DISCOVERY, PRODUCTION AND PURIFICATION OF THE Na^+, K^+ ACTIVATED ATPASE INHIBITOR, L-681,110 FROM THE FERMENTATION BROTH OF *STREPTOMYCES* SP. MA-5038

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The maximum yield for the production of L-681,110 by *Streptomyces* sp. MA-5038 (ATCC 31587) was observed after 5 days' incubation at 28°C and pH about 8.3. L-681,110 was isolated from the fermentation broth by acetone extraction of the mycelia, absorption to Amberlite XAD-2 resin and two separations by thin-layer chromatography. The structure of L-681,110 was found to consist of a sixteen-membered lactone with a new type of substitution. The inhibition of ATPase, activity against *Caenorhabditis elegans* and stimulation of γ-amino-butyric acid release indicate that L-681,110 possesses some characteristics of both oligomycin and avermectin. L-681,110 was also active against tapeworm and ticks in an *in vivo* assay.

L-681,110 was discovered during the screening of fermentation broths for inhibitors of Na^+, K^+ activated ATPase. It was produced by *Streptomyces* sp. MA-5038[1]. The activity was associated with the mycelia. L-681,110 belongs to a new class of sixteen-membered macrocyclic lactones with a C_{14} side chain which incorporates a tetrahydropyran ring having both a hemiketal hydroxy and fumarate half-ester group as substituents. The detailed spectroscopic evidence and structure determination of L-681,110 have been published[2]. In this paper, we present the production, isolation and biological activity of L-681,110.

Materials and Methods

Culture Conditions

The microorganism used in this study, *Streptomyces* sp. MA-5038 (ATCC 31587) was obtained from a soil isolation program at the MSDRL, CIBE Laboratories, Madrid, Spain. A lyophil was inoculated into a seed flask containing 55 ml of seed medium with the following composition (g/liter): Dextrose 1.0, soluble starch 10, beef extract 3, yeast autolysate 5, NZ-amine E 5, $MgSO_4 \cdot 7H_2O$ 0.05, KH_2PO_4 0.18, Na_2HPO_4 0.19 and $CaCO_3$ 0.5, pH 7.0~7.2. Two ml of one day old seed culture was then inoculated into a production flask containing 50 ml of production medium with the following composition (g/liter): tomato paste 20, primary yeast 10, dextrin 20 and $CoCl_2 \cdot 6H_2O$ 0.005, pH 7.2~7.4. The flask was shaken on a rotary shaker at 220 rpm and 28°C for 4 days.

Assay of Na^+, K^+ Activated ATPase Inhibitory Activity

Na^+, K^+ activated ATPase from either porcine cerebral cortex or canine kidney was purchased from Sigma Chemical Co. Adenosine triphosphate (ATP), 99% pure was also obtained from Sigma Chemical Co. The methods as described by LEDEAUT *et al.*[3] and MARTIN and DOTY[4] were adapted for screening fermentation broths. The reaction mixture contained 5 mM adenosine triphosphate disodium salt, 0.05 M Tris-HCl pH 7.6, 0.1 M NaCl, 0.015 M KCl, 0.005 M $MgSO_4$ and 0.002 M EDTA. Routinely, 50 μl of the supernatant of 50% aqueous acetone extract of the whole broth and 0.9 ml of

reaction mixture was mixed and the enzyme reaction was initiated with 50 μl of enzyme (about 0.01 unit). The assay mixture was incubated at 37°C for 20 minutes, and the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid and cooled in an ice bath for 5 minutes. Two ml of water saturated 2-butanol was then added as was 0.5 ml of 3% ammonium molybdate and the tube was vortexed immediately. One ml of organic phase was transferred to another tube and 1.0 ml of acid alcohol (0.36 N sulfuric acid in alcohol) was then added as was 50 μl of 1.3% $SnCl_2$. The tube was vortexed and the blue color was measured at 750 nm.

In Vitro [^{3}H]GABA Release Assay

The method was as described by PONG *et al.*[5] P_2 synaptosomal fraction from fresh rat brain was used for all of the studies. [^{3}H]GABA (γ-aminobutyric acid, 70 ~ 100 Ci/mmol) was obtained from New England Nuclear. L-681,110 A_1 was tested at a concentration of 20 μM and avermectin B_{1a}[6] was tested at a concentration of 10 μM.

Assay of the Mobility of *Caenorhabditis elegans*

The detailed method was as described by PONG *et al.*[5] *C. elegans* N_2 (wild type) was used for the assay. The concentrations of L-681,110 for the test ranged from 10 to 100 μg/ml. The concentration of avermectin for the test ranged from 0.1 to 2 μg/ml. The compounds were dissolved in dimethyl sulfoxide, and then they were diluted into the assay mixture.

