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Biosynthesis of $\Delta^{6,7}$-anhydroerythromycin *via* enoyl reductase inhibition by isonicotinic hydrazide (INH)

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Abstract

Isonicotinic hydrazide (INH) was added to a fermentation of mutant of Saccharopolyspora erythraea *ATCC 11912 to inhibit the activity of 2,4,6,8-tetramethyl-7,9-dihydroxy-2-en-5-on-undecyl-ACP enoyl reductase, an enzyme which catalyses enoyl reduction process of an intermediate in the erythronolide biosynthesis, and produce $\Delta^{6,7}$-anhydroerythromycin derivative. The optimum INH additional concentration of above 0.1% was able to inhibit the enzyme activity, and produce $\Delta^{6,7}$-anhydroerythromycin derivative as shown by FT-infrared spectrophotometric analysis of the product after its purification using thin layer chromatography.*

Keywords: *Isonicotinic hydrazide (INH) – enoyl reductase – $\Delta^{6,7}$-anhydroerythromycin – mutant* Saccharopolyspora erythraea *ATCC 11912.*

Introduction

Erythromycin is unstable in an acidic condition at where it will decompose into its components and lose its antibacterial activity. The decomposition process is initiated by a nucleophilic attack of the C_6-hydroxy group to the C_9-carbonyl group of erythromycin to form an internal ether, and this will be continued with a dehydration process that produces 6,9-hemiketal-8,9-anhydroerythromycin, this product in further releases water and forms 6,9,9,12 spiroketal-erythromycin before it splits into the components (Sakakibara and Omura, 1984). Structural modification on the C_6-hydroxy and/or C_9-carbonyl of erythromycin has been done to avoid this acidic decomposition, either chemically or genetically (Baltz, 1986; Kirst and Sides, 1989; Donadio *et al.*, 1993; Mun'im, 1997), as well as using hybrid biosynthetic technique (Jenie *et al.* 1998 a and 1998b).

The C_9-carbonyl, as well as C_{11}- and C_{12}-hydroxyls, dimethylamino, and $C_{3''}$-methoxyl groups, is a functional group of erythromycin as an antibiotic; in the contrary the C_6-hydroxy group contributes a weak antibiotic activity. Therefore modification or omitting C_6-hydroxy group will not considerably affect the antibiotic activity.

The fourth step of the biosynthesis of erythronolide B (the aglycone part of erythromycin) consists of 4 processes: condensation, β-keto-reduction, dehydration, and enoyl-reduction (Donadio *et al.*, 1991). The last process is catalyzed by 2,4,6,8-tetramethyl-7,9-dihydroxy-2-en-5-on-undecyl-ACP enoyl reductase which needs NADPH as the coenzyme. Inhibition of this reductase will

not change the adduct of 2,4,6,8 tetramethyl-7,9-dihydroxy-2-en-5-on-undecyl-ACP, which in last step of the biosynthesis will form $\Delta^{6,7}$-anhydroerythromycin derivative instead of the erythromycin.

Isonicotinic hydrazide (INH) was found to inhibit the activity of enoyl reductase in the biosynthesis of mycolic acid within the *Mycobacterium tuberculosis* (Magliozo and Marcinkevicience, 1997). It was found by Rozwarski *et al.* (1998) that the enoyl reductase inhibition is due the covalently bonded of NADH by activated-INH so that it cannot be bonded by the active site of the enoyl-reductase. However INH also inhibits the activity of β-ketoacyl ACP synthase as reported by Mdluli *et al.* (1998); which means that the biosynthesis of the polyketides, included the erythronolides, is inhibited.

An addition of INH into *Sac. erythraea* fermentation hopefully inhibit the activity of the 2,4,6,8-tetramethyl-7,9-dihydroxy-2-en-5-on-undecyl-ACP enoyl reductase (the only enoyl reductase in the erythronolide B biosynthesis) because its coenzyme, NADPH (Donadio *et al.*, 1992), has a base moiety (nicotinamide) which is structurally identical to that of the NADH. This inhibition will in turn inhibit the enoyl reduction process in the erythronolide B biosynthesis, and produce $\Delta^{6,7}$-anhydroerythromycin derivative in last step of the biosynthesis instead of the erythromycin. However the yield quantity might be low due the inhibition of β-ketoacyl synthase of the erythronolide B biosynthesis.

