DEACYLATION OF ECHINOCANDIN B BY *ACTINOPLANES UTAHENSIS*

LaVerne D. Boeck, David S. Fukuda, Bernard J. Abbott
and Manuel Debono

Lilly Research Laboratories, Lilly Corporate Center,
Indianapolis, Indiana, U.S.A.

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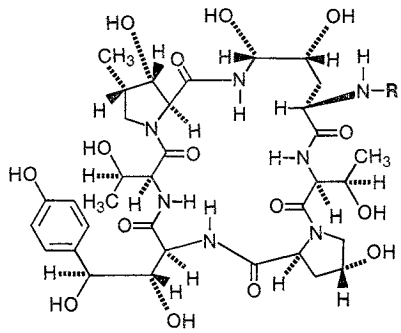
Echinocandin B (ECB) is a lipopeptide antifungal agent produced by several species of *Aspergillus*. The lipid side chain of cyclic lipopeptides is known to be an important determinant of their antibiotic activity and toxicity. Deacylation of another lipopeptide antibiotic, A21978C, had formerly been accomplished with *Actinoplanes utahensis*. In spite of the structural dissimilarities between the peptide cores and acyl side chains of A21978C and ECB, *A. utahensis* also removed the linoleoyl acyl unit from the amino terminus of ECB to yield the bioinactive cyclic peptide core, or "nucleus". The ECB nucleus, which contained a new titratable group at the *N*-terminus, was subsequently employed for chemical reacylation with other side chains to yield a variety of novel ECB analogs. One of these, cilofungin (LY121019), containing an *N*-(4-*n*-octyloxybenzoyl)acyl unit, is currently undergoing clinical evaluation.

In contrast to the numerous therapeutically effective antibacterial antibiotics available, few efficacious antifungal agents are known. As a consequence, vigorous pursuit of new antifungal leads from the ongoing soil screen in our laboratories resulted in the isolation and identification of several members of the echinocandin complex. Produced by species of both *Aspergillus nidulans* and *Aspergillus rugulosus*, these include echinocandins B (ECB, the major factor), C, D and A30912H[1]. The echinocandins are lipopeptides that possess a cyclic peptide nucleus bearing a fatty acid acyl group attached to the *N*-terminus. Comparative studies of ECB and tetrahydro-ECB demonstrated that the different fatty acid acyl side chains of these otherwise identical cyclic peptide antibiotics were important determinants of their antifungal activity and toxicity[2]. This information suggested the desirability of evaluating other side chains to determine their structure-activity relationships.

Fig. 1. Structures of ECB, ECB nucleus, and cilofungin (LY121019).



ECB	$R=CO(CH_2)_7CH=CHCH_2CH=CH(CH_2)_4CH_3$
ECB nucleus	$R=H$
LY121019 (*N*-(4-*n*-octyloxybenzoyl)-ECB)	$R=CO\text{-}C_6H_5\text{-}p\text{-}OC_8H_{17}$

The preliminary requirement for initiation of such a program was the availability of the ECB peptide core, or "nucleus", for chemical reacylation with unique side chains. These laboratories recently reported the deacylation of A21978C, an acidic lipopeptide antibiotic complex produced by *Streptomyces roseosporus*, which inhibits Gram-positive bacteria[3]. The cyclic peptide nucleus resulting from A21978C deacylation by *Actinoplanes utahensis* served as the starting intermediate for chemical reacylation with new fatty acid acyl chains[4]. One of the semi-synthetic analogs subsequently prepared, daptomycin (LY146032), demonstrated a therapeutic index superior to A21978C[5].

From the standpoint of deacylation potential, the cyclic ECB lipopeptide differed from the cyclic A21978C lipopeptide in amino acid composition, nature of the fatty acid side chain, and the relative insolubility of ECB in aqueous fermentation broths. However, ECB was also successfully deacylated with *A. utahensis* to produce the polypeptide nucleus subsequently employed in synthesis of numerous semisynthetic ECB analogs[6]. One of these, cilofungin (formerly known as LY121019), *N*-(4-*n*-octyloxybenzoyl)-ECB (Fig. 1), possesses potent anti-*Candida* activity with reduced toxicity[7] and is being evaluated clinically.

This paper reports details of the ECB deacylation technique.

Materials and Methods

Culture Growth and Deacylation Procedure

Stock Actinoplanaceae cultures, preserved in the vapor phase of liquid nitrogen, were introduced into a medium composed of sucrose 2.0%, pre-cooked oatmeal 2.0%, distiller's grain 0.5%, yeast 0.25%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, and $FeSO_4 \cdot 7H_2O$ 0.0002% in deionized water. After incubation for 72 hours, the resulting mycelial suspension was transferred (2%) into medium PM3. This medium contained sucrose 2.0%, peanut meal 1.0%, K_2HPO_4 0.12%, KH_2PO_4 0.05%, and $MgSO_4 \cdot 7H_2O$ 0.025% in tap water. *A. utahensis* cultures were grown in the PM3 medium for a period of 60~90 hours before being used to deacylate ECB. Both growth stages contained 50 ml of media in wide-mouth 250-ml Erlenmeyer flasks and were incubated at 30°C on a gyrotatory shaker orbiting at 250 rpm.

