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Biotransformation of lantadene A (22β-angeloyloxy-3-oxoolean-12-en-28-oic acid), the pentacyclic triterpenoid, by *Alcaligenes faecalis*

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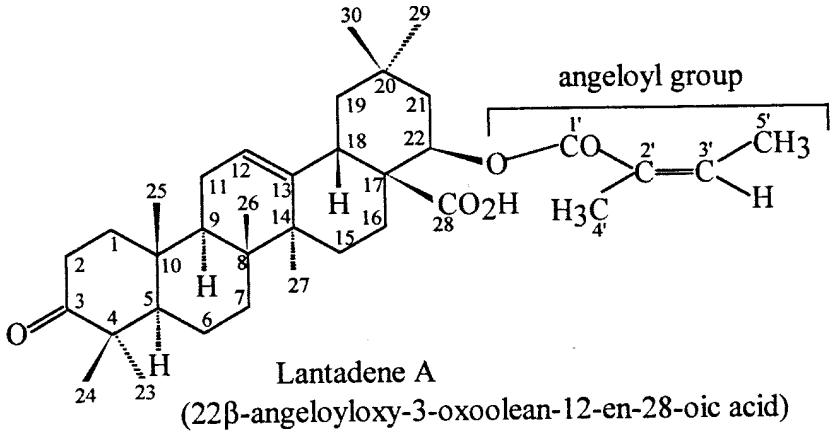
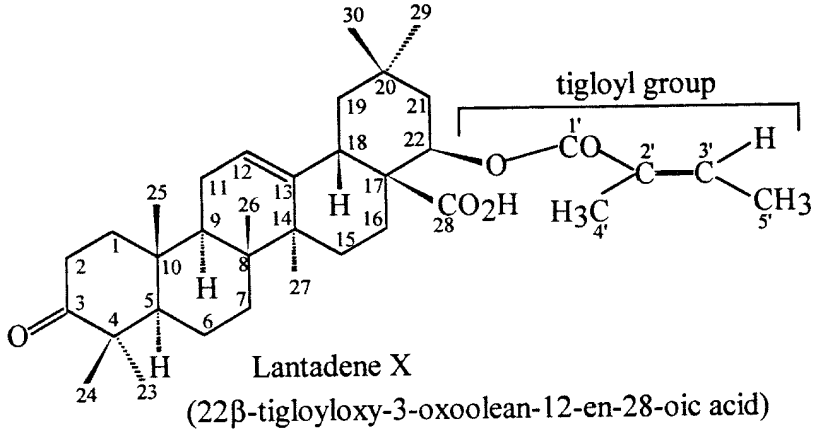
Abstract

A bacterial strain capable of biotransformation of lantadene A (22β-angeloyloxy-3-oxo-olean-12-en-28-oic acid), the pentacyclic hepatotoxin of lantana (*Lantana camara* var. *aculeata*) has been isolated from soil using lantadene A as the sole carbon source. The organism is Gram negative, rod shaped, motile, catalase positive and has been identified as *Alcaligenes faecalis*. The isolate has been found to be specific for lantadene A and did not utilize lantadene B. In studies using sucrose as an additional carbon source, *A. faecalis* elicited biotransformation of lantadene A to its trans isomer 22β-tigloyloxy-3-oxoolean-12-en-28-oic acid, designated as lantadene X and two other minor metabolites which could not be isolated in pure state.

