VOL. 52 NO. 10, OCT. 1999 THE JOURNAL OF ANTIBIOTICS pp. 862 — 872

A Novel (6*S*)-4,6-Dimethyldodeca-2*E*,4*E*-dienoyl Ester of Phomalactone and Related α-Pyrone Esters from a *Phomopsis* sp. with Cytokine Production Inhibitory Activity

STEPHEN K. WRIGLEY*, ROYA SADEGHI[††], SANGEETA BAHL[a], ANDREW J. WHITING, A. MARTYN AINSWORTH, STEVEN M. MARTIN, WERNER KATZER[†††], ROBERT FORD, DAVID A. KAU, NEIL ROBINSON[a], MARTIN A. HAYES[†], CLAIRE ELCOCK, THOMAS MANDER[†] and MICHAEL MOORE[a]

TerraGen Discovery (UK) Ltd.,
545 Ipswich Road, Slough, SL1 4EQ, UK
[a]Xenova Ltd.,
240 Bath Road, Slough, SL1 4EF, UK

(Received for publication March 23, 1999)

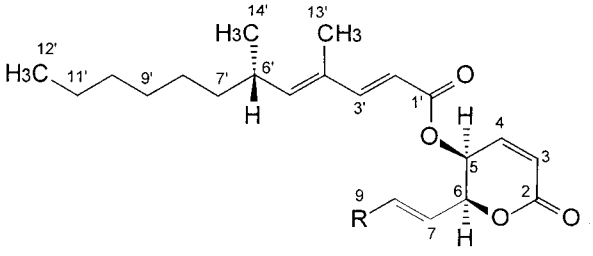
A series of novel 6-substituted 5,6-dihydro-5-hydroxy-α-pyrone esters, **1**~**3**, isolated from fermentations of a *Phomopsis* sp. (Xenova culture collection no. X22502) have been identified as inhibitors of lipopolysaccharide (LPS)-induced cytokine production. These include the (6*S*)-4,6-dimethyldodecadien-2*E*,4*E*-dienoyl ester of phomalactone, **1**, and two analogues bearing a prop-2*E*-enoic acid moiety at the 6-position of the α-pyrone ring. (6*S*)-4,6-Dimethyl-2*E*,4*E*-dienoic acid, **4**, and a hydroxylated analogue, **5**, were also isolated and characterised. The most potent cytokine production inhibitor was **1**, which inhibited LPS-induced tumour necrosis factor α (TNFα) production by U937 cells and LPS-induced interleukin 1β (IL-1β) production by peripheral blood mononuclear cells (PBMC) with IC_{50} values of 80 nM and 190 nM respectively. The effect of **1** in PBMC was selective for IL-1β relative to TNFα. The inhibition of IL-1β production by **1** involved a post-translational mechanism of action at the level of IL-1β secretion as demonstrated by the lack of an effect on cell-associated IL-1β production. **1** showed no effect on the activity of caspase 1 in cytosolic extracts from the THP1 monocytic cell line.

The pro-inflammatory cytokines tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β) are known to be of great importance in the regulation of immune responses.[1] Both cytokines are important targets for the discovery of potentially useful anti-inflammatory agents for the treatment of conditions such as Crohn's disease, multiple sclerosis and rheumatoid arthritis. TNFα and IL-1β have similar actions and are produced in response to similar stimuli. The regulation of secretion of IL-1β differs from that of TNFα in that it is secreted by a mechanism independent of the Golgi and the endoplasmic reticulum because it does not have a signal sequence.[2,3]

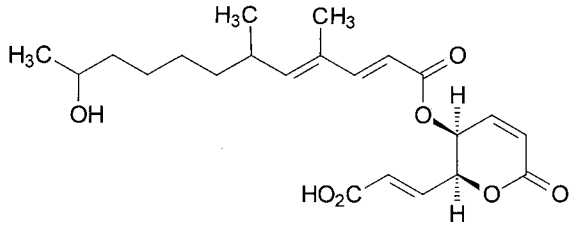
We have reported the development of a high-throughput screen for identification of inhibitors of TNFα production by U937 cells.[4] This assay has been used to conduct a screening programme concentrated on microbial samples and resulted in the identification of a series of novel 5,6-substituted 5,6-dihydro-α-pyrones produced in fermentations of a *Phomopsis* sp., which exhibit potent cytokine production inhibitory activity. On further evaluation using normal cells, the most potent of these, **1**, was found to be selective for inhibition of IL-1β relative to TNFα production. The taxonomy of the producing organism, fermentation, purification and structure elu-

Present addresses: [†] Glaxo Wellcome, Gunnels Wood Road, Stevenage, SG1 2NY, UK.
[††] Pfizer Ltd., Ramsgate Road, Sandwich, Kent, CT13 9NJ, UK.
[†††] Biotechon, Tegelerweg 33, Berlin-Charlottenburg, Germany.

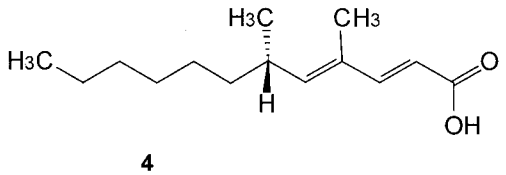
Fig. 1. The Structures of compounds **1**~**5**.



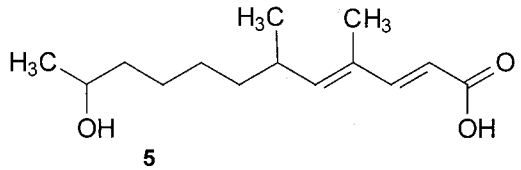
1 R = CH_3
2 R = CO_2H



3



4



5

cidation of these compounds **1**~**3**, and the related fatty acids, **4** and **5** are reported herein, together with further biological studies of **1**. The structures of compounds **1**~**5** are shown in Fig. 1.