In Vivo Tapeworm Assay

The compound was given by gavage in polyethylene glycol 400 - dimethyl sulfoxide (1 : 2) to artificially infected laboratory rodents harboring 21 day old *Hymenolepis diminuta*. Six or seven days after treatment, the animals were sacrificed and the entire intestine was removed. The intestine was flushed with tap water over a 200-mesh sieve (Newark Wire Cloth) and the intestinal debris was examined for scolices and strobila.

Tick Infestations in Guinea Pigs

Hartley male guinea-pigs, weighing 600 ~ 800 g, were infected with nymphs of *Rhipicephalus sanguineus* contained in plastic capsules attached to the shaved flanks of the animals. The nymphs were allowed to attach to the shaved flanks of the animals. The nymphs were allowed to attach to the host one day before treatment of the host with a single topical treatment. The aqueous vehicle contained 5% acetone and 0.2% Triton X-100. Observations were made on the number of nymphs that engorged and detached from the host and the number that subsequently molted[6,7].

Results

Production of L-681,110 by *Streptomyces* sp.

The production of L-681,110 by *Streptomyces* sp. MA-5038 was studied. Both the effect of incubation time and the initial pH of the production medium on the yield of L-681,110 were monitored by inhibition of Na^+, K^+ activated ATPase and confirmed by silica gel thin-layer chromatography. The results are shown in Figs. 1 and 2. The production of L-681,110 reached a maximum after 5 days' incubation and the optimal initial pH for the production of L-681,110 is around 8.0 which was also optimum for growth of the producing organism.

Isolation and Characterization of L-681,110

The culture broth (900 ml) was mixed with an equal volume of acetone and filtered through Celite. The filtrate was evaporated under vacuum to remove the acetone. The aqueous suspension was passed through an Amberlite XAD-2 resin column (6 × 15.5 cm). The column was washed with water and eluted with 600 ml of methanol. The methanol eluate was evaporated and further purified by a preparative thin-layer chromatography on six silica gel thin-layer plates (60F-254, 2 mm). The plates were developed in $CHCl_3$ containing 17% methanol. The UV absorbing bands were scrapped from the

Fig. 1. Production of L-681,110 by *Streptomyces* sp. *versus* time of incubation.
● L-681,110 produced (μg/ml), △ glucose concentration (mg/ml), ○ cell dry weight (mg/ml).

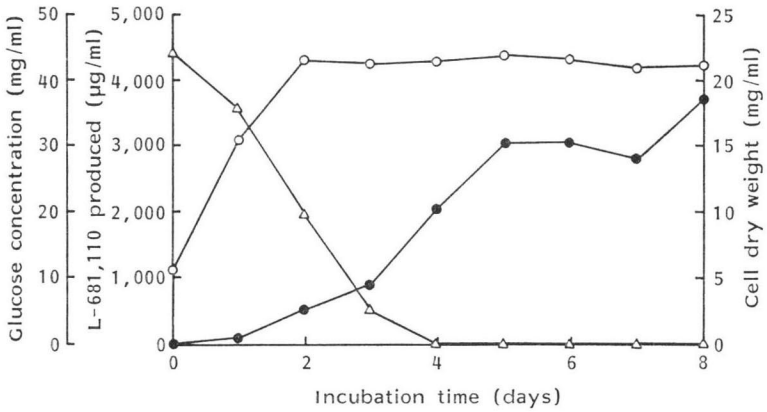
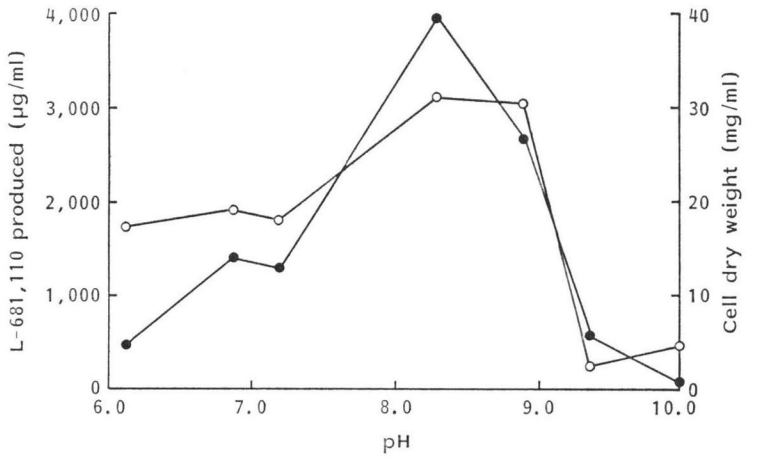