Introduction

Lantadenes are the pentacyclic triterpenoids present in the leaves of the hepatotoxic plant *Lantana camara* (Hart et al. 1976; Sharma et al. 1988; Sharma & Sharma 1989). Lantadene A followed by lantadene B (Figure 1) are the major constituents in different taxa of lantana plants (Hart et al. 1976; Sharma et al. 1991b; Sharma et al. 1997b). Lantadene A induced hepatotoxicity in guinea pigs and sheep (Pass et al. 1979; Sharma et al. 1991a). In addition, lantadenes and other triterpenoids have a number of biological activities like inhibition of activation of Epstein–Barr virus, anti-AIDS, anti-inflammatory, antimicrobial, antitumor and antimutagenic (Fujioka et al. 1994; Nick et al. 1994; Pensupart et al. 1994; Wang, 1994; Inada et al. 1995; Liu, 1995; Barre et al. 1997; Inada et al. 1997; Safayhi & Sailer 1997; Verma et al. 1997). The current strategy for the management of plant toxicosis problem in grazing animals includes isolation of microbes with capacity for biodegradation of toxins and transferring this property to rumen anaerobes (Gregg et al. 1994; Selinger et al. 1996). Further, in view of a number of biological properties of pentacyclic triterpenoids, lantadenes are attractive natural products for biotransformation to new drugs (Hutchinson 1994; Scott 1994). Cyclic triterpenoids are recalcitrant to biodegradation and biotransformation (Krasnobajew, 1984). Biodegradation of many xenobiotics and recalcitrant natural products, though usually slow, has been achieved by enrichment techniques (Cook et al. 1983). Indeed, we have, earlier reported an organism *Pseudomonas picketii* which elicited biodegradation of lantadene A (Sharma et al. 1997a). In further search of microbial systems for biotransformation of lantadene A, we report here another organism *Alcaligenes faecalis* which utilized lantadene A as sole carbon source and elicited biotransformation in the presence of sucrose as cosubstrate.





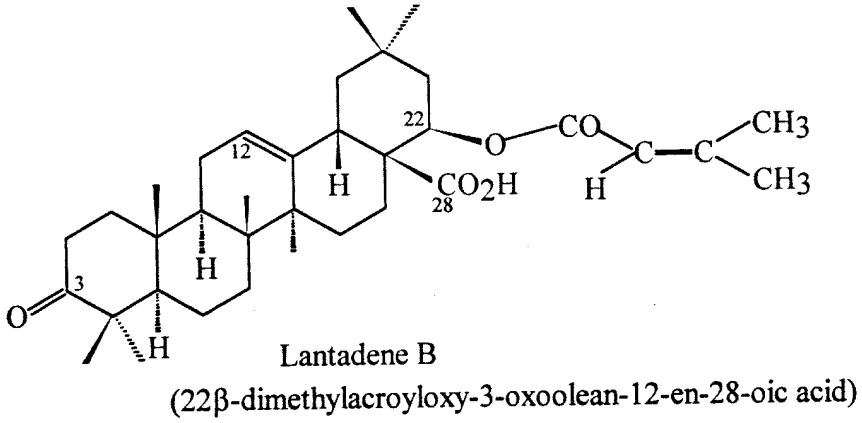


Figure 1. Chemical structures of lantadene A, lantadene X (metabolite M1) and lantadene B.