Results

Taxonomy of Fungus X22502

This fungus was identified as a strain of the coelomycete *Phomopsis* sp. on the basis of its morphological characteristics following inoculation of its A-conidia (see below) at the centre of Petri dishes containing 2% malt extract (Difco Laboratories) agar. At 24°C it produced a dense, dark grey-olivaceous mycelium with a white lobate margin (colony colours according to a standard mycological identification chart.)[5] After 7 days the mycelium attained a diameter within the range 2.5~3.5 cm. Conidiomatal development was stimulated by exposure to near-UV light. Conidiomata were solitary, carbonaceous, unilocular, ostiolate and measured 1.5~2.0 mm wide and 1.25~2.0 mm high. Conidiogenous cells were borne on branched conidiophores which lined the conidiogenous cavity. These cells were hyaline, obclavate to cylindrical, integrated, phialidic and measured 16~20 μm×1.5~2.0 μm. Conidia were hyaline, aseptate and generally of three types: A-conidia (5.5~7.5 μm×1.5~3.0 μm) were ellipsoid to fusiform, usually with acute apices and a guttule at each end; B-conidia (20~30 μm×<1 μm) were hamate and filiform; C-conidia (9.5~11.5 μm×1.5~3.0 μm) were obclavate with acute apices and usually at least three guttules. All three conidial types could be found within a single conidioma.

The strain is accommodated in the form genus *Phomopsis* (Saccardo) Bubák based on the above morphological characters. The observed microscopical characters and lack of host plant data did not allow further classification to species.

Fermentation

The increases in dry cell weight (DCW) and titres of metabolites **2** and **1** that occurred during a typical fermentation of *Phomopsis* sp. X22502 are shown in Fig. 2.

Structure Elucidation

The physico-chemical properties, ^{1}H and ^{13}C NMR spectral data for compounds **1**~**5** are summarised in Tables 1 to 3 respectively. All five compounds were isolated as colourless oils.

Fig. 2 Typical fermentation timecourse for the production of **2** and **1** by *Phomopsis* sp. X22502.

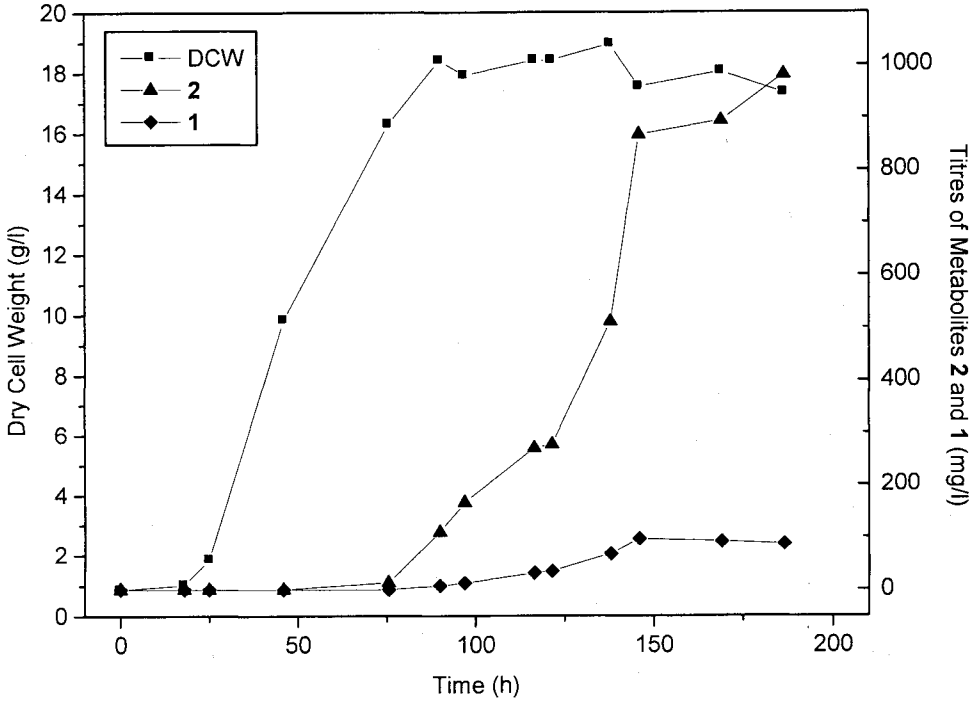


Table 1. Physico-chemical properties of compounds **1**~**5**.

	1	**2**	**3**	**4**	**5**
DCI-MS (*m/z*)	378 (MNH_4^+) 361(MH^+)	408 (MNH_4^+) 391 (MH^+)	424 (MNH_4^+) 407 (MH^+)	242 (MNH_4^+) 225 (MH^+)	258 (MNH_4^+) 240 (M^+)
High resolution EI-MS (*m/z*)					
Found	360.2315	390.2050	406.2014	224.1776	240.1714
Calcd	360.2302	390.2043	406.1992	224.1777	240.1726
Molecular formula	$C_{22}H_{32}O_4$	$C_{22}H_{30}O_6$	$C_{22}H_{30}O_7$	$C_{14}H_{24}O_2$	$C_{14}H_{24}O_3$
UV λ_{max} nm	204, 274	206, 276	205, 274	265	266
IR (KBr) υ cm^{-1}	3390, 2960, 2928, 1720, 1615, 1560, 1250, 1180, 1010, 980	3391, 2960, 2928, 2855, 1717, 1620, 1570, 1396, 1285, 1250, 1161, 1024, 980	3400, 2900, 1714, 1620, 1286, 1247, 1156, 1106, 980	2900, 2685, 2589, 1687, 1618, 1459, 1417, 1285, 1207, 1028, 984, 940, 852, 700	3363, 1692, 1622, 1285, 1198, 1029, 983

The major component isolated from fermentations of *Phomopsis* sp. X22502 was **2**. This compound is soluble in organic solvents such as methanol and DMSO and is insoluble in water and very non-polar solvents such as hexane. The ^1H and ^{13}C NMR spectra of **2** exhibited 22 carbon and 29 proton signals respectively. DEPT spectra revealed the presence of three CH_3, five CH_2, ten CH and four quaternary carbons. An HMQC (^1H-^{13}C correlation) experiment established the one bond connectivities of the proton and carbon atoms. Consideration of the chemical

Table 2. ^{1}H (400 MHz) NMR spectral data for compounds **1**~**5**.

