THIELOCINS A1α AND A1β, NOVEL PHOSPHOLIPASE A_2 INHIBITORS FROM ASCOMYCETES

Sir:

Phospholipase A_2 (PLA_2, EC 3.1.1.4) specifically hydrolyze the sn-2 ester bond of glycerophospholipids to liberate arachidonate which profoundly influence inflammatory reactions. The reguratory molecules of this enzyme activity would be implicated in the control of a wide range of physiological and pathological states such as inflammation, asthma, ischemia, psoriasis, pancreatitis and rheumatoid arthritis[1]. In the course of a screening for PLA_2 inhibitors in our laboratories[2], we found novel potent inhibitors named thielocins A1α and A1β† in the culture broth of an ascomycetes, *Thielavia terricola* RF-143. In this communication, we report production, isolation, structural determination and inhibitory activity on PLA_2.

A slant culture of the organism was inoculated into a medium containing glucose 2%, Polypepton 1%, meat extract 0.3%, yeast extract 0.2% and NaCl 0.1%. Aliquots (800 ml) of the inoculated culture cultivated at 28°C for 3 days on a rotary shaker were introduced into a fresh medium (20 liters) of the same composition and cultivated at 28°C for 1 day (aeration: 20 liters/minute, agitation: 150 rpm). A 6-liter portion of the seed culture was transferred into a production medium (180 liters) containing potato decoction (200 g/liter) and sugar (20 g/liter). Fermentation was effected at 28°C (agitation: 370 rpm) over 7 days with aeration at 150 liters/minute. The culture broth (146 liters) was adjusted to pH 3 by addition of dil HCl and filtered. After addition of NaCl (7 kg), the filtrate was extracted with EtOAc (36 liters). The mycelial cake was extracted twice with 80% acetone (18 liters each) and the extracts were combined and concentrated to 7 liters *in vacuo*. The concentrate was extracted with EtOAc (18 liters) at pH 2.5 after addition of water (6 liters). The EtOAc extract was evaporated under a reduced pressure to give a crude mass which was then applied to MCI gel CHP-20P column (8 liters). The column was eluted with a linear gradient prepared from acetone - phosphate buffer 20 mM (pH 7.5) containing NaCl 3% (3 : 7) and acetone - phosphate buffer 20 mM (pH 7.5) (7 : 3). The active eluate was concentrated *in vacuo* to give a crude powder (9.0 g) which was then subjected to silica gel column eluted with $CHCl_3$ - MeOH - H_2O (62 : 25 : 4). After concentration of the active fraction, the resultant crude powder (2.9 g) was purified by a reverse phase HPLC (Nucleosil $5C_{18}$) eluted with CH_3CN - 0.1% H_3PO_4 (6 : 4) to afford A1α (260 mg) and A1β (470 mg) as a colorless powder. The culture of a thielocin-producing strain of *Thielavia terricola* RF-143 has been deposited as FERM BP-2196.

Table 1. Physico-chemical properties of thielocins A1α and A1β.

	A1α	A1β
Appearance	Colorless powder	Colorless powder
MP (°C)	244 ~ 247	190 ~ 194
MW ($(M+H)^+$) *m/z*	997.3836 (Δ 0.7 mmu)	997.3856 (Δ 0.2 mmu)
Molecular formula	$C_{54}H_{60}O_{18}$	$C_{54}H_{60}O_{18}$
Elementary analysis	$C_{54}H_{60}O_{18} \cdot H_2O$	$C_{54}H_{60}O_{18} \cdot H_2O$
Calcd:	C 63.91, H 6.11	C 63.91, H 6.11
Found:	C 63.63, H 6.06	C 63.52, H 6.28
UV λ_{max}^{MeOH} nm	End absorption	End absorption
IR (KBr) cm^{-1}	3490, 1740 ~ 1675	3560 ~ 3440, 1744 ~ 1675
CD (MeOH)	$[\Theta]_{265 \sim 360}$ zero	$[\Theta]_{230 \sim 300}$ zero
Solubility	Less soluble in most org. solvent Insoluble in petroleum ether ether, water	More soluble in most org. solvent Insoluble in petroleum ether ether, water
TLC (Rf value)[a]	0.5	0.5
HPLC (Rt)[b]	7.0	4.4

[a] SiO_2: $CHCl_3$ - MeOH - H_2O (4 : 7 : 2).
[b] Nucleosil $5C_{18}$, CH_3CN - 0.1% H_3PO_4 (55 : 45), flow rate; 2 ml/minute, detected at 220 nm.

† Thielocins A1α and A1β have been patented: EP-395,418 (Oct. 31, 1990). We now designate α-isomer claimed in the patent specification as A1β; β-isomer as A1α.

Fig. 1. Structures of thielocins A1α and A1β and their derivatization.