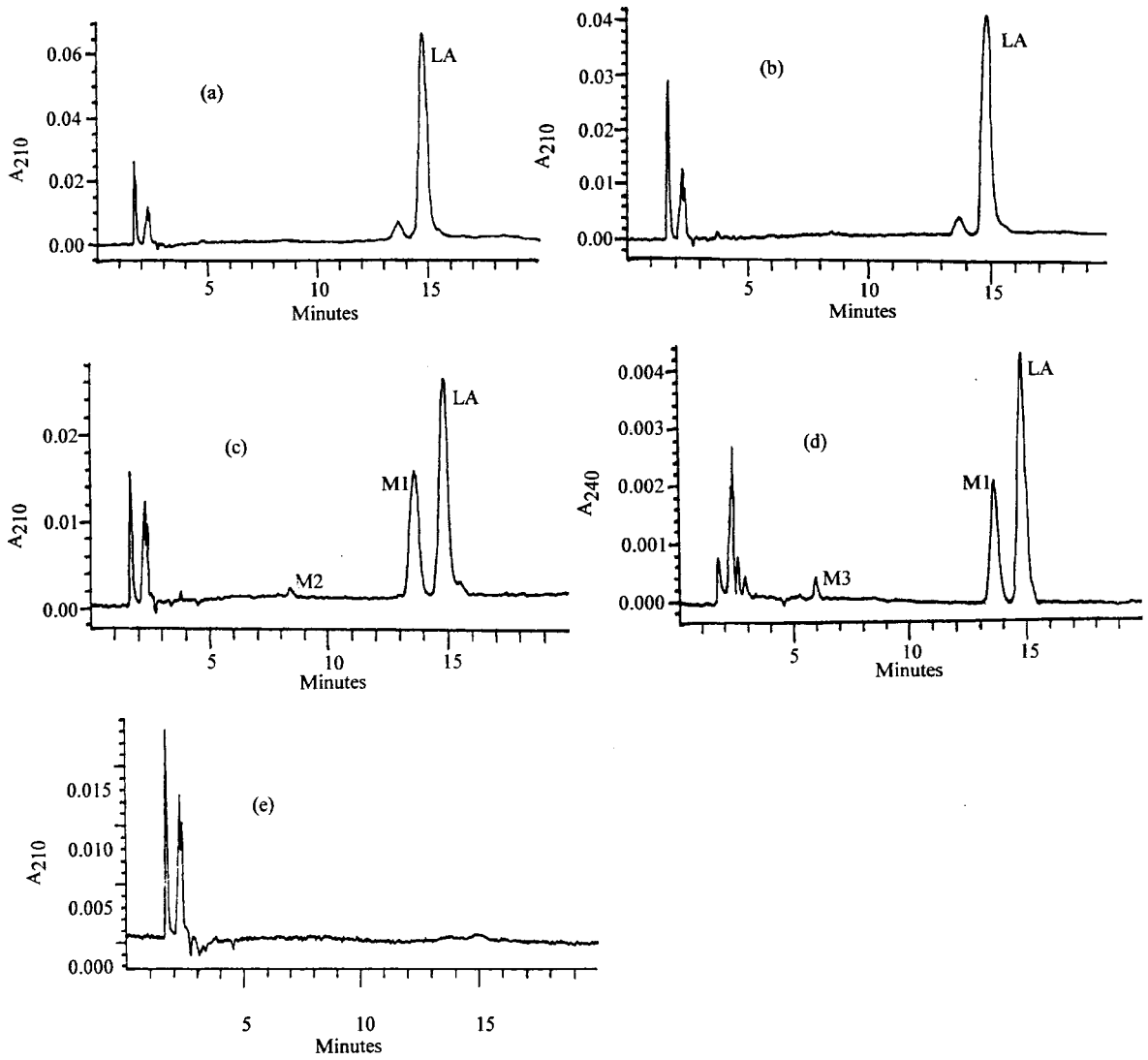


Figure 2. HPLC profile of the extracts of different fermentation sets for the residual lantadene A and metabolites. LA, lantadene A. M1, M2 and M3, metabolites. (a) Profile of the extracts of the set: BSS + LA + Sucrose (20 mM), control, uninoculated); (b) Profile of the extract of the set: BSS + LA (inoculated); (c) Profile of the extract of the set: BSS + LA + Sucrose (20 mM, inoculated); absorbance at 210 nm; (d) Profile of the extract of the set: BSS + LA + Sucrose (20 mM, inoculated); absorbance at 240 nm; (e) Profile of the extract of the set: BSS + Sucrose (20 mM, inoculated).

Materials and methods

Preparation of lantadene A

Lantadene A (LA) and lantadene B (LB) were prepared from the leaves of*Lantana camara* var. *aculeata* (Barton et al. 1954; Barton et al. 1956; Sharma et al. 1991a). For the enrichment and fermentation studies, LA sample was dissolved in acetone and suitable aliquots were transferred into 250 ml Erlenmeyer flasks. The solvent was removed *in vacuo* at 40 °C and the residue was mixed with a basal salt solution.