Position	δ_H/ppm in MeOH-d_4: coupling constants expressed in Hz are given in parentheses				
	1	**2**	**3**	**4**[a]	**5**
2					
3	6.29 (1H, d, 9.8)	6.35 (1H, d, 9.7)	6.35 (1H, d, 9.8)		
4	7.18 (1H, dd, 9.8, 5.6)	7.21 (1H, dd, 9.7, 5.6)	7.21 (1H, dd, 9.8, 5.8)		
5	5.48 (1H, dd, 5.6, 3.1)	5.64 (1H, dd, 5.6, 3.0)	5.67 (1H, dd, 6.3, 5.3)		
6	5.19 (1H, dddd, 7.2, 3.0, 0.9, 0.9)	5.49 (1H, m)	5.52 (1H, m)		
7	5.72 (1H, ddq, 15.4, 7.2, 1.7)	6.91 (1H, dd, 15.7, 5.0)	6.98 (1H, dd, 15.7, 4.7)		
8	6.05 (1H, dqd, 15.4, 6.6, 1.1)	6.32 (1H, dd, 15.7, 1.8)	6.31 (1H, dd, 15.7, 1.9)		
9	1.84 (3H, ddd, 6.8, 1.7, 0.8)				
1′OH				11.70 (1H, br s)	
2′	5.95 (1H, d, 15.7)	5.88 (1H, d, 15.6)	5.87 (1H, d, 15.6)	5.77 (1H, d, 15.6)	5.85 (1H, d, 15.6)
3′	7.41 (1H, dd, 15.6, 0.8)	7.41 (1H, dd, 15.6, 0.6)	7.40 (1H, d, 15.5)	7.39 (1H, d, 15.6)	7.40 (1H, d, 15.7)
4′					
5′	5.83 (1H, br d, 9.2)	5.83 (1H, br d, 9.8)	5.83 (1H, brd, 9.9)	5.72 (1H, d, 9.8)	5.75 (1H, d, 9.8)
6′	2.65 (1H, m)	2.66 (1H, m)	2.67 (1H, m)	2.53 (1H, m)	2.65 (1H, m)
7′	1.4 (10H, m)	1.3~1.5 (10H, m)	1.3~1.55 (8H, m)	1.2~1.4 (10H, m)	1.4~1.6 (8H, m)
8′	1.4 (10H, m)	1.3~1.5 (10H, m)	1.3~1.55 (8H, m)	1.2~1.4 (10H, m)	1.4~1.6 (8H, m)
9′	1.4 (10H, m)	1.3~1.5 (10H, m)	1.3~1.55 (8H, m)	1.2~1.4 (10H, m)	1.4~1.6 (8H, m)
10′	1.4 (10H, m)	1.3~1.5 (10H, m)	1.3~1.55 (8H, m)	1.2~1.4 (10H, m)	1.4~1.6 (8H, m)
11′	1.4 (10H, m)	1.3~1.5 (10H, m)	3.77 (1H, m)	1.2~1.4 (10H, m)	3.78 (1H, m)
12′	0.95 (3H, t, 6.9)	0.97 (3H, t, 6.9)	1.23 (3H, d, 6.1)	0.87 (3H, t, 6.7)	1.20 (3H, d, 6.2)
13′	1.81 (3H, d, 1.2)	1.87 (3H, d, 1.1)	1.88 (3H, d, 1.0)	1.78 (3H, d, 0.8)	1.90 (3H, s)
14′	1.09 (3H, d, 6.6)	1.08 (3H, d, 6.6)	1.08 (3H, d, 6.6)	0.98 (3H, d, 6.6)	1.10 (3H, d, 6.6)

[a] Measured in $CDCl_3$.

Table 3. ^{13}C (100 MHz) NMR spectral data for compounds **1**~**5**.

Position	δ_C/ppm in MeOH-d_4				
	1	**2**	**3**	**4**[a]	**5**
2	165.5	164.6	164.0		
3	126.1	125.6	125.2		
4	143.4	143.4	142.8		
5	65.6	64.9	64.2		
6	81.4	79.6	78.9		
7	125.5	139.6	141.2		
8	138.8	128.2	125.5		
9	18.2	170.7	168.8		
1′	168.0	168.0	167.6	173.1	171.0
2′	115.5	115.3	114.8	114.7	116.8
3′	153.2	153.5	153.1	152.1	151.6
4′	133.1	133.3	132.9	131.2	132.8
5′	151.5	151.7	151.2	149.8	149.7
6′	34.8	35.0	34.6	33.2	34.4
7′	38.6	38.7	38.3	37.1	38.4
8′	33.2	33.4	28.9	31.8	28.7
9′	30.7	30.9	27.0	29.3	27.0
10′	28.9	29.0	40.2	27.3	40.2
11′	23.9	24.1	68.6	22.5	68.6
12′	14.6	14.8	23.5	13.9	23.6
13′	12.7	12.9	12.5	12.1	12.6
14′	20.9	21.1	20.7	20.2	20.9

[a] Measured in $CDCl_3$.

Fig. 3A. Long range ^{1}H-^{13}C correlations observed in the HMBC NMR spectrum of **2**.

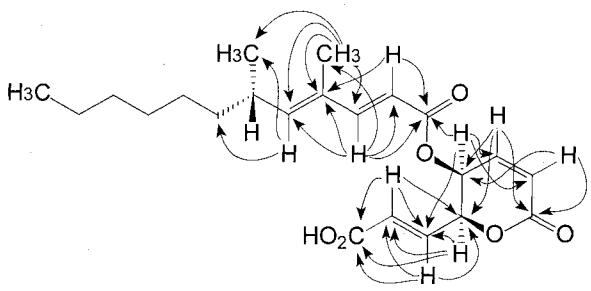
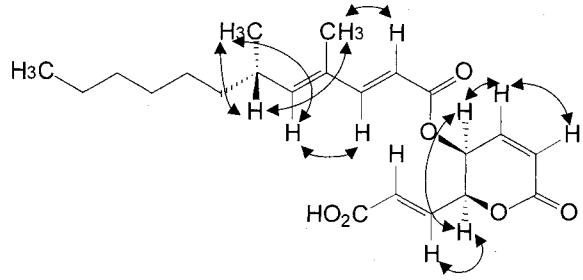


Fig. 3B. NOE's observed in the NOESY NMR spectrum of **2**.