Fig. 2. Production of L-681,110 by *Streptomyces* sp. *versus* pH of medium.
● L-681,110 produced (μg/ml), ○ cell dry weight (mg/ml).



plates and eluted with $CHCl_3$ containing 12% methanol. The eluate was evaporated and the residue was further purified by a second silica gel thin-layer chromatography. The plates were developed in methylene chloride containing 10% methanol and 1% concentrated NH_4OH for 20 hours. The bands were eluted with $CHCl_3$ containing 10% methanol. The total purification of L-681,110 is summarized in Table 1. The final yield of L-681,110 A_1 was 539 mg and that of A_2 was 54 mg. The purification was monitored by inhibition of Na^+, K^+ activated ATPase. The I_{50} of A_1 and A_2 for Na^+, K^+ activated ATPase were estimated to be 7 μg/ml (1×10^{-5} M) and 79 μg/ml (1.4×10^{-4} M, Table 1). The structure was determined to be a sixteen-membered macrolide as shown in Fig. 3[2]. The molecular weight of A_1 is 720 with a molecular formula of $C_{38}H_{60}O_{12}$. It has UV (CH_3OH) 210 nm (ε 26,300), 246 (35,590), 284.5 (15,880) and mass spectrum *m/z* 586, 568, 525. The molecular weight of A_2 is 730 with a molecular formula of $C_{40}H_{62}O_{12}$ and A_2 has UV (CH_3OH) 209 nm (ε 24,170), 246 (32,870), 283 (14,120) and mass spectrum *m/z* 734 (M^+), 586, 568, 525. B_1 was also isolated as a by-product[2]. The molecular weight of B_1 is 636 with a molecular formula of $C_{36}H_{60}O_9$ and B_1 has UV (CH_3OH) 246 nm (ε 38,200), 284.5 (16,950) and mass spectrum *m/z* 618 ($M^+ - H_2O$), 600, 586, 568, 525.

Table 1. Purification of L-681,110 (A_1 and A_2).

Component	Weight (mg)	I_{50} (μg/ml)
Crude extract	7,500	80
XAD-2-purified	1,500	21.4
1st TLC purified	859	12
2nd TLC purified	539 (A_1)	7 (A_1)
	54 (A_2)	79 (A_2)

Table 2. Activity of L-681,110, ouabain, oligomycin and avermectin B_1 against Na^+, K^+ activated ATPase.

Compound	I_{50} (μM)
Ouabain	0.56±0.02
Oligomycin	4.1±0.1
L-681,110 A_1	13±1.4
Avermectin B_1 (94% B_{1a}+6% B_{1b})	929±97

Table 3. Activity of L-681,110 against *C. elegans*.

Sample	Component	I_{50} (μg/ml)
L-681,110	A_1	32.5
	B_1	15
Avermectin	83% B_{1a} 9% B_{1b}	0.4

Fig. 3. The proposed structure L-681,110.
Component A_1 R=H
A_2 R=OCH_3
B_1 R=H and substitution of OCH_3 for fumarate moiety

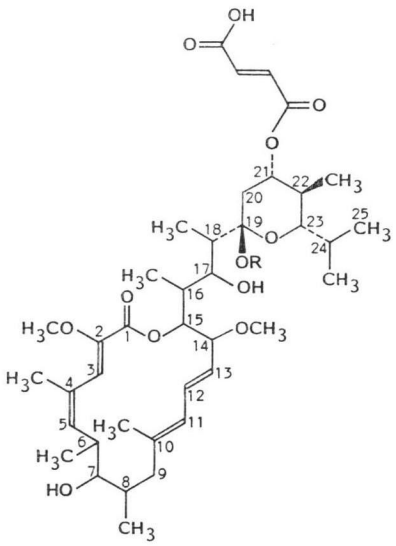
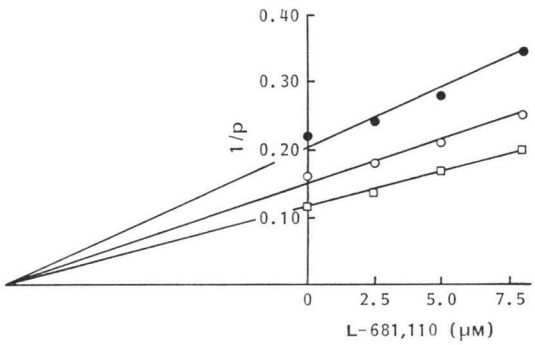


Fig. 4. The kinetic study of L-681,110 against Na^+, K^+ activated ATPase.

DIXON's plot of 1/p (phosphate released) *versus* the concentrations of L-681,110 at 0.5 mM ATP (●), 1 mM ATP (○) and 2 mM ATP (□). All samples were run in triplicate. The results were expressed in the average of the triplicate.