Soil sample and preparation of inoculum

A soil sample (10 g) collected from the vicinity of lantana bushes near the laboratories of the Indian Veterinary Research Institute, Regional Station Palampur, India, was mixed with sterile phosphate-buffered saline (PBS, pH 7.2, 50 ml). The mixture was stirred and left undisturbed until the heavier particles settled

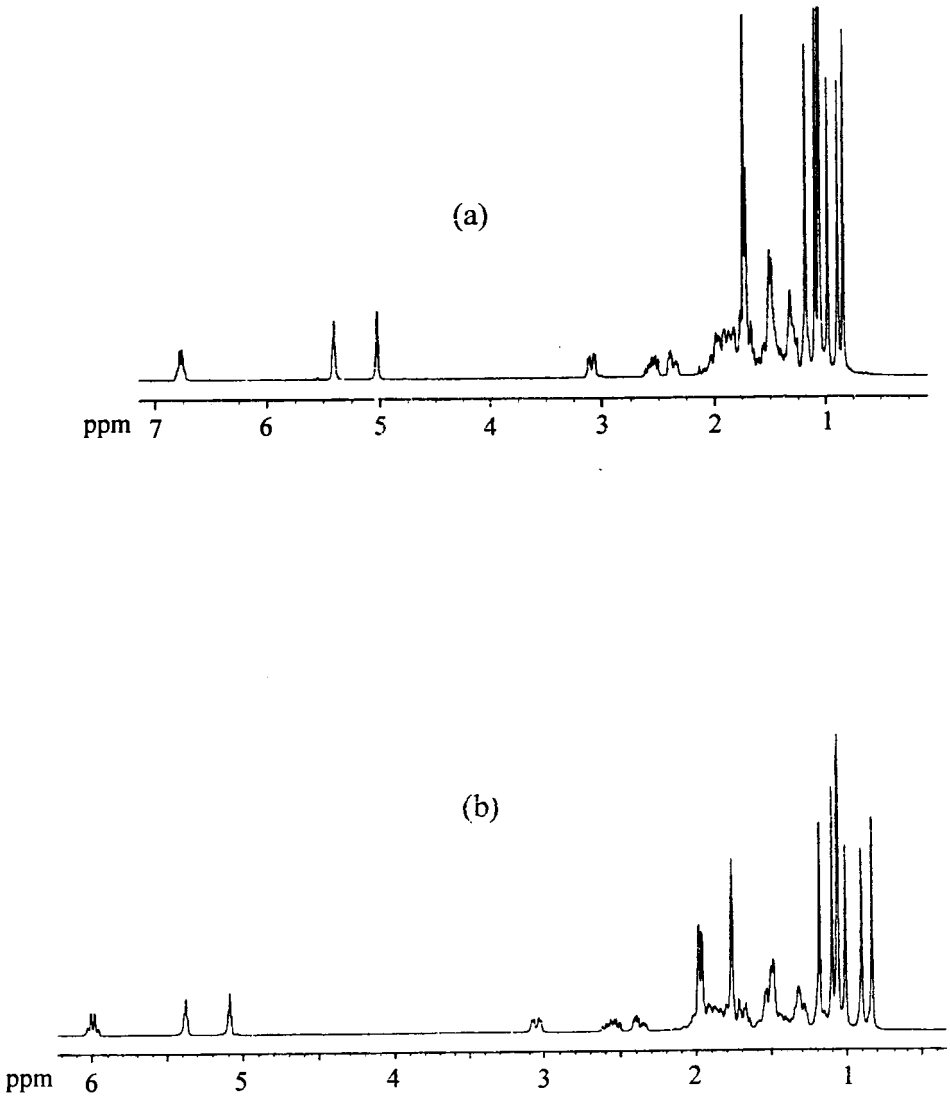


Figure 3. ^{1}H-NMR spectra of (a) lantadene X, (b) lantadene A.

down. The supernatant was decanted off and used as inoculum for enrichments (Harder 1981; Cook et al. 1983).