shift data indicated that the 64.9 and 79.6 ppm CH carbons and the quaternary carbons at 164.6, 168.0 and 170.7 ppm were attached to oxygen. The presence of seven double bonds was deduced from the observation of eight olefinic (115.3, 125.6, 128.2, 133.3, 139.6, 143.4, 151.7 and 153.5 ppm) and three carbonyl (164.6, 168.0 and 170.7 ppm) carbons. Mass spectrometric data indicated a molecular weight of 390 and a likely molecular formula of $C_{22}H_{30}O_6$. Inspection of the ^{1}H-^{1}H coupling constants and 2D-COSY data, and consideration of ^{1}H and ^{13}C chemical shift data suggested the presence of a 5,6-dihydro-α-pyrone fragment bearing an oxygen substituent at the 5-position and an olefinic side chain at the 6-position. The ^{1}H-^{1}H coupling constants around this system matched very closely with those previously reported for other metabolites containing this moiety such as phomalactone,[6,7] asperlin[8] and phomopsolides A and B[9] suggesting a similar stereochemical arrangement. This fragment was confirmed by HMBC ^{1}H-^{13}C data which also led to the identification of a 4,6-dimethyldodeca-2,4-dienoyl side chain attached to the 5-position of the pyrone ring by an ester linkage. The protons at the 6-position of the pyrone ring, and of the olefinic moiety attached to it all showed correlations to the carbonyl at 170 ppm, which was deduced to be a carboxyl carbon. This was later confirmed by derivatisation experiments (data not reported here). A NOESY experiment established the stereochemistry of the olefinic groups of the 4,6-dimethyldodeca-2,4-dienoyl side chain as 2*E*, 4*E*, consistent with the coupling constant of 15.6 Hz between H-2′ and H-3′. The ^{1}H-^{13}C correlations observed in the HMBC experiment and NOE interactions observed in the NOESY experiment are shown in Fig. 3.

Mass spectrometric studies of compound **1** indicated a molecular weight of 360 and a molecular formula of $C_{22}H_{32}O_4$. The ^{1}H and ^{13}C NMR spectra of **1** were very similar to those of **2** except for the signals associated with the olefinic side chain at the 6-position of the pyrone ring. These were now consistent with a prop-2*E*-enyl moiety. Thus **1** is a novel 4,6-dimethyldodeca-2*E*,4*E*-dienoyl ester of phomalactone. The ^{1}H and ^{13}C NMR data associated with the phomalactone moiety of **1** matched very closely with those reported for phomalactone.[6,10]

The only previous reports of a 4,6-dimethyldodeca-2*E*,4*E*-dienoyl side chain concern aranorosin and related metabolites from *Pseudoarachniotus roseus*,[11,12] where it is present as an amide moiety. Compound **4** had a molecular weight of 224 and molecular formula $C_{14}H_{24}O_2$. Its ^{1}H and ^{13}C NMR spectra were as expected for 4,6-dimethyldodeca-2*E*,4*E*-dienoic acid. Both enantiomers of 4,6-dimethyldodeca-2*E*,4*E*-dienoic acid have been synthesised during the total synthesis of aranorosin.[13] Comparison of the reported $[\alpha]_D$ values for these enantiomers with that measured for **4** ($[\alpha]_D^{20} = +41°$ (*c* 0.7, CH_2Cl_2)) indicated that **4** had *S* stereochemistry at position 6. This is in contrast to aranorosin which has *R* stereochemistry at the 6-position of its 4,6-dimethyldodeca-2*E*,4*E*-dienoyl fragment.[13] Compound **1** has been synthesised by esterification of **4** with phomalactone obtained from a fermentation of a *Paecilomyces* sp. (WRIGLEY *et al.*, in preparation).

Compound **3** had a molecular weight of 406, and molecular formula $C_{22}H_{30}O_7$, indicating the presence of one extra oxygen compared to **2**. Inspection of its NMR spectra indicated that this was present as an extra hydroxyl substituent in the 4,6-dimethyldodeca-2*E*,4*E*-dienoyl side chain, at position 11.

Compound **5** was similarly found to be an analogue of 4,6-dimethyldodeca-2*E*,4*E*-dienoic acid hydroxylated at the

11 position.

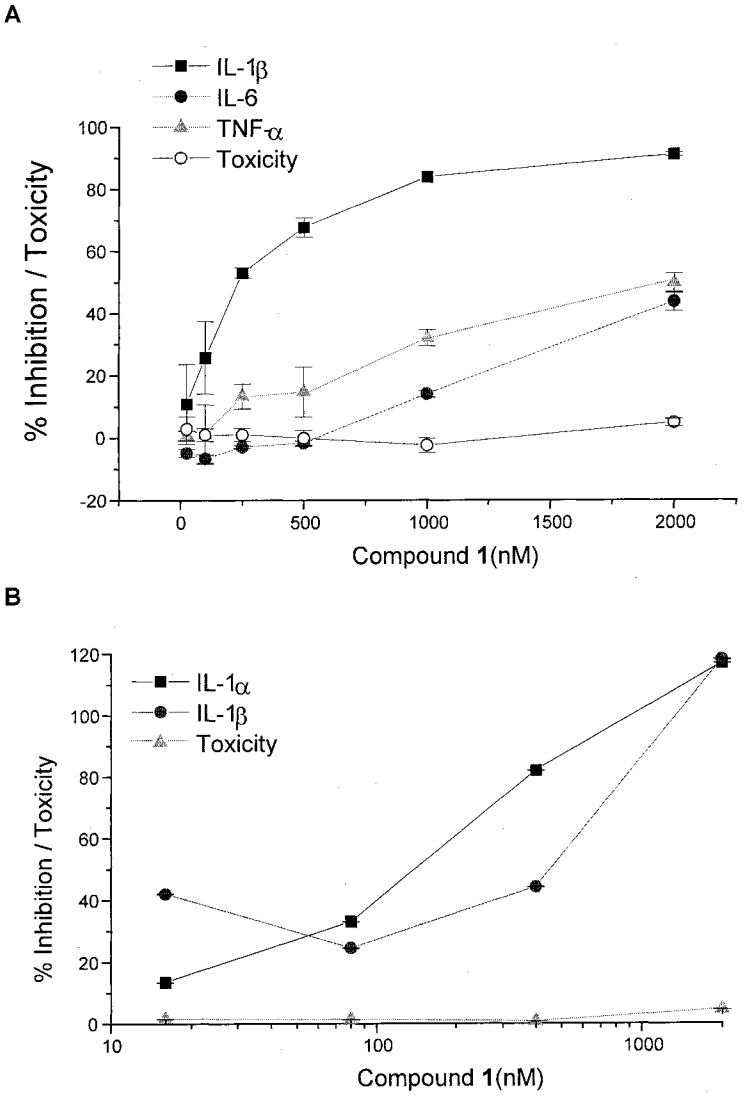
Biological Activity

The IC_{50} values of **1**~**5** for inhibition of LPS-induced TNFα production from U937 cells, the assay used for screening and to guide purification, are shown in Table 4. Further evaluation was based on the effects on normal peripheral blood mononuclear cells (PBMC) and the IC_{50} values of **1**~**5** for inhibition of LPS-induced IL-1β production from PBMC are also shown in Table 4. Compound **1** was the most potent studied, with IC_{50} values

Table 4. IC_{50} values of compounds **1**~**5** for the inhibition of LPS-induced cytokine production.

