent system. Biotransformation products in the reaction mixture were detected by bioautography against *Bacillus subtilis* ATCC 6633 after PPC.

**Identification of Fermentation Products**

Antibiotics produced by blocked mutants of *M. olivasterospora* were purified and identified by the methods reported previously\(^{7,10}\).

**Results**

**Isolation of Mutants Blocked in the Biosynthesis of Astromicin**

Many blocked mutants were isolated from the ASTM producing microorganism, *M. olivasterospora*. As shown in Table 1, a total number of 42,940 colonies were examined, and 182 blocked mutants (0.4%) were obtained. Treatment with \(^{60}\)Co and EMS induced these mutants at high frequency. Many mutants were devoid of, or low, in sporulating ability or inferior in growth, and some colonies were mucoid or dwarfish.

**Complementation Pattern between Blocked Mutants Isolated from *M. olivasterospora***

To determine the blocked step in 29 selected mutants, the cosynthetic ability in pairs of mutants was examined; that is, the ability or inability to produce ASTM in complementation experiments.
When these 29 blocked mutants were tested in 812 pairs for their cosynthetic ability, 25 could be classified into 10 groups according to their complementation patterns (Fig. 3). Each pair of mutants showing complementation on the agar plate was proved to produce ASTM in cofermentation in fluid medium. The remaining 4 mutants showed complex complementation patterns.

Table 1. Isolation frequencies of mutants blocked in astromicin biosynthesis.

<table>
<thead>
<tr>
<th>Mutagens</th>
<th>Isolation frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG</td>
<td>0.19 (18/9,453)</td>
</tr>
<tr>
<td>EMS</td>
<td>0.70 (26/3,706)</td>
</tr>
<tr>
<td>MMS</td>
<td>0.40 (10/2,669)</td>
</tr>
<tr>
<td>UV</td>
<td>0.17 ( 2/1,183)</td>
</tr>
<tr>
<td>N-UV (with 8-MOP)</td>
<td>0.33 (21/6,451)</td>
</tr>
<tr>
<td>60Co</td>
<td>0.79 (72/9,151)</td>
</tr>
<tr>
<td>2AP</td>
<td>0.13 ( 3/1,983)</td>
</tr>
<tr>
<td>NaNO2</td>
<td>0.00 ( 0/1,969)</td>
</tr>
<tr>
<td>NTG+60Co</td>
<td>0.46 (12/2,618)</td>
</tr>
<tr>
<td>NTG+UV</td>
<td>0.33 ( 7/2,139)</td>
</tr>
<tr>
<td>EMS+2AP</td>
<td>0.68 (11/1,618)</td>
</tr>
<tr>
<td>None</td>
<td>— ( 0/4,068)</td>
</tr>
<tr>
<td>Total</td>
<td>0.42 (182/42,940)</td>
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Mutagen-treated suspensions of spores or mycelial fragments were diluted and spread on ATCC No. 5 agar plate. After about 3 weeks incubation of the plate at 30°C, each colony developed was picked, and the production of antibiotics was tested by the agar piece method14).

Isolation and Identification of Biosynthetic Intermediates Produced by the Blocked Mutants

From each of the 10 groups of mutants classified by the cosynthesis experiments, a representative strain was chosen for isolation and identification of its major products. They are shown in Table 2. It was found by TLC that KY11558 accumulated scyllo-inosamine. From KY11554, two major products, FU-10 and FOX-2, were isolated. Major products of KY11581, KY11583 and KY11556 were fortimicins (FTM)-AO, FTM-KL₁ and FTM-KK₁, respectively. KY11560 produced 3-O-demethylfortimicins such as FTM-KJ, FTM-KS and FTM-AN in addition to the major product, FTM-AP. KY11555 produced FTM-KH in large amounts. From KY11557 and KY11582, FTM-KR and FTM-B, respectively, were isolated as major products. No product could be identified from KY11559, a mutant which serves as a convertor of all other mutants.