Methodology

Microbes: mutant *Saccharopolyspora erythraea* ATCC 11912 (an erythronolides producer). **Media:** The sporulation as stated in Weber *et al.* (1985), while the inoculation and fermentation media were as shown in Sudibyo (1998). **Chemicals:** Isonicotinic hydrazide (INH), standard erythromycin A, all are Merck products. **Instruments:** Rotary shake incubator, ultracentrifuge, CAMAG TLC-scanner, Shimadzu FT-IR Spectrophotometer. **Fermentation.** Shake cultures of *Sac. erythraea* ATCC 11912 added with 0.01; 0.1; 0.2; 0.25; and 0.5% and without INH were incubated at 28°C, and agitated at 180 rpm for 48 hours. After centrifugation for the cell separation, all the supernatants were adjusted into pH 8 before extraction of the macrolides using chloroform. The chloroform was evaporated, and the residues were purified through preparative thin layer chromatographic (TLC) method using eluent of ethanol: methanol: triethylamine = 170 : 30 : 1. The TLC isolates were analyzed on an FT-IR-spectrophotometer.

Results and Discussion

There was one component of macrolides resulted from the fermentation without the addition of INH; and two main components from the fermentation with additional INH in all concentrations (Table 1).

Table 1. The time retention peak and its percentage of the under curve area of macrolide components of the *Sac. erytlxraea* fermentation isolates' chromatograms.

Additional INH (%)	Component I		Component II	
	Retention time (R_f)	Under curve area (%)	Retention time (R_f)	Under curve area (%)
0	0.67	100	-	-
0.01	0.77	73.12	0.42	26.88
0.10	0.83	6.12	0.60	85.57
0.20	0.81	2.68	0.60	85.17
0.25	0.81	3.84	0.61	80.61
0.50	0.82	7.32	0.64	89.22

It was shown in the Table 1 that the component I resulted from the fermentation of *Sac. erythraea* ATCC 11912 without additional INH having lower retention time (R_f) of 0.67. This R_f value is not in accord with that of erythronolide B (0.80) at the similar eluent (Corcoran, 1981). This means that component I might be different derivative of macrolide. The most possibility was that the mutant *Sac. erythaea* has been reverted and produced its erythromycin which having a bigger molecule than erythronolide B so that it had a lower R_f value than that of erythronolide B. Based on the Corcoran's TLC-analysis result on the supernatant of *Sac. erythraea* ATCC 11635 (the wild type) (Corcoran, 1981), it was also shown that all erythromycins having lower Rfs than those of deoxyerythronolide B and erythronolide B. These facts support that component I could be an erythromycin derivative (that might be mixed with the erythronolide B and showed a broadening spectrogram); which means that the *Sac. erythraea* ATCC 11912 fermented in this research might has been reverted and produces one of its erythromycins.

The addition of INH in the fermentation of *Sac. erythraea* ATCC 11912 resulted a new component of macrolide (component II) with average retention time of 0.60. This component II-R_f is lower than that of the component 1, which means that component II is less polar than component I. If the additional INH to the fermentation did inhibit the activity of the *Sac. erythraea* 2,4,6,8-tetramethyl-7,9-dihydroxy-2-en-5-on-undecyl-ACP enoyl reductase, therefore component II was a $\Delta^{6,7}$-anhydroerythromycin derivative which had no C_6-hydroxyl group in the ring system and became less polar than erythromycins. It is showed in the table that additional INH of 0.1 % into the fermentation was an optimum concentration for producing component II.

To confirm whether the component I is a derivative of erythromycins, and component II was a $\Delta^{6,7}$-anhydroerythromycin derivative, FT-IR analysis was conducted to the TLC isolates of the both components.

The IR spectrograms of component I and component II resulted respectively from fermentation with no and with the additional INH were showed in subsequently Figure 1 and 2. While the conclusion of those both spectrograms is described in Figure 2.

It is showed in the Table 2 that component II produced stretching vibrations of bonding of -C-H (Sp^2) and -C=C- at wave numbers of 3111 and 1604 cm^{-1} respectively. These bands were not shown by the IR-spectrogram of component I. The most possibility of those bonding-vibrations are due to the -C_6H=C_7 (CH_3)- group of the $\Delta^{6,7}$-anhydroerythromycin derivative (and might also the $\Delta^{6,7}$-anhydroerythronolide B) in the isolate.

It is concluded therefore that the additional INH into the fermentation potentially inhibited the activity of the 2,4,6,8-tetramethyl-7,9-dihydroxy-2-en-5-on-undecyl-ACP enoyl reductase of the *Sac. erythraea so* that it produced $\Delta^{6,7}$-anhydroerythromycin derivative.