Compound	IC_{50} (μM)	
	LPS-induced TNFα production in U937 Cells	LPS-induced IL-1β production by Monocytes
1	0.08	0.19
2	2	2
3	31	>6
4	54	>6
5	52	>6

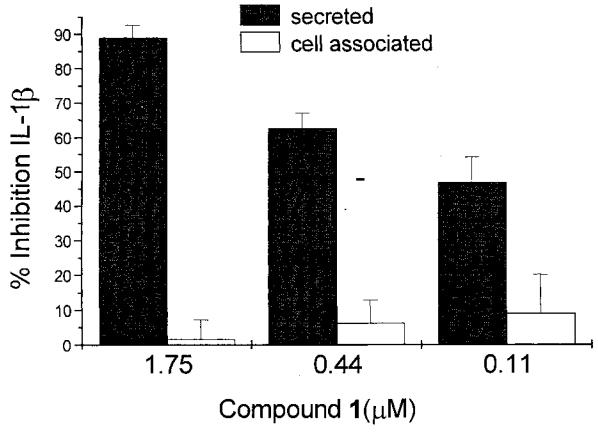
Fig. 4. Inhibition of IL-1β and IL-1α production by **1** in PBMC.



Cells were exposed to ranges of concentration between 0.025~2 μM **1** and stimulated with 1 ng/ml LPS as described. A: Comparison of effects on IL-1β, TNF-α, IL-6 and cell cytotoxicity. Cytotoxicity determined using the redox potential sensitive dye, resazurin. B: Comparison of inhibition of IL-1α and IL-1β production with minimal

for inhibition of TNFα production from U937 cells and IL-1β production from PBMC of 80 nM and 190 nM respectively. Compound **2** also demonstrated significant cytokine production inhibitory properties with IC_{50}s of 2 μM in both assays. The more polar **3** only demonstrated moderate activity while the activity of the fatty acids **4** and **5** was weak.

Fig. 5. Effect of **1** on cell associated IL-1β expresion.



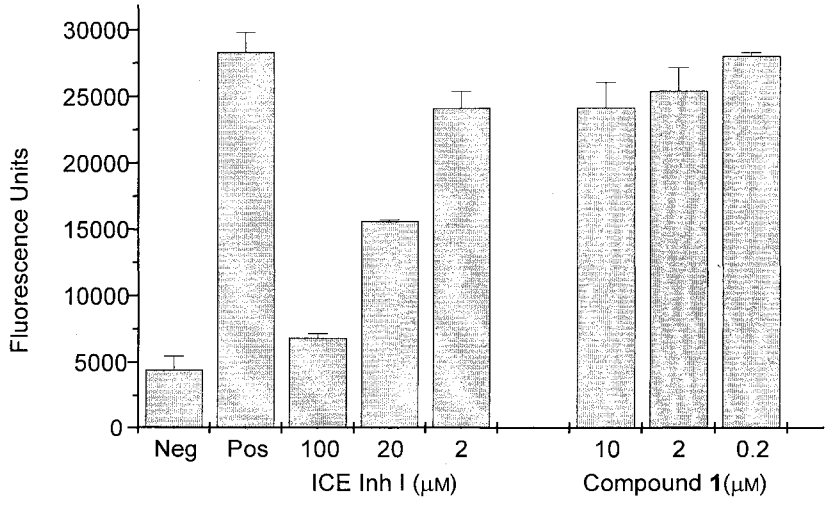
Compound **1** has no significant effect on cell associated IL-1β expression. Effects of **1** at concentrations between 0.11~1.75 μM on IL-1β secretion in cell culture supernatant compared to cell associated IL-1β from cell lysates by ELISA.

Compound **1** was consistently five to tenfold more potent for the inhibition of IL-1β relative to TNF-α and IL-6 production in culture supernatant from LPS activated PBMC (Fig. 4A). Cytotoxicity was observed at concentrations above 2 μM. Studies of the effect of **1** on interleukin 1α (IL-1α) secretion indicated that it inhibited both IL-1β and IL-1α production with similar potency (Fig. 4B). No significant change in the levels of cell associated IL-1β expression was observed in the presence of **1** at concentrations up to 1.75 μM indicating that **1** inhibited IL-1β secretion by a post-translational mechanism (Fig. 5). Studies of the effect of **1** on the activity of caspase 1 (interleukin 1β converting enzyme (ICE))-like enzyme in cell extracts from THP1 monocytic cells using published methods[14] indicated that, in contrast to ICE inhibitor 1 (Ac-Tyr-Val-Ala-Asp-CHO) it minimally modulated caspase 1 like activity. Inhibition of ICE-like activity by ICE inhibitor 1 was dose dependent with maximum inhibition observed at 100 μM inhibitor concentration (Fig. 6). ATP is known to promote IL-1 secretion by a post-translational mechanism.[15,16]

Discussion

A series of α-pyrone esters and their corresponding

Fig. 6. Effects of ICE inhibitor I (Ac-Tyr-Val-Ala-ASP-CHO) and **1** on caspase 1-like activity from THP1 monocytic extract.