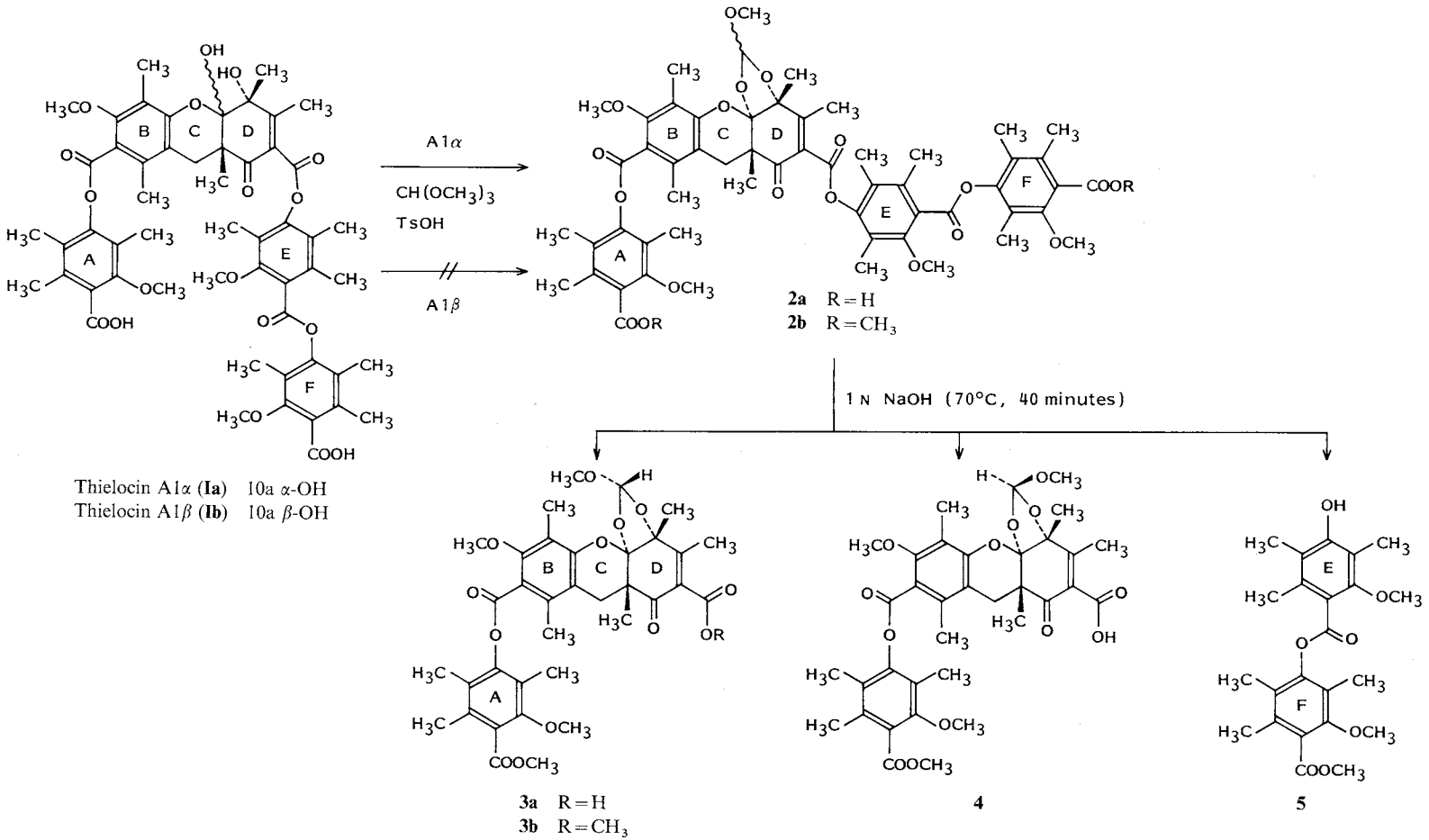
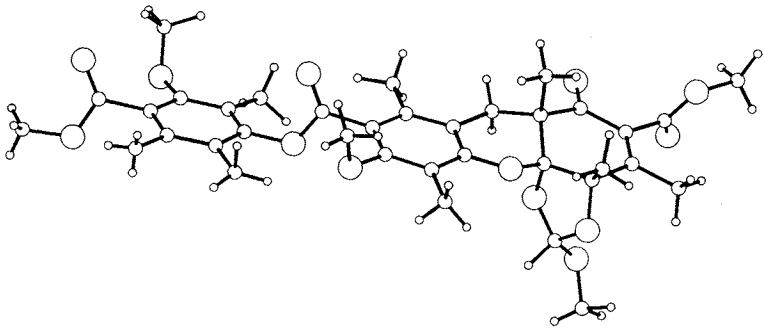


Fig. 2. Perspective view of **3b**.