$Ki=11.2\times10^{-6}$ M$=1.1\times10^{-5}$ M.



Inhibition of Na^+, K^+ Activated ATPase

In the kinetic study of enzyme inhibition, L-681,110 was preincubated for 10 minutes at 37°C with the enzyme. The reaction was initiated by the addition of three different concentrations of ATP. The *Ki* of L-681,110 was determined by the DIXON's plot[9] and it was estimated to be 1.1×10^{-5} M as shown in Fig. 4. The I_{50}'s of ouabain[8], oligomycin[10], L-681,110 and avermectin B_{1a}[11~14] are shown in Table 2. L-681,110 is about one twentieth as potent as ouabain, one third as potent as oligomycin and about seventy times more potent than avermectin at inhibiting Na^+, K^+ activated ATPase.

Activity of L-681,110 against *C. elegans*

L-681,110 A_1, B_1 and avermectin were compared for activity against *C. elegans*. The results are shown in Table 3. The I_{50} of L-681,110 A_1 is estimated to be 32.5 μg/ml and that of L-681,110 B_1 is about 15 μg/ml. They are about 1/49 to 1/80 of the potency of avermectin. No activity can be detected with oligomycin under the same concentration.

Fig. 5. Stimulation of release of GABA from brain synaptosomes by L-681,110 A_1 (20 μM) (▲), avermectin B_{1a} (10 μM) (○) and baseline (□).

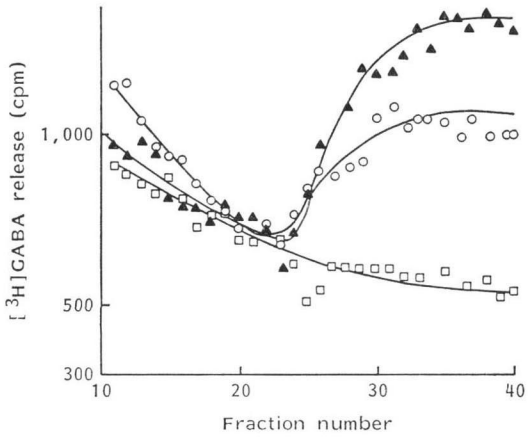


Table 4. Activity of L-681,110 and avermectin against ticks, *R. sanguineus*.

Sample	Concentration (μg/ml)	Efficacy of treatment (%)	ED_{50} (μg/ml)
L-681,110 A_1	Topical 900	57	771
Avermectin B_{1a}	4.1	77	1.8

Stimulation of Release of GABA from Brain Synaptosomes

Both L-681,110 A_1 and avermectin B_{1a} exhibited very good stimulation of release of GABA from brain synaptosomes, when they were tested at a concentration of 10 μM to 20 μM (Fig. 5). Ouabain and oligomycin, the potent Na^+, K^+ activated ATPase inhibitors, do not show any activity at a concentration of 20 μM.

Tapeworm Activity

L-681,110 A_1 was tested in artificially infected rodents as described in "Materials and Methods". It was active at 12.5 mg/kg but not at 7.5 mg/kg. Ouabain, oligomycin and avermectin B_1 were inactive at both dosage levels.

Activity of L-681,110 against Ticks

Purified L-681,110 A_1, was tested at 900 μg/ml, where it reduced the number of viable ticks by 57%. This is about one four-hundredth of the activity of avermectin B_1. As shown in Table 4, the ED_{50} for purified L-681,110 A_1 is about 771 μg/ml and that of avermectin B_1 is about 1.8 μg/ml. The results of the ticks assay are statistically significant with $P<0.025$ for L-681,110 A_1 and $P<0.001$ for avermectin B_1.

Discussion

Cardiac glycosides, the potent inhibitor of Na^+, K^+ activated ATPase have been implicated as a cardiotonic agent[15,16] and an insecticide[17,18]. Obviously we screen the broths for the inhibitor of Na^+, K^+ activated ATPase with the hope that the inhibitor will show some cardiotonic activity and some protection against insects. L-681,110 shows some activity against tapeworms and ticks, but does not have any *in vitro* cardiotonic activity (unpublished observations). L-681,110 shares features of oligomycin in its ability to inhibit Na^+, K^+ activated ATPase and of avermectin in its ability to inhibit *C. elegans* and to stimulate the release of GABA from brain synaptosomes. Neither oligomycin nor avermectin have activity against the tapeworm, *Hymenolepis diminuta*, whereas L-681,110 exhibits significant activity against this parasite. There are some structural similarities among the three compounds, L-681,110, avermectin and oligomycin. This may explain their broad activities. It would require more extensive studies with these compounds and other analogues to determine which structural features are responsible for the various activities.

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