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L-741,494, A FUNGAL METABOLITE THAT IS AN INHIBITOR OF INTERLEUKIN-1β CONVERTING ENZYME

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ABSTRACT.—γ-Pyrone-3-acetic acid (L-741,494) is a novel metabolite produced by a culture of the fungal genus *Xylaria*. This substance is a water-soluble, competitive, irreversible inhibitor of Interleukin-1β