Negative control (Neg) fluorescence indicates substrate alone, in the absence of added cell lysate as a source of caspase 1-like activity. Positive control (Pos) indicates total caspase 1-like activity in fluorescence units detected in cell extracts from THP1 cell line. Samples were treated with 100, 20 and 2 μM Ice Inhibitor I (Ice Inh I) or with 10, 2 and 0.2 μM **1** as indicated, in the presence of substrate and THP1 cell extract as a source of caspase 1-like activity.

free fatty acids have been isolated from *Phomopsis* sp. X22502 and studied for their cytokine production inhibitory properties. The most potent compound in the series, **1**, is a novel ester of the known fungal metabolite phomalactone, (6*S*)-4,6-dimethyldodeca-2*E*,4*E*-dienoyl phomalactone. Phomalactone itself has been reported to have immunosuppressive properties, although these have not been studied in detail.[6] Compound **1** is significantly more potent than phomalactone in terms of inhibition of cytokine production, as will be discussed in a subsequent publication (WRIGLEY *et al.*, in preparation). Compounds **2** and **3** are analogues of **1** bearing a prop-2*E*-enoic acid side chain at the 6-position of the α-pyrone ring in place of the propenyl moiety present in **1**. No such analogues of phomalactone bearing an acidic side chain have been previously reported.

Compound **1** was studied for its inhibition of production of the pro-inflammatory cytokines IL-1β and TNFα. In normal PBMC **1** was selective for inhibition of IL-1β production, and our studies of its mechanism of action show that it acts *via* inhibition of IL-1β secretion. This conclusion was based on the lack of inhibition of IL-1β mRNA expression (data not shown) and cell-associated IL-1β production by **1**. Compound **1** also showed no effect on the activity of caspase 1.

Caspase 1 is primarily important for the regulation of the cleavage of IL-1β precursor to its mature form and is also thought to be important in facilitating the release of both membrane associated IL-1β and IL-1α.[17,18] However, it is not known if the catalytic activity responsible for the cleavage of the precursor form is also involved in the release of IL-1 from the cell membrane. The assay for the caspase 1 activity using THP1 cell extract does not necessarily differentiate between these two activities and the possibility that **1** affects this latter activity of caspase 1 remains.

IL-1, like the acidic and basic fibroblast growth factors (FGF) and interleukin 18 (IL-18), is expressed without a signal sequence and secreted by a process independent of the Golgi and endoplasmic reticulum.[19,20] Common regulatory mechanisms may contribute to the release of IL-1β, IL-18 and FGF from the cell membrane. Addition of ATP at concentrations from 0.25~1 mM enhanced LPS-induced IL-1β release and overcame the **1**-mediated inhibition of IL-1β production, suggesting that **1** acts *via* an ATP-dependent mechanism (data not shown). Thus it is possible that **1** can inhibit IL-18 and FGF as well as IL-1 secretion. Further studies of the effects of **1** on FGF and IL-18 secretion would address this issue.

Experimental

Source of Organism

The microfungus designated Xenova culture collection number X22502 was isolated from foam in a tropical forest stream, Thailand, and accessed by the collection in March 1991. The strain X22502 was deposited under the Budapest Treaty at the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, on 19 March 1996, and was assigned the reference number CBS 313.96.

Fermentation

A 1.5 ml cryovial containing 1 ml of macerated vegetative mycelium suspended in a 10% glycerol solution was retrieved from storage at −135°. A preculture was produced by aseptically placing 1 ml of starting material in a 250 ml baffled Erlenmeyer flask containing 40 ml of nutrient solution S (1.5% glycerol, 1.5% soya bean peptone, 1% D-glucose, 0.5% malt extract, 0.3% NaCl, 0.1% $CaCO_3$, 0.1% Tween 80, 0.1% Junlon PW110 [suppliers: Honeywell and Stein Ltd., Times House, Throwley Way, Sutton, Surrey, SM1 4AF] adjusted to pH 6) shaken at 240 rpm for 3 days at 25°C.

An intermediate culture was generated by aseptically transferring the preculture to 2 liters of nutrient solution S in a 3 liter fermenter. The fermenter was agitated at 500 rpm, aerated at 0.5 vvm, and the temperature controlled at 25°C for 3 days.

A production culture was generated by aseptically transferring an intermediate culture to a 75 liter fermenter containing 50 liters of nutrient solution P (3.6% molasses, 0.2% casein hydrolysate, 0.004% phytic acid, 0.09% calcium chloride, 0.1% Tween 80, adjusted to pH 5). The production fermenter was stirred at 350 rpm, aerated at 0.5 vvm, and temperature controlled at 25°C. After 5½ days incubation the fermentation was stopped and the culture was harvested.

Dry cell weights (DCW) were determined during the fermentation as follows: whole culture (10 ml) was pipetted into a preweighed centrifuge tube and centrifuged at 3000 *g* for 15 minutes. After removal of the supernatant the biomass was lyophilised. The tube and dried biomass were then reweighed to give the DCW.

Purification

A 50 liter fermentation broth was harvested by filtration using a Schenk Niro 430 filter press, the clarified filtrate was discarded and the retained biomass was extracted with 25 liters of recirculating methanol for 24 hours. The methanolic extract was harvested *via* filtration through the filter press and evaporated to an aqueous concentrate using

a thin film evaporator.

The aqueous concentrate (10 liter) was then extracted with 2×7 liters of ethyl acetate - hexane (1 : 1). The solvent extracts were pooled and evaporated to a gum under reduced pressure and redissolved in 50 ml of ethyl acetate - hexane (1 : 1). Purification was achieved by normal phase chromatography using a Biotage Flash 75 L chromatography system and a Flash 75 KP-Sil silica (32~62 μm 60 Å) column (ID 7.5×30 cm length) and an isocratic mobile phase (ethyl acetate - hexane 1 : 1, 200 ml/minute flow rate). 1 liter fractions were collected and analysed by thin layer chromatography on silica gel plates [Merck 5554, 20 cm×20 cm, 0.2 mm thickness of silica gel $60F_{254}$] using the same mobile phase as the developing solvent.

Compounds **1** (Rf 0.89), **2** (Rf 0.53) and **4** (Rf 0.75) rich fractions were pooled, evaporated to dryness under reduced pressure and subjected to further purification by preparative reversed phase HPLC using a Waters NovaPak C_{18} (100 Å 5 μM) column (ID 2.5×20 cm length) and an isocratic mobile phase (80% acetonitrile: 20% water plus 0.1% v/v glacial acetic acid, flow rate 50 ml/minute). Wavelength monitoring was at 278 nm. The peaks collected at 11~14 minutes, 19~21 minutes and 30~32 minutes were evaporated to dryness to yield **1** (0.9 g), **2** (7.5 g), and **4** (0.35 g) respectively.