In order to maintain a constant pH during deacylation, additional phosphate buffer was incorporated into the broth, to a final concentration of 0.1 M, at the desired pH immediately prior to substrate addition. After ECB addition, incubation was continued for an appropriate period, usually 24 hours unless otherwise indicated. This procedure was similar to that previously described for the deacylation of A21978C[3], with one major exception. ECB is an antifungal agent that does not possess antibacterial activity. This lack of natural protection from bacteria provided an opportunity for the outgrowth of chance bacterial contaminants during the deacylation process if non-sterile preparations of ECB were employed. This problem was magnified by the slow rate of ECB deacylation, which necessitated an extended incubation period with *A. utahensis*. The low aqueous solubility of ECB, inhibition of the deacylase by other solubilizing agents, and the presence of insoluble impurities in semi-pure preparations made filter sterilization of ECB solutions generally impractical. Therefore, lyophilized ECB preparations were micronized and sterilized in a conventional ethylene oxide atmosphere prior to incorporation of the dry powders into *A. utahensis* fermentations.

Sampling Procedures

Because of the extremely low solubility of ECB in aqueous media, <5% of the exogenous ECB pulsed into *A. utahensis* fermentations was detectable in broth filtrates or centrates. Quantitation of the remaining ECB required recovery from the fermentation biomass by solvent extraction. This was accomplished by extracting the broth solids, after centrifugation at $1,000 \times g$, with a volume of methanol equal to the initial broth volume.

Removal of the linoleoyl side chain from ECB concurrently eliminated the aqueous insolubility

of ECB, allowing the ECB nucleus to be monitored by direct HPLC quantitation in broth centrates after deacylation by *A. utahensis*.

Microbiological Monitoring of Bioactivity

The antifungal activity of ECB was monitored by a conventional disc-plate agar diffusion assay employing *Candida albicans*, NIH B207.

Acylation of Paper Chromatograms

Developed paper chromatograms of *A. utahensis* broth samples that contained inactivated ECB were removed from the developing chambers and dried. The chromatograms were then buffered by spraying with a 2% solution of aqueous $NaHCO_3$, redried, and sprayed with an active ester of a fatty acid solubilized in petroleum ether.

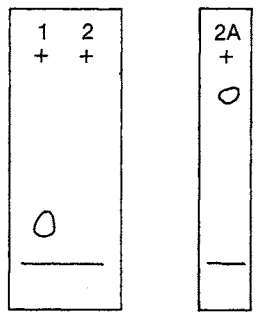
Results and Discussion

Deacylation of ECB

Fermentation broths of *A. utahensis* possessed no intrinsic antibiotic activity. The addition of ECB, an antifungal agent, to the whole broth immediately instilled antifungal activity which decreased progressively during continued incubation with *A. utahensis*. This reduction in bioactivity suggested modification, possibly deacylation, of the ECB by *A. utahensis*. Because ECB did not possess characteristic UV fluorescence, the nucleus also could not be monitored by UV. Further, an ECB nucleus standard was not available at this time. In order to determine whether the ECB inactivation actually resulted from removal of the linoleic acid acyl side chain, the inactivated culture broth was acylated with myristoyl chloride. Acylation restored antifungal activity, suggesting the presence of an authentic nucleus on which the natural linoleoyl side chain had been replaced by a myristoyl acyl unit.

Additional putative evidence of deacylation was obtained by monitoring samples through descending adsorption chromatography on Whatman No. 1 paper in butanol - acetic acid - H_2O (3 : 1 : 1), with bioautography vs. *Neurospora crassa*. The Rf value of ECB in this system was 0.82. The bioinactive ECB nucleus was not directly detectable by this method. However, when developed chromatograms containing samples of inactivated ECB culture broths were acylated with myristoyl chloride prior to bioautography, new zones of inhibition with an Rf value of 0.2 were observed (Fig. 2). This likewise indicated restoration of bioactivity through reacylation of the bioinactive nucleus.

Fig. 2. Bioautographic chromatograms of ECB, bioinactivated ECB (nucleus), and bioinactivated ECB acylated with myristoyl chloride.



Solvent system was butanol - acetic acid - H_2O (3 : 1 : 1). Lane 1: ECB, lane 2: bioinactivated ECB, lane 2A: duplicate of lane 2 acylated with myristoyl chloride prior to bioautography.