Enrichment

Enrichment studies were done at pH 6.5 using LA as the sole carbon source. Basal salt solution (BSS) contained (g/L) sodium nitrate, 2.0; potassium dihydrgen phosphate, 1.0; magnesium sulphate, 0.5; potassium chloride, 0.5 and ferrous sulphate, 0.01. Solid medium contained 0.025% LA and 2% noble agar. LA suspension in BSS was sterilized by autoclaving in 250 ml Erlenmeyer flasks. LA is heat stable and did not undergo any decomposition or autooxidation under these conditions (Sharma et al. 1997a). The inoculum (1 ml) was added to the sterilized medium and the flasks were incubated at 30 °C and 120 rpm in the orbital shaker for 7 days. Fresh medium was subcultured nine times for the selection of the microorganism specific for LA. The culture was further purified by streaking on solid medium containing LA as the sole carbon source. The pure culture was transferred to liquid medium containing LA in BSS.

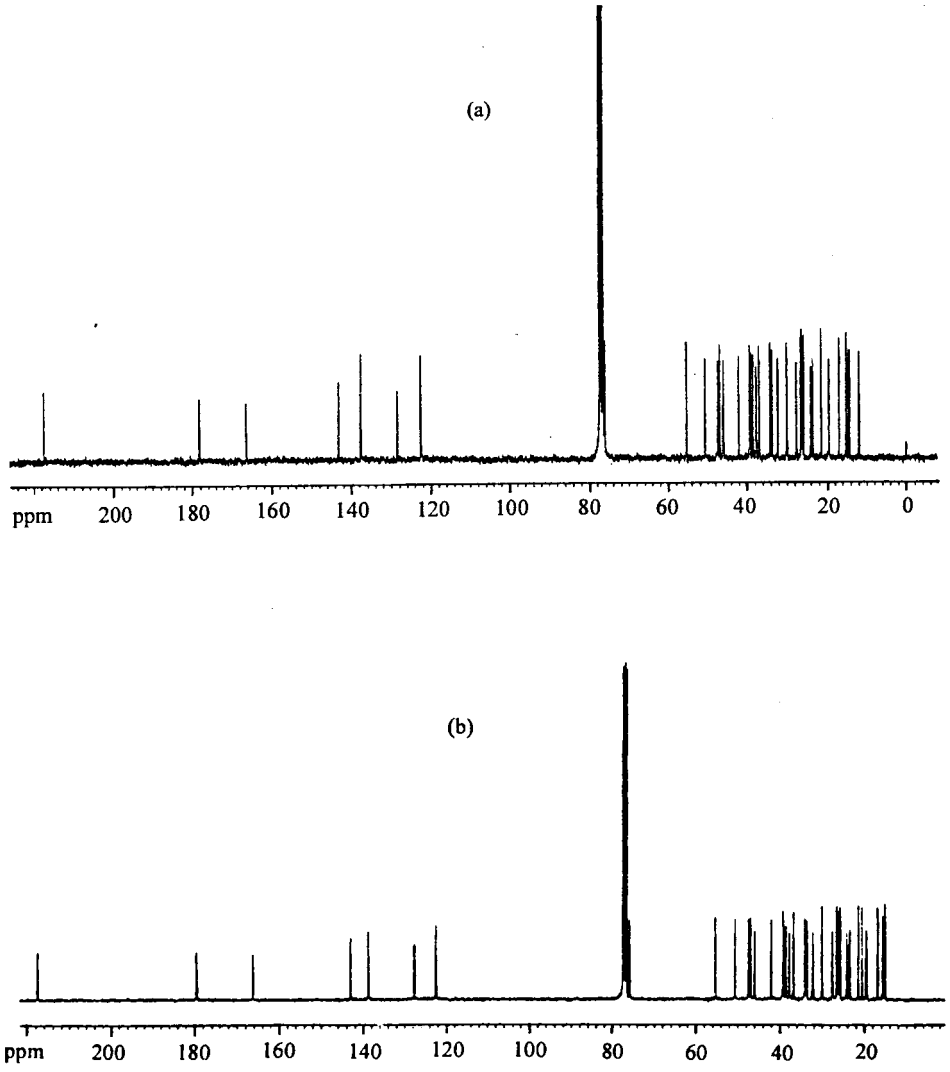


Figure 4. ^{13}C-NMR spectrum of (a) lantadene X, (b) lantadene A.