Physico-chemical properties of A1α and A1β are summarized in Table 1. The molecular formulae of A1α and A1β were determined both as $C_{54}H_{60}O_{18}$ by high resolution liquid secondary ion (HRLSI)-MS and elemental analysis. Thielocins A1α and A1β are isomers which are interconvertible in 20 mM phosphate buffer containg 10% DMSO at pH 7.25 to form an equilibrium mixture (84:16). The ^{1}H NMR spectrum (400 MHz, $CDCl_3$-CD_3OD (20:1)) showed the signals (δ) of one methylene (2.74 (1H, d, J=16 Hz), 3.3 (1H, d, J=16 Hz)), four methoxy (3.83 (9H, s), 3.35 (3H, s)), 14 methyl groups (1.33, 1.89, 2.23, 2.24, 2.26, 2.27, 2.40, 2.41 (3H each, s), 2.27, 2.30, 2.32 (6H each, s)) for A1α; those of one methylene (2.72 (1H, d, J=16 Hz), 3.38 (1H, d, J=16 Hz)), four methoxy (3.78 (1H, s), 3.81 (1H, s), 3.82 (6H, s)), 14 methyl groups (1.52, 1.75, 2.13, 2.20, 2.21, 2.22, 2.25, 2.34, 2.37, 2.40 (3H each, s), 2.24, 2.28 (6H each, s)) for A1β.

Upon treatment with $CH(OCH_3)_3$-TsOH, thielocin A1α provided a cyclic orthoester **2a**: MP 198~200°C; $C_{56}H_{62}O_{19}$ ($(M+Na)^+$, m/z 1,061.3763, Δ1.6 mmu), while A1β did not; indicating their vicinal hydroxyl groups are in *cis* relationship in A1α, *trans* in A1β. Alkaline hydrolysis (1 N-NaOH, 70°C, 40 minutes) of the dimethyl ester **2b**: $C_{58}H_{66}O_{19}$ ($(M+H)^+$, m/z 1,067.4281, Δ 0.8 mmu), obtained by treatment of **2a** with trimethylsilyldiazomethane ($TMSCHN_2$), provided two diastereoisomers **3a**: $C_{35}H_{40}O_{13}$ ($(M+H)^+$, m/z 669.2534, Δ 1.0 mmu) and **4**: $C_{35}H_{40}O_{13}$ ($(M+H)^+$, m/z 669.2554, Δ 0.9 mmu) together with a known depside **5**[3]: MP 169~170°C; $C_{23}H_{28}O_7$ ($(M+H)^+$, m/z 417.1911, Δ 0.0 mmu). Treatment of **3a** with $TMSCHN_2$ gave **3b**: MP 181~183°C; $C_{36}H_{42}O_{13}$ ($(M+H)^+$, m/z 683.2705, Δ 0.4 mmu). The structure of **3b** was determined by X-ray analysis. The crystals obtained from aq ethanol are monoclinic, space group $P2_1/c$, with a=13.694 (1), b=8.858 (1), c=34.255 (4) Å, β=122.42 (1)°, Z=4. The structure was solved by direct methods using MULTAN 87[4]. The final R value was 0.049. A perspective drawing of **3c** was given in Fig. 2. From the above results the structures of thielocins A1α and A1β were determined to be **Ia** and **Ib**, respectively as shown in Fig. 1.

Studies of PLA_2 inhibitory activity[2] of thielocin A1β revealed its specificity on the group II PLA_2[5] from various sources, giving IC_{50} values of 0.0033 μM for rat platelet[6], 21.0 μM for rat pancreas[7], 2.0 μM for bee venom, 63.0 μM for porcine pancreas and 7.1 μM for *Naja naja* venom, respectively. The details will be reported elsewhere in due course.

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References

1) PRUZANSKI, W. & P. VADAS: Phospholipase A_2-a mediator between proximal and distal effectors of inflammation. Immunology Today 12: 143~146, 1991
2) TANAKA, K.; H. ITAZAKI & T. YOSHIDA: Cinatrins; a novel family of phospholipase A_2 inhibitors. II. Biological activities. J. Antibiotics 45 (1): 1992, in press
3) KITAHARA, N.; H. HARUYAMA, T. HATA & S. TAKAHASHI: The structures of thielavins A, B and C. Prostaglandin synthetase inhibitors from fungi. J. Antibiotics 36: 599~600, 1983
4) DEBAERDEMAEKER, T.; G. GERMAIN, P. MAIN, C. TATE & M. M. WOOLFSON: A System of Computer Programs for the Automatic Solution of Crystal Structures from X-ray Diffraction Data. Univ. of York, England, and Louvain, Belgium, 1987
5) HEINRIKSON, R. L.; E. T. KRUEGER & P. S. KEIM: Amino acid sequence of phospholipase A_2 from the venom of *Crotalus adamanteus*. A new classification of phospholipase A_2 based upon structural determinations. J. Biol. Chem. 252: 4913~4921, 1977
6) HAYAKAWA, M.; I. KUDO, M. TOMITA, S. NOJIMA & K. INOUE: The primary structure of rat platelet phospholipase A_2. J. Biochem. 104: 767~772, 1988
7) ONO, T.; H. TOJO, K. INOUE, H. KAGAMIYAMA, T. YAMANO & M. OKAMOTO: Rat pancreatic phospholipase A_2: Purification, characterization, and N-terminal amino acid sequence. J. Biochem. 96: 785~792, 1984