Biotransformation of Intermediates to ASTM with Resting Cells of the Blocked Mutants

The biotransformation of major products isolated from the mutants was studied with resting cells of the blocked mutants. The results are shown in Table 3. It is evident that convertors can
convert all major products accumulated by the secretor into ASTM. The results of biotransformation experiments support without contradiction the order of blocked sites in the pathway of mutant biosynthesis deduced from the results of cosynthesis.

Biotransformation of Cyclitols by an Inosamine Idiotroph of *M. olivasterospora*

The results of cosynthesis experiments show that the mutant KY11559 serves as a convertor with mutants of all other groups imply that KY11559 is blocked at the earliest step in the ASTM biosynthetic pathway. Since this mutant requires inosamine for ASTM production, it was presumed to lack the ability to biosynthesize fortamine, the aminocyclitol moiety of ASTM. Thus, KY11559 was classified as an “inosamine (ISM)-idiotroph”.

Biotransformation of various cyclitols was carried out with the ISM-idiotroph, KY11559. As shown in Table 4, KY11559 converted scyllo-inosose, scyllo-inosamine and FU-10 to ASTM. On the other hand, myo-inositol, 1,4-inosadiamine, 1,3-inosadiamine, fortamine-KH (aminocyclitol of FTM-KH) and fortamine-B (aminocyclitol of FTM-B) could not be converted to ASTM. These results suggest that fortamine is made by the following process; synthesis of scyllo-inosamine via scyllo-inosose, transformation to FU-10 in the form of monoaminocyclitol, and then amination at position 4.

**Biosynthetic Pathway of ASTM and Blocked Site in Mutants of *M. olivasterospora***

On the basis of the experimental findings described so far, the authors propose the extended biosynthetic pathway shown in Fig. 4 for ASTM.

**Discussion**

ASTM is a pseudodisaccharide aminoglycoside of a new type. Sporaricin\textsuperscript{22}, istamycin\textsuperscript{23} and dactiminicin\textsuperscript{24} are recently found to be aminoglycosides belonging to the same group. These antibiotics
Table 3. Biotransformation of biosynthetic precursors to astromicin with resting cells of the blocked mutants.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>KY11559</th>
<th>KY11558</th>
<th>KY11554</th>
<th>KY11581</th>
<th>KY11583</th>
<th>KY11556</th>
<th>KY11560</th>
<th>KY11555</th>
<th>KY11557</th>
<th>KY11582</th>
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<tr>
<td>FU-10</td>
<td>+</td>
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<td>FTM-AO</td>
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</table>

The reaction mixture containing 500~1,000 µg of a substrate and 150 mg (dry weight) of washed mycelia in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5) was incubated at 30°C for about 20 hours. The biotransformation product, ASTM was detected by TLC and PPC.

Symbols: +, converted to ASTM; −, not converted to ASTM.
Fig. 4. Proposed biosynthetic pathway for fortamine and astromicin.

\[ \text{Scylo-inosose} \rightarrow \text{Scylo-inosamine} \rightarrow \text{FU-10} \]

\[ \text{KY11559} \rightarrow \text{KY11558} \rightarrow \text{KY11550} \]

\[ \text{AO} \rightarrow \text{KL}_1 \rightarrow \text{KK}_1 \]

\[ \text{KY11554} \rightarrow \text{KY11581} \rightarrow \text{KY11583} \]

\[ \text{AP} \rightarrow \text{KH} \rightarrow \text{KR} \]

\[ \text{KY11556} \rightarrow \text{KY11560} \rightarrow \text{KY11555} \]

\[ \text{B} \rightarrow \text{Astromicin} \]
are closely akin in chemical structure, and it has been interesting problem to know the biosynthetic origin of these antibiotics. In the previous paper\(^7\), part of the pathway for ASTM biosynthesis was deduced from the results of biotransformation experiments. In the present study, the ASTM biosynthetic pathway was established in more detail by isolating many blocked mutants and investigating their properties.