Purification of Putative ECB Nucleus

Initial purification samples were monitored with the acylation reaction to determine the presence of acylable material presumed to be nucleus. This procedure permitted development of a purification scheme involving adsorption of the bioinactive ECB from fermentation centrates onto Diaion HP-20 resin and further purification through reverse-phase HPLC. As a result, the putative ECB nucleus was successfully isolated from *A. utahensis* fermentation broths containing bioinactivated ECB (Fig. 3).

Paper chromatography was later replaced by an analytical HPLC system that was thereafter employed for the identification and quantitation of both ECB and the ECB nucleus (Fig. 4).

Structure Proof of ECB Nucleus

Characterization of the putative nucleus was accomplished by several techniques. The UV and IR spectra of ECB and the inactivated ECB were found to be virtually identical except that the IR spectrum of the latter lacked an adsorption band at 2900 cm^{-1}. This band is characteristic of the carbon-hydrogen stretch of fatty acid chain methylene groups. Titration data indicated ECB contained no titratable groups while the nucleus contained one titratable component. Formation of one such unit would be expected through replacement of the acyl fatty acid side chain with a proton, producing a primary amino group. Amino acid analyses of ECB and inactivated ECB were identical. The inactivated ECB was then reacylated with linoleic acid, the side chain naturally present on ECB. The NMR spectra of the natural ECB and the inactivated ECB reacylated with linoleic acid were identical. These data indicated that, except for the formation of a primary amino group at the *N*-terminus through addition of a proton, removal of the linoleoyl side chain of ECB by *A. utahensis* did

Fig. 3. Purification scheme for isolation of ECB nucleus.

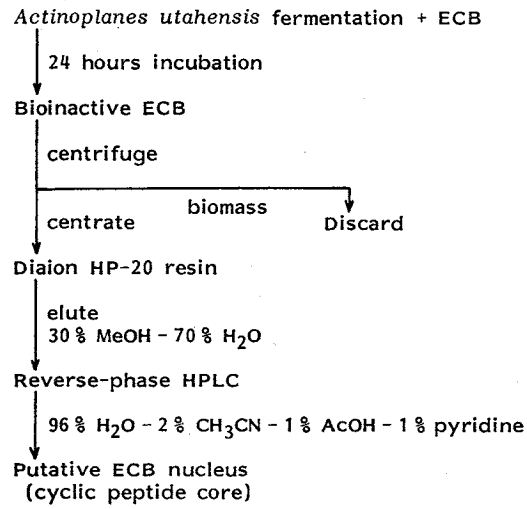
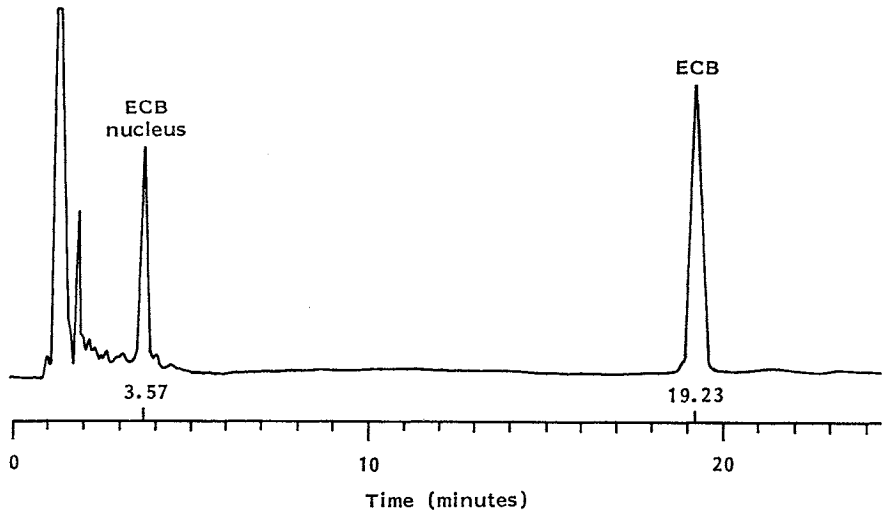


Fig. 4. HPLC of ECB and ECB nucleus.



Column: NOVA C18, 8×100 mm; flow rate: 2 ml/minute; detection: 225 nm (UV) (Waters Associates); dual mobile phases: A=3% CH_3CN - 0.5% $NH_4H_2PO_4$ - H_2O, B=50% CH_3CN - 0.5% $NH_4H_2PO_4$ - H_2O; gradient: 5% B for 3 minutes, Waters Associates gradient curve No. 6 for 12 minutes, 100% B for 7 minutes.

not otherwise alter the cyclic peptide core. Reacylation reversibly restored the bioactivity and chemical integrity of natural ECB.

