NOTES

**Production, Isolation and Biological Properties
of TMC-120A, B and C,
Novel Inhibitors of Eosinophil Survival from
Aspergillus ustus TC 1118**

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(Received for publication April 30, 1999)

Eosinophils are believed to contribute proinflammatory effects in allergic inflammation, including asthma.¹⁾ The cytokines such as hematopoietins, interleukin-5 (IL-5), IL-3 and GM-CSF have been shown to stimulate production of eosinophils in the bone marrow,²⁾ and the same cytokines inhibit eosinophilic apoptosis and prolong survival when eosinophils are cultured.^{3~5)} In allergic individuals, large numbers of eosinophils are accumulated and release

activated oxygen and toxic granule-derived proteins such as major basic protein and eosinophil cationic protein.¹⁾ Consequently, agents that inhibit the prolongation of eosinophil survival will be another candidate for development of drugs to treat bronchial asthma.

In the course of our screening for inhibitors of IL-5 mediated prolongation of eosinophil survival, a fungus, *Aspergillus ustus* (Bain.) Thom & Church TC 1118 was found to produce three novel isoquinoline alkaloids, designated as TMC-120A (1), B (2) and C (3). We report here the production, isolation and biological activities of TMC-120A, B and C, and their derivatives.

A loopful of spores of the producing strain, *Aspergillus ustus* was inoculated into 500-ml Erlenmeyer flasks containing 70 ml of a seed medium and incubated on a rotary shaker at 220 rpm at 27°C for 5 days. The resultant seed culture (300 ml) was transferred into a 50-liter jar fermentor containing 30 liters of a production medium having the same composition as the seed medium. The fermentation was carried out at 27°C for 3 days with agitation of 200~600 rpm and aeration of 15 liters/minute to keep the dissolved oxygen concentration at 10% of saturation.

Fig. 1. Structures of TMC-120A (1), B (2), C (3) and derivatives (4, 5).

The typical time course of TMC-120s production in a 30-liter jar is shown in Fig. 2. The pH and packed volume (PCV) gradually increased from the initiation of the fermentation to 120 hours. The glucose concentration was gradually decreased, and reached 0.4 mg/ml at 120 hours. Compound **2** was produced in the early stage of the fermentation, followed by the production of **1** and **3**. The maximum production of **2**, **1**, and **3** was observed at 48, 72, and 120 hours, respectively, suggesting that **1** and **3** were derived from **2** by biological or chemical transformation. The total production of **1**, **2** and **3** reached the maximum at 72 hours.

The isolation procedure for TMC-120s is summarized in Fig. 3. The fermentation broth of *Aspergillus ustus* was extracted with 1-butanol, and the extract was purified by solvent partition and repeated column chromatography. Finally, compounds **2** and **3** were recrystallized with organic solvents to afford needles. The structure determination has been reported in a separate paper.⁶⁾

In order to obtain a preliminary information on structure-activity relationships, **2** was reduced with sodium borohydride, giving two diastereomers, **4** (*E* isomer) and **5** (*Z* isomer). Structures of **4** and **5** were determined by comparison of ¹H and ¹³C NMR data with those of **1**, together with the NOESY experiments of **4** and **5** as shown in Fig. 4.

Fig. 2. Time course of TMC-120s production.

Fig. 3. Isolation and purification procedure for TMC-120A (**1**), B (**2**) and C (**3**).

Fig. 4. The NOESY experiments of **4** and **5**. Table 1. Inhibitory effects of **1**~**5** on the survival of guinea pig peritoneal eosinophils.

IC ₅₀ (μM)				
1	2	3	4	5
13.7	2.0	> 39	> 41	> 41

TMC-120B (**2**) showed moderate inhibitory activity against the IL-5 mediated prolongation of eosinophil survival (IC₅₀=2.0 μM), but **1**, **3**, **4** and **5** which are reductive analogs of **2**, were less active than **2** or inactive (Table 1). These results suggested that the α,β-unsaturated ketone of **2** played an important role for the activity, involving the alkylation of biological nucleophiles in a MICHAEL-type addition. TMC-120B showed no cytotoxicity against human leukemia HL-60 at the concentration of 42 μM.

Experimental

Microorganism

The producing fungal strain TC 1118 was isolated from rhizosphere of grass collected in Kawaguchi-shi, Saitama, Japan. It was identified as *Aspergillus ustus* (Bain.) Thom & Church on the basis of the following distinct

characteristics; dull gray conidial area, yellow reverse side of colonies, small vesicles of 6~18 μm in diameter, long brownish stipes, and globose to subglobose, rough-walled conidia of 4.0~5.5×2.5~3.0 μm from biseriate aspergilla.

Fermentation

Seed and production medium contained glucose 0.5%, glycerol 2.0%, soybean meal 2.0%, yeast extract 0.2%, CaCO₃ 0.4% and NaCl 0.25% (pH 6.5). Growth of the organism was evaluated as packed cell volume (PCV) by centrifuging the fermentation broth. The PCV was recorded as % of total broth volume. The production of TMC-120s during the fermentation, was analyzed by HPLC of the 1-butanol extract of the fermentation broth.

HPLC Analysis

Analytical HPLC was carried out on a Hewlett-Packard HP-1090 equipped with a diode array detector. The conditions for HPLC analysis were as follows; column: YMC-Pack ODS-AM AM-301-3 (4.6×100 mm), mobile phase: 55% aq CH₃CN, flow rate: 1.2 ml/minute, detection: UV at 254 nm. Under these conditions, TMC-120A, B and C eluted at 3.9, 4.2 and 2.1 minutes, respectively.