Compounds **3** and **5** were isolated from the fractions eluting from the Flash 75 KP-Sil silica column after those containing compounds **1**, **2** and **4**. These fractions were concentrated and purified by semi-preparative reversed phase HPLC on two Prep Nova-Pak HR C_{18} radial compression cartridge columns (40 mm×10 cm, 6 mM particle size, 60 Å pore size, (Waters WAT037704) connected in series in a Prep-Pak Holder Assembly with Extension (Waters) along with a Prep Nova-Pak HR C_{18} Guard-Pak (Waters WAT037854) eluted isocratically with a mobile phase comprising acetonitrile - Water (39 : 61) containing 0.05% v/v trifluoroacetic acid at a flow rate of 50 ml/minute. The eluate was monitored at 278 nm and the peaks eluting after 8.0 and 13.3 minutes were collected and evaporated to dryness to yield **5** (31 mg) and **3** (102 mg) respectively.

Determination of Physico-chemical Properties

UV/visible spectra were measured on a Perkin-Elmer Lambda 17 UV/visible spectrometer. IR spectra were recorded in KBr on a Nicolet 5PC FTIR spectrometer. Low resolution EI-MS and DCI-MS were obtained on a Finnigan Mat 95 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 308K on a Bruker ACF400 spectrometer at 400 MHz and 100 MHz respectively. All chemical shifts (δ) are quoted in ppm and are referenced to external TMS (0 ppm). Standard techniques were used to obtain the DEPT, COSY-45, HMQC, HMBC and NOESY spectra. In HMQC experiments the $^{1}J_{CH}$ was optimised for 145 Hz. In HMBC experiments the long range coupling constant $^{3\sim5}J_{CH}$ was optimised for 5 Hz. A mixing time of 1 second was used in the NOESY experiment.

TNF-α Release from U937 Cells

The screening assay used to detect compounds **1**~**5** measured TNF-α release using a known method.[4] The human histolytic lymphoma U937 cell line was obtained from a commercial source (ECACC, Salisbury, UK) and maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 5% fetal bovine serum (FBS). The cells were pretreated with 25 ng/ml phorbol myristate acetate (PMA) for six hours and then exposed to dose ranges of samples or compounds to be tested followed by the addition of 1 ng/ml LPS. After 18 hours incubation at 37°C with 5% CO_2 the cell culture supernatants were harvested and stored at −70°C, until required for determination of TNF-α secretion[4] by dissociation enhanced lanthanide fluorescence immuno assay. The effect of the compounds on cell cytotoxicity was measured using the tetrazolium salt, XTT (2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide salt).

Cytokine Production from Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were separated from Buffy coats on Lymphoprep and resuspended in RPMI 1640 medium with 5% FBS. Cells were exposed to a concentration range of 0.01~2.5 μM **1** and 1 ng/ml LPS and incubated at 37°C with 5% CO_2 for 20 hours. Cell culture supernatant was then harvested for determination of cytokines, IL-1α, IL-1β, TNF-α and IL-6. A human IL-1α ELISA kit (TCS biologicals Ltd, Bucks, UK), and an in-house IL-1β sandwich ELISA, using a monoclonal anti-IL-1β (R & D Systems, UK) as coating antibody and a polyclonal anti-IL-1β (R & D Systems, UK) as detection antibody, were used respectively for quantification of IL-1α and IL-1β concentrations. The IL-1β ELISA did not distinguish between mature and precusor forms of IL-1β. Similarly TNF-α and IL-6 concentrations in the cell culture supernatant were quantified using sandwich ELISAs for each cytokine. For TNF-α a monoclonal anti-TNF-α was used as a coating antibody and a polyclonal anti-TNF-α as a detection antibody.[4] The IL-6 sandwich ELISA consisted of a

monoclonal anti-IL-6 coating antibody and a polyclonal goat anti-IL-6 antibody (kind gift of Dr. S. POOLE, NIBSC) for detection.[21] For cytotoxicity assessment the cells were incubated at 37°C with 5% CO_2 in the presence of the redox potential sensitive dye, resazurin (BDH, UK) for 20 hours and optical density reading at 570/600 nm determined.

Alternatively the cells were lysed following the addition of 200 μl lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.5% Triton X100, 0.1 mM AEBSF protease inhibitor) for determination of cell associated IL-1β levels.

Caspase 1 Activity in Cytosolic Extracts from THP1 Monocytic Cell Line

THP1 cell extract were prepared essentially as described.[14] Briefly, 2×10^8 cells resuspended in 1.2 ml extraction buffer (50 mM PIPES-NaOH-pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$ 1 mM DTT, 20 μM cytochalasin B, 1 mM. PMSF, 1 μg/ml Leupeptin, 1 μg/ml pepstatin A, 50 μg/ml anti-pain, 10 μg/ml chymopain) were lysed by four repeated freezing on dry ice and thawing, with a hand homogenizer. The cell suspension was then spun at 14000 rpm for 15 minutes and the lysate diluted 1 : 5 in standard buffer (100 mM HEPES-KOH pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml ovalbumin). Cell extracts were incubated at 30°C in the presence of 0.2~10 μM **1** or 2~100 μM ICE inhibitor I (Ac-Tyr-Val-Ala-Asp-CHO, BACHEM, Cat No. H8410) for 30 minutes and the caspase 1 specific fluorogenic tetra-peptide substrate, Ac-Trp-Glu-His-Asp-AMC[22] (BACHEM, Germany) at the optimal concentration of 10 μM added and incubated for a another 60 minutes at 30°C.[22] Changes in fluorometric reading quantified as a measure of Capase 1 like activity.

Chemicals and Biochemical Reagents

Unless stated otherwise, all reagents were obtained from Sigma Chemical Co., UK.

Acknowledgments

We are grateful to Drs. MASASHI MATSUI, TOSHIO TATSUOKA and YASUNORI TAWARAGI of SUNTORY Limited, Institute for Biomedical Research for discussions during the course of this work. We would like to acknowledge TREVOR GIBSON and STEVE HOARE for providing Mass Spectra and MOHAMMED LATIF for NMR spectra. We would also like to thank LIZ CRUTCH for assistance in preparation of this manuscript.