Identification

The culture was identified by the MTCC and Gene Bank Division of the Institute of Microbial Technology, Chandigarh, India.

Fermentation studies

BSS (20 ml) containing LA (3 mg) was autoclaved in 250 ml Erlenmeyer flasks. The flasks were inoculated with the liquid culture of the isolate, *A. faecalis* (12 μg protein) and incubated for 15 days at 30 °C and 120 rpm in the orbital shaker. The fermentation broth was extracted with ethyl acetate for assay of residual lantadenes and putative metabolites. In parallel studies, 20 mM sucrose was used as cosubstrate for the metabolism of LA. A liquid culture of the isolate was also used to ascertain if it could utilize LB as the sole carbon for its growth.

Analytical methods

Bacterial growth was measured by protein estimation using bovine serum albumin as a standard (Lowry et al. 1951; Cook et al. 1983). Fermentation broths were acidified to pH 2.0 using 1N HCl and extracted twice with equal volumes ethyl acetate. The extracts were combined and dried using sodium sulphate. Suitable aliquots of ethyl acetate extract were dried *in*

Table 1. Lantadene A content and microbial growth (microbial protein) on incubation with *Alcaligenes faecalis*

Experimental set	Lantadene A (mg)	Loss of lantadene A (%)	Protein (mg)
BSS + LA (uninoculated) (control)	2.8 ± 0.1	–	0.0
BSS + LA (inoculated)	1.7 ± 0.4	40.9 ± 15.2	0.3 ± 0.01
BSS + LA + sucrose (20 mM) (inoculated)	0.8 ± 0.3	70.4 ± 9.4	20.3 ± 1.9

Incubation time, 15 days. Amount of LA added to each set was 3 mg.
The values are mean ±S.D. of three observations. The organism did not utilize LB.

vacuo, the residue was redissolved in methanol and filtered through Whatman stainless steel filtration assembly and Millipore membrane filter (0.22 μm). Twenty μl aliquots were used for the HPLC analysis of lantadenes and the putative metabolites using Waters HPLC system: column C18 (Nov-Pak 4μ, 4.6 × 250 mm); 510 pump, Rheodyne injector, 490 E multichannel detector and Millennium 2010 data processor. The elution was done using the mobile phase methanol : acetonitrile : water : acetic acid (71 : 20 : 9 : 0.01) at a flow rate of 1 ml/min. LX and LB were not resolved in this mobile phase. The mobile phase for the resolution of LX and LB was acetonitrile : water : acetic acid (70 : 30 : 0.01) at a flow rate of 1.5 ml/min. The monitoring was done at 210 and 240 nm (Sharma et al. 1997b). All the chemicals were of analytical or HPLC grade.

Chemical characterisation of metabolite M1

The metabolite M1 was purified on silica gel (60–120 mesh) column using the solvent mixture petroleum ether (60–80 °C): ethyl acetate: acetic acid (95 : 3 : 2). The fractions enriched in M1 were pooled and the solvent was removed *in vacuo*. The residue was crystallised using methanol and subjected to spectroscopic analysis. Assignment of ^{13}C NMR δ-values of LA and M1 were made with the help of DEPT (distortionless enhancement by polarization transfer) studies, known chemical shift rules (Stothers 1972; Wehrli & Wirthlin 1980) and by comparison with spectra of appropriate pure pentacyclic triterpenoids (Mahato & Kundu 1994).