New derivatives of ECB were prepared by chemical reacylation of the purified nucleus with various acyl groups. Details of their preparation and structure-activity relationships, including the *N*-(4-*n*-octyloxybenzoyl) derivative known as cilofungin (LY121019), are reported separately[6,8].

Effect of Broth pH on Bioconversion of ECB to Nucleus

The pH of *A. utahensis* broth was known to affect bioactivity reduction, deacylation rate, and nucleus conversion efficiency of A21978C. Conversion of the lipopeptide antibiotic to the cyclic peptide nucleus through deacylation resulted in a concurrent loss of antibiotic activity. However, bioactivity reduction also occurred through alkaline hydrolysis of the cyclic peptide and/or through non-specific degradation under certain conditions. Therefore, bioactivity reduction alone did not unambiguously indicate quantitative conversion to the nucleus[3]. The putative deacylation suggested by bioactivity reduction was consequently confirmed by quantitative HPLC measurements of the nucleus actually formed.

As broth pH values were increased between 5.5 and 6.5 during the deacylation of ECB, the rate of bioactivity reduction likewise increased. At pH 7.0 to 8.0, bioactivity was virtually eliminated (Table 1). ECB conversion to the ECB nucleus proceeded most efficiently, with the highest levels of nucleus production, at pH 6.5. Conversion efficiency at lower pH values was good, but progressed much more slowly. At higher pH values, increasingly larger percentages of the ECB and/or nucleus were lost, either through opening of the peptide ring or through undetermined methods of degradation.

Stability of ECB and ECB Nucleus

The bioactivity loss during deacylation at various pH values suggested stability evaluation of ECB and ECB nucleus in the absence of the deacylating culture. ECB was stable in refrigerated methanolic extracts of the *Aspergillus* biomass, retaining 65% of the initial activity after a holding period of 21 days.

In phosphate buffer, levels of both ECB and ECB nucleus were only slightly reduced at pH 6.0. The rate of loss approximately doubled with each increase of 0.5 pH unit, with 43% of the ECB and 59% of the nucleus being lost within 24 hours at pH 8.0 (Table 2). The ECB nucleus was less stable at all pH values than the parent compound.

Aqueous Solubility of ECB

Purified ECB was only slightly soluble in aqueous agents but was highly soluble in ethanol and PEG 300. However, ethanol levels exceeding 2.5% in the reaction medium reduced the

Table 1. Effect of *Actinoplanes utahensis* broth pH on bioconversion of ECB to nucleus.

Broth pH	Bioactivity reduction[a] (%)	Nucleus produced (μg/ml)	Conversion efficiency[b] (%)
5.5	48	340	80
6.0	70	478	78
6.5	90	710	89
7.0	>98	591	68
7.5	>98	451	52
8.0	>98	302	36

[a] Initial ECB concentration was 1,980 μg/ml. Preparation was 76% pure.
[b] 24 hours incubation at 30°C.

Table 2. Effect of pH on stability of ECB and ECB nucleus in aqueous buffer[a].

pH value	Reduction (%)	
	ECB	ECB nucleus
6.0	2	3
6.5	4	7
7.0	9	16
7.5	19	29
8.0	43	59

[a] 24 hours, 30°C, 10^{-1} M phosphate.

Table 3. Effect of pH and borate addition on solubility of ECB in *Actinoplanes utahensis* fermentation broth.

pH value	Suspending agent		ECB solubility[a] (%)
	A. utahensis filtrate	Borate (0.01 M)	
6.5	+		4.1
7.3		+	98.1
7.3	+	+	97.5
6.5	+	+	3.8

[a] Pure preparation, addition level 1.25 μg/ml.

Table 4. Effect of substrate purity on bioconversion of ECB to ECB nucleus by *Actinoplanes utahensis*.

Substrate purity (% ECB)	Nucleus produced (μg/mg biomass/hour)
6	0.7
34	4.3
67	8.9
97	12.7

deacylation rate, as did PEG 300 levels exceeding 1%. When ECB was solubilized in either of these agents and pulsed into aqueous media at low solvent levels, the ECB immediately precipitated from the media. While studying the effects of various buffering agents, ECB was found to be soluble in 10^{-2} M borate under slightly alkaline conditions (K. MICHEL; personal communication). Addition of borate to the *A. utahensis* fermentation medium likewise resulted in almost total solubilization of the ECB, but only when the pH value was maintained at or above approximately 7.3 (Table 3).

Effect of Substrate Purity on Conversion of ECB to ECB Nucleus

Because deacylation of A21978C has been accomplished with semi-pure preparations, the effect of substrate purity on deacylation of ECB was also investigated. ECB was deacylated and converted to ECB nucleus even in highly impure preparations containing as little as 6% of the compound. However, ECB preparations with higher purity were deacylated more rapidly (Table 4).

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