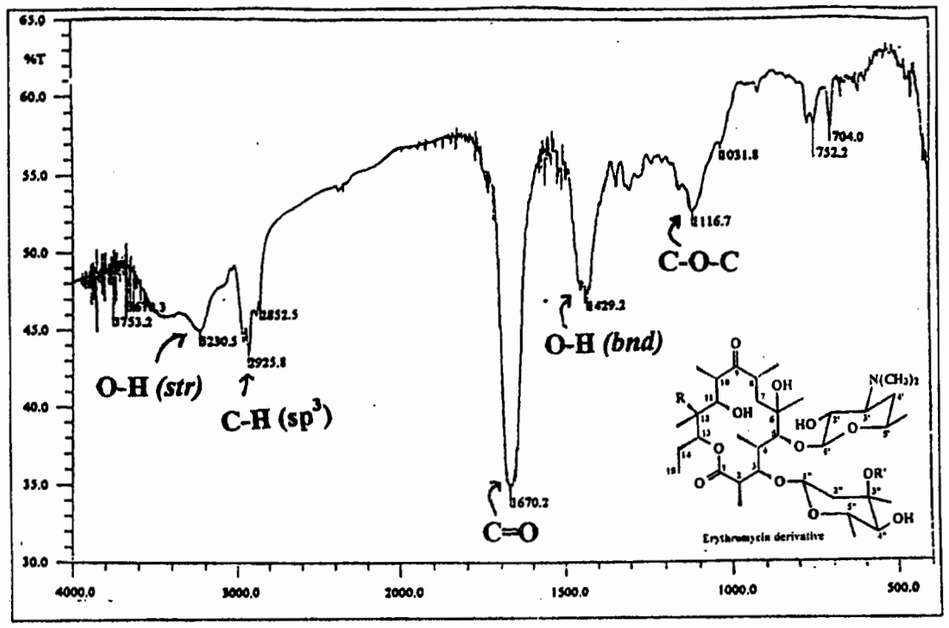

Figure 1. The IR-spectrogram of the component I which was isolated from the fermentation of *Sac erythraea* ATCC 11912 with no additional INH.

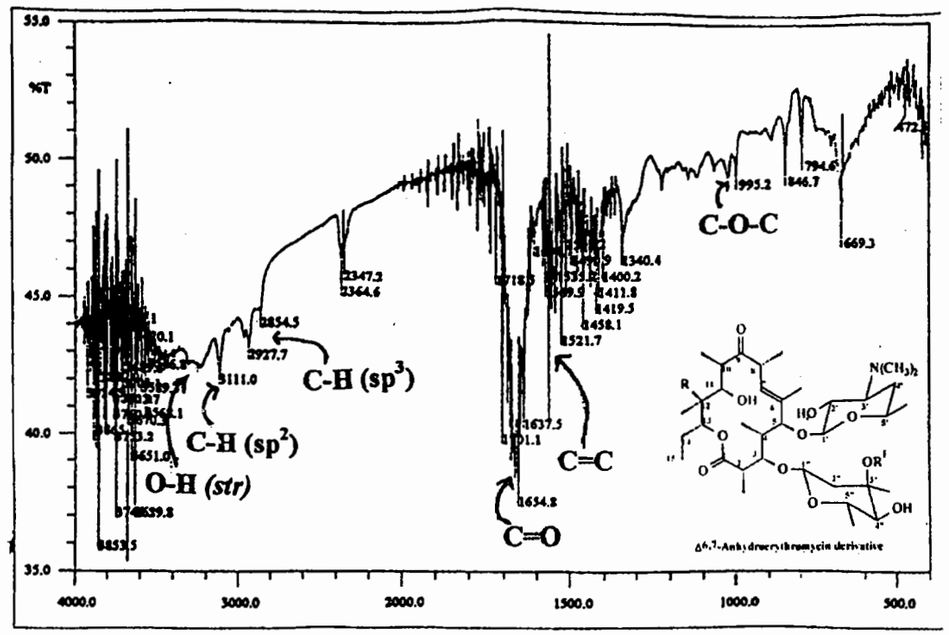

Figure 2. The IR-spectrogram of the component II which was isolated from the fermentation of *Sac. erythraea* ATCC 11912 with the additional INH.

Table 2. The IR-spectrogram bands of components I and II which were isolated respectively from the fermentation of *Sac. erythraea* ATCC 11912 with no and with the additional INH.

Bonding vibration of:	Component I (with no INH) Wave number (cm^{-1})	Component II (with 0.1-0.5% additional INH) Wave number (cm^{-1})
O-H	3230.5 (stretching), 1429.2 (bending)	3500 - 3200 (stretching), 1419 (bending)
C-H (sp^2)	-	3111
C-H (sp^3)	2975 – 2825.5	3000 - 2854
C=O	1670	1654
-C=C-	-	1604
-C-O-C-	1116.7	995.2

The study of the resistance of component II ($\Delta^{6,7}$-anhydroerythromycin derivative) to an acidic condition using FT-IR spectrophotometric analysis and microbiological test found out that component II was more stable in an acidic condition than erythromycin A (Sudibyo *et al.* 1999). Structural elucidation using 600MHz-^{1}HNMRspectrometer confirmed that component II was $\Delta^{6,7}$-anhydroerythromycin D (Jenie *et al.* 1999).

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