References

1) AREND, W. P. & J. M. DAYER: Inhibition of the production and effects of interleukin 1 and tumour necrosis factor α in rheumatoid arthritis. Arth. Rheum 38: 151~160, 1995
2) SINGER, I. I.; S. SCOTT, G. L. HALL, G. LIMJUCO, J. CHIN & J. A. SCHMIT: Interleukin 1β is localised in the cytoplasmic ground substance but is largely absent from the Golgi apparatus and plasma membranes of stimulated human monocytes. J. Exp. Med. 167: 389~407, 1988
3) STEVENSON, F. T.; F. TORRANO, R. M. LOCKSLEY & D. H. LOVETT: Interleukin 1: the patterns of translation and intracellular distribution support alternative secretory mechanisms. J. Cell Physiol. 152: 223~231, 1992
4) MANDER T.; S. HILL, A. HUGHES, P. RAWLINS, C. CLARK, G. GAMMON, B. FOXWELL & M. MOORE: Differential effects on TNFα production by pharmacological agents with varying molecular sites of action. Int. J. Immunopharmacol. 19: 451~462, 1997
5) ANON: Flora of British Fungi: Colour identification chart. HMSO, Edinburgh, UK, 1969
6) KRIVOBOK, S.; F. THOMASSON, F. SEIGLE-MURANDI, R. STEIMAN & C. BOTTEX-GAUTHIER: 6-Allyl-5,6-dihydro-5-hydroxy-pyran-2-one, a lactone produced by a new *Drechslera* species: specified ^1H and ^{13}C NMR assignments, mutagenic and immunomodulating testings. Pharmazie 49: 605~607, 1994
7) EVANS, R. H.; G. A. ELLESTAD & M. P. KUNSTMANN: Two new metabolites from an unidentified *Nigrospora* species. Tetrahedron Letts: 1791~1794, 1969
8) FUKUYAMA, K.; Y KATSUBE, A. NODA, T. HAMASAKI & Y. HATSUDA: Absolute configurations of asperlin, a metabolite of *Aspergillus nidulans*, and its related compounds. Bull. Chem. Soc. Japan 51: 3175~3181, 1978
9) GROVE, J. F.: Metabolic products of *Phomopsis oblonga* Part 2 Phomopsolide A and B, tiglic esters of two 6-substituted 5,6-dihydro-5-hydroxypyran-2-ones. J. Chem. Soc. Perkin Trans. 1: 865~869, 1985
10) MURAYAMA, T.; T SUGIYAMA & K. YAMASHITA: Synthesis of natural (+)-phomalactone, (+)-acetylophomalactone, (+)-asperlin and their isomers. Agric. Biol. Chem. 51: 2055~2060, 1987
11) FEHLHABER, H.-W.; H. KOGLER, T. MUKHOPADHYAY, E. K. S. VIJAYAKUMAR, K. ROY, R. H. RUPP & B. N. GANGULI: Aranorosin, a novel antibiotic from *Pseudoarachniotus roseus* II. Structure elucidation. J. Antibiotics 41: 1785~1794, 1988
12) ROY, K.; E. K. S. VIJAYAKUMAR, T. MUKHOPADHYAY, S. Q. CHATTERJEE, R. G. BHAT, J. BLUMBACH & B. N. GANGULI: Aranorosinol A and aranorosinol B, two new metabolites from *Pseudoarachniotus roseus*: production, isolation, structure elucidation and biological properties. J. Antibiotics 45: 1592~1598, 1992
13) WIPF, P.; Y. KIM & P. C. FRITCH: Total synthesis and structure assignment of the antitumour antibiotic aranorosin. J. Org. Chem. 58: 7195~7203, 1993
14) ENARI, M.; R. V. TALANIAN, W. W. WONG & S. NAGATA: Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 380: 723~726, 1996
15) FERRARI, D.; P. CHIOZZI, S. FATZONI, S. HANAN & F. D. VIRGILIO: Purinergic modulation of interleukin-1β release from microbial cells stimulated with bacterial endotoxin. J. Exp. Med. 185: 579~582, 1997

16) PERREGAUX, D. G.; R. E. LALIBERTE & C. A. GABEL: Human monocyte interleukin 1β post-translational processing. J. Biol. Chem. 271: 29830~29838, 1996
17) SINGER, I. I.; S. SCOTT, J. CHIN, E. K. BAYNE, G. LIMJUCO, J. WEIDNER, D. K. MILLER, K. CHAPMAN & M. J. KOSTURA: The interleukin-1β-converting enzyme (ICE) is localised on the external cell surface membranes and in the cytoplasmic ground substance of human monocytes by immuno-electron microscopy. J. Exp. Med. 182: 1447~1459, 1995
18) HAWARD A. D.; O. C. PALYHA, P. R. GRIFFIN, E. P. PETERSON, A. B. LENNY, G. J. F. DING, D. J. PICKUP, N. A. THORNBERRY, J. A. SCHMIDT & M. J. TOCCI: Human IL-1β processing and secretion in recombinant baculovirus-infected sf9 cells is blocked by the cowpox virus serpin crmA. J. Immunol. 154: 2321~2332, 1995
19) TARANTINI, F.; S. GAMBLE, A. JACKSON & T. MACIAG: The cysteine residue responsible for the release of fibroblast growth factor-1 residues in a domain independent of the domain for phophatidylserine binding. J. Biol. Chem. 270: 29039~29042, 1995
20) OKAMURA, H.; H. TSUTSUI, T. KOMATSU, M. YUTSUDO, A. MAKURA, T. TANIMOTO, K. TORIGOE, T. OKURA, Y. NUKADA, K. HATTORI, K. AKITA, M. NAMBA, F. TANABE, K. KONISHI, S. FUKUDA & M. KURIMOTO: Cloning of a new cytokine that induces IFN-γ production by T Cells. Nature 378: 88~90, 1995
21) BROWN, D.; R. SADEGHI, J. STEEL, M. MOORE & G. GAMMON: Detection of interleukin-1 signal transduction inhibitors: action of protein kinase inhibitors. Int. J. Immunopharmacol. 17: 887~894, 1995
22) RANO, T. A.; T. TIMKEY, E. P. PETERSON, J. ROTONDA, D. W. NICHOLSON, J. W. BECKER, K. T. CHAPMAN & N. A. THORNBERRY: A combinatorial approach for determining protease specificities: application to interleukin-1β converting enzyme (ICE). Chemistry and Biology 4: 149~155, 1997