Results and discussion

A set of flasks containing lantadene A as the sole carbon source was started for enrichment at 30 °C and 120 rpm in the orbital shaker. The culture was subcultured into fresh medium containing LA as the sole carbon source for the selection of the organism which utilized lantadene A. The growth of the isolate on solid medium containing LA as the sole carbon source was very slow. Very few colonies appeared on the LA-agar plates by 48 h. Distinct growth was seen on incubation for 7 days on LA-agar medium. Earlier workers have also observed that growth of isolates on solid medium containing recalcitrant compounds as the sole carbon source is very slow or no growth occurs at all (Cook et al. 1983; Stucki et al. 1983; Sharma et al. 1997a). The culture lost the degradative capacity on transfer to nutrient agar plates. For maintaining the degradative capacity, the isolate was maintained in liquid medium in the presence of LA as the sole carbon source (Cook et al. 1983; Sharma et al. 1997a).

The LA degrading isolate was Gram negative, motile, and rod shaped. The growth on MacConkey agar showed the isolate to be a non-lactose fermenter. The isolate gave the following positive tests: catalase, cytochrome oxidase, lysine decarboxylase and ornithine decarboxylase. It was able to hydrolyse starch and produce H_2S. The isolate was identified *Alcaligenes faecalis* (MTCC 3134).

For investigations on the biotransformation of LA, two parallel sets of flasks, one containing LA as the sole carbon source and the other containing 20 mM sucrose as cosubstrate were started. Uninoculated controls were incubated simultaneously to check autooxidation. The residual LA content and the microbial growth, on incubation with *A. faecalis* for 15 days, are given in Table 1. There was 41% loss of LA within 15 days when incubations were done in the presence of LA as the sole carbon source. There was complete loss of LA when the incubations were done for as long as 2 months. No metabolite could be detected when LA was used as the sole carbon source (Figure 2b), imply-

ing thereby that *A. faecalis* caused mineralization of lantadene A in the absence of any cosubstrate.

There was 70% loss of LA within 15 days when incubations were done in the presence of 20 mM sucrose (Table 1, Figure 2c). Three compounds M1, M2 and M3 with retention time 13.7, 8.4, and 6.0 min in the mobile phase methanol:acetonitrile : water : acetic acid (71 : 20 : 9 : 0.01) were detected from the extracts of the fermentation sets which contained sucrose as the cosubstrate (Figure 2 c,d). No peak corresponding to M1, M2, M3 was observed in the control fermentation set BSS + sucrose inoculated with *A. faecalis* (Figure 2e). This implied that the compounds M1, M2, M3 did not result from the bacterial mass but were the biotransformation products of LA. M1 had a retention time comparable to that of LB in mobile phase methanol:acetonitrile : water : acetic acid (71 : 20 : 9 : 0.01) but differed in channel ratio (A_{210}/A_{240}). However, M1 could be resolved from LB in the HPLC analysis using the mobile phase acetonitrile : water : acetic acid (70 : 30 : 0.01), at the flow rate 1.5 ml/min. The retention times of M1, LB and LA were 37.1, 39.1 and 41.9 min respectively. M3 did not absorb at 210 nm and could be detected during monitoring at 240 nm (Figure 2d). The chemical structure of M1, the major metabolite was elucidated as 22β-tigloyloxy-3-oxoolean-12-en-28-oic acid (Figure 1) by spectroscopic methods and the compound has been designated as lantadene X (LX). The UV and IR spectra of LX were similar to those of LA. The mass spectrum of LX showed the molecular ion peak at m/z 552 which is the same as that of LA. The ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of LX (Figure 3a) displayed discernible peaks at δ 0.84 (3H, s), 0.89 (3H, s), 0.98 (3H, s), 1.05 (3H, s), 1.06 (3H, s), 1.09 (3H, s), 1.18 (3H, s), 1.71 (3H, 5′-H), 1.76 (3H, d, J = 7Hz, 4′-H), 5.02 (1H, t, 22-H), 5.41 (1H, t, 12-H) and 6.77 (1H, q, J = 7Hz, 3′-H). The ^{1}H NMR spectrum of LA (Figure 3b) showed peaks at δ 0.83 (3H, s), 0.89 (3H, s), 1.00 (3H, s), 1.05 (3H, s), 1.06 (3H, s), 1.09 (3H, s). 1.18 (3H, s), 1.76 (3H, s, 5′-H), 1.96 (3H, d, J = 7Hz, 4′-H).