Reduction of **2** with Sodium Borohydride

To a solution of **2** (39.1 mg, 0.164 mmol) in ethanol (20 ml), sodium borohydride (7.8 mg, 0.206 mmol) was added. After stirring at room temperature for 5 hours, the reaction mixture was neutralized by addition of 1 N HCl. The resulting mixture was concentrated and purified by

preparative HPLC (50% aqueous CH_3CN) to separate **4** and **5**, which were further purified by a Sephadex LH-20 column (22×440 mm) with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (1 : 1) to yield **4** (7.6 mg, 19.1%) and **5** (16.1 mg, 40.4%), respectively.

4: Pale yellow solid; UV λ_{max} (log ϵ): 344 (3.72), 305 (3.50), 293 (sh, 3.46), 241 (4.22), 225 (4.38), 210 (4.49) nm; IR ν_{max} : 3350~3200, 2955, 2920, 2870, 1640, 1570, 1460, 1425, 1385, 1375, 1355, 1270, 1160, 1080, 985 cm^{-1} ; ESI-MS (positive) m/z 244 ($\text{M}+\text{H}$)⁺, 226 ($\text{M}+\text{H}-\text{H}_2\text{O}$)⁺, 210, 188, 173; ESI-MS (negative) m/z 242 ($\text{M}-\text{H}$)⁻; HRESI-MS m/z found 242.1147 ($\text{M}-\text{H}$)⁻, calcd. for $\text{C}_{15}\text{H}_{16}\text{NO}_2$: 242.1181; ^1H NMR in CDCl_3 : δ (ppm); 9.28 (s, H-9); 7.60 (d, 8.5 Hz, H-4); 7.39 (s, H-6); 7.21 (d, 8.5 Hz, H-5); 5.26 (d, 3.6 Hz, H-3); 4.52 (dd, 6.6 Hz, 3.6 Hz, H-2); 2.65 (s, H-10); 2.03 (m, H-11); 1.07 and 1.06 (d, 6.7 Hz, H-13 and 12); ^{13}C NMR in CDCl_3 : δ (ppm); 157.0 (s, C-9b); 152.5 (s, C-7); 146.8 (d, C-9); 138.5 (s, C-5a); 127.4 (d, C-4); 122.4 (s, C-3a); 118.6 (d, C-6); 118.3 (d, C-5); 114.5 (s, C-9a); 97.9 (d, C-2); 75.5 (d, C-3); 31.5 (d, C-11); 24.2 (q, C-10); 17.7 and 17.6 (q, C-13 and 12).

5: Pale yellow solid; UV λ_{max} (log ϵ): 343 (3.79), 305 (3.53), 293 (sh, 3.48), 241 (4.29), 225 (4.47), 210 (4.57) nm; IR ν_{max} : 3200, 2950, 2870, 1640, 1570, 1425, 1385, 1350, 1280, 1265, 1255, 1170, 1160, 1125, 1085, 1065, 1000, 940 cm^{-1} ; ESI-MS (positive) m/z 244 ($\text{M}+\text{H}$)⁺, 226 ($\text{M}+\text{H}-\text{H}_2\text{O}$)⁺, 210, 188, 173; ESI-MS (negative) m/z 242 ($\text{M}-\text{H}$)⁻; HRESI-MS m/z found 242.1155 ($\text{M}-\text{H}$)⁻, calcd. for $\text{C}_{15}\text{H}_{16}\text{NO}_2$: 242.1181; ^1H NMR in CDCl_3 : δ (ppm); 9.19 (s, H-9); 7.66 (d, 8.3 Hz, H-4); 7.20 (s, H-6); 7.07 (d, 8.3 Hz, H-5); 5.22 (d, 5.5 Hz, H-3); 4.20 (dd, 10.5 Hz, 5.5 Hz, H-2); 2.54 (s, H-10); 2.54 (m, H-11); 1.40 and 1.24 (d, 6.6 Hz, H-13 and 12); ^{13}C NMR in CDCl_3 : δ (ppm); 157.0 (s, C-9b); 151.0 (s, C-7); 145.9 (d, C-9); 138.2 (s, C-5a); 128.5 (d, C-4); 124.3 (s, C-3a); 118.8 (d, C-6); 118.0 (d, C-5); 114.1 (s, C-9a); 95.0 (d, C-2); 72.1 (d, C-3); 27.6 (d, C-11); 23.3 (q, C-10); 19.9 and 19.5 (q, C-13 and 12).

Measurement of Eosinophil Survival

Eosinophils were obtained from peritoneal lavage fluid

of guinea pigs sensitized with polymixin B. The cells were purified by centrifuge on a discontinuous Percoll density gradient. The purified eosinophils (>90% purity, $7.5 \times 10^3/\text{well}$) were suspended in 100 μl of RPMI-1640 medium containing 5% (v/v) fetal calf serum, recombinant IL-5 (0.1 ng/ml) and various concentrations of drugs, and incubated at 37°C for 5 days in a humidified atmosphere containing 5% CO_2 . The drugs were dissolved in DMSO and added to the medium. The final concentration of DMSO was adjusted to 0.1% (v/v). The control medium contained the same amount of DMSO. After incubation, the cells were washed with PBS and the viability of the cells were determined by their ability to take up fluorescein diacetate.

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