Of the variety of mutagens tried, \(\gamma\)-rays and EMS induced blocked mutants and auxotrophic mutants with high frequency (0.8~1.0\%). Treatment with \(^{60}\)CO, EMS and MMS (lower in frequency of mutant isolation) induced some mutants blocked at different steps on the ASTM biosynthetic pathway. Most of mutants obtained by N-UV and UV irradiation accumulated FU-10 (mutants blocked at step C in Fig. 3). THOMA\(^{14}\) reported that EMS and MMS were effective as mutagens with *Penicillium chrysogenum* (penicillin-producer), but not practical for *Streptomyces*. Our study demonstrated that \(\gamma\)-rays and alkylating agents were suitable mutagens for *M. olivasterospora*.

Mutants blocked in the pathway of antibiotic biosynthesis have recently been called idiotrophs\(^{25}\), and their practical usefulness has been recognized. Studies with idiotrophs have contributed to increased knowledge of antibiotic biosynthesis\(^{8,19,20,28,30}\) as well as to the production of new antibi-otics\(^{25,28-24}\). For instance, 2-deoxystreptamine-(DOS)-idiotrophs used to study aminoglycoside biosynthesis\(^{32}\) have contributed to the elucidation of DOS biosynthesis in *M. purpurea*\(^{32}\), *M. sagamiensis*\(^{19}\) and *B. circulans*\(^{28}\). There is no previous information on the biosynthesis of the ASTM
aminocyclitol, fortamine (1,4-diaminocyclitol). The isolation of an inosamine idiotroph of *M. olivasterospora*, KY11559, and the results of bioconversion and cosynthesis experiments with this mutant indicate that fortamine is formed from scyllo-inosamine (via scyllo-inosose), which is already attached to β-glucosamine in FU-10 and is then aminated at position 4. Part of this biosynthetic route, that is, \(\text{scyllo-inosose} \rightarrow \text{scyllo-inosamine}\), is seen in streptidine biosynthesis. Though it has not been known whether scyllo-inosose is synthesized from myo-inositol in *M. olivasterospora*, fortamine and streptidine are most likely formed by a partially common route.

In the case of kanamycin, gentamicin, sagamicin and butirosin, it has been known that 1,3-diaminocyclitol (DOS) itself is incorporated into the antibiotics. A similar process has been reported for incorporation of the cyclitol (streptidine) in streptomycin biosynthesis. However, in the case of ASTM, the amination at position 4 introducing the second amino group of fortamine occurred after formation of the disaccharide. The results of biotransformations with the ISM-idiotroph (Table 4) and accumulation of FU-10 and scyllo-inosamine by KY11554 and KY11558, respectively, strongly supported this conclusion.

SU-1, 2 and 3 are antibiotics having a monoaminocyclitol. These antibiotics are 1-deamino-1-hydroxyl derivatives of gentamicin C3, C1a and sagamicin, respectively, and are produced by mutants of *M. sagamiensis*. KASE et al. have reported that, instead of DOS, 2-deoxy-scyllo-inosamine (DOI) was incorporated into the aminocyclitol moiety. Biosyntheses of ASTM and of these antibiotics differ in the point that the monoaminocyclitol precursor of ASTM is converted into a diaminocyclitol after forming a disaccharide.

In addition to this unique feature in the introduction of amino functions, the biosynthesis of fortamine is characterized by further modification of the aminocyclitol moiety after pseudodisaccharide formation. The only known example of the modification of an aminocyclitol unit after combination with other subunits in the biosynthesis of aminoglycoside antibiotics is the transfer of an acyl group to the NH₂ group at position 1 in butirosin biosynthesis. In ASTM biosynthesis, by contrast, 5 conversions viz methylation at positions 3 and 4, glycylation at position 4 and epimerization at positions 3 and 4, occur as a series of reactions subsequent to formation of the amino functions at positions 1 and 4.

Besides *Micromonospora* and *Streptomyces*, *Saccharopolyspora* and *Dactylosporangium* are known to produce antibiotics of the ASTM group. It would be very interesting to elucidate whether these related antibiotics are synthesized by a common biosynthetic route.

References