The ^{13}C NMR chemical shifts of LX and LA (Figures 4) are shown in Table 2. It is evident that the ^{1}H and ^{13}C NMR data of LX and LA are similar for the ring systems but differ in the values of the acyl moieties. This difference is rationalized by assuming the biotransformation of the angeloyl group of LA to the tigloyl group (Figure 1) by the strain of *A. faecalis*. M2 and M3 could not be characterized due to paucity of materials. The isolate did not grow on LB, an isomer of LA (Figure 1, Table 1). It appears that LA which has an angeloyl acyl group with *cis* configuration at C-22 is converted to tigloyl moiety with *trans* configuration (Figure 1) by a *cis* to *trans* isomerase generated by *A. faecalis*. A similar *cis-trans* isomerisation has been observed for the conversion of maleylacetoacetate to fumarylacetate (Rodwell 1993). LX is a novel pentacyclic triterpenoid and its biological activity is not known so far.

Table 2. ^{13}C NMR chemical shifts δc ($\pm$0.1) of metabolite M1 (lantadene X) and lantadene A in $CDCl_3$

Carbon No.	Lantadene X	Lantadene A
1	39.2	39.1
2	34.1	34.1
3	217.5	217.5
4	47.5	47.5
5	55.4	55.3
6	19.6	19.5
7	32.3	32.2
8	39.3	39.3
9	46.9	46.9
10	36.8	36.8
11	23.6	23.5
12	122.6	122.5
13	143.2	143.1
14	42.1	42.0
15	27.6	27.6
16	24.0	24.2
17	50.7	50.6
18	38.5	38.5
19	46.0	46.0
20	30.0	30.0
21	36.8	36.8
22	76.2	75.9
23	26.5	26.4
24	21.5	21.4
25	17.0	16.9
26	21.5	20.5
27	26.2	26.1
28	178.3	179.6
29	33.7	33.6
30	25.8	25.8
1′	166.6	166.3
2′	128.5	127.7
3′	137.7	138.8
4′	11.9	15.1
5′	14.4	15.6

A. faecalis is known to degrade compounds with aromatic structures, e.g., phenanthrene, aryl group containing xenobiotics and chlorobenzoates (Kiyohara et al. 1990; Dimkov and Topalova 1993; Grover et al. 1993). *A. faecalis* KK314 degraded polyvinyl alcohol (Matsumura et al. 1994). In addition, *A. faecalis* degraded phenol, *p*-cresol but not resorcinol (Shivaraman 1992). The present study demonstrates the biotransformation of LA, a compound without any aromatic nucleus (Figure 1), by *A. faecalis*. The property of microbial biotransformation of plant toxins has been used for reducing forage toxicity in animals by genetic manipulation of gut microflora (Gregg et al. 1994; Gregg 1995). For example, a *Moraxella* sp. capable of degradation of the plant toxin fluoroacetate has been isolated from soil by enrichment (Kawasaki et al. 1981). Subsequently, the fluoroacetate dehydrogenase gene of *Morexalla* sp. has been successfully incorporated into rumen anaerobe *Butyrivibrio fibrisolvens* (Gregg et al. 1994; Gregg 1995). Thus, potential exists for future utilization of LA utilizing capacity of *A. faecalis* for gene manipulation of rumen bacteria (Gregg 1995). In addition, the isolate can be exploited for preparation of bioactive products by biotransformation of lantadene A (Hutchinson 1994, Scott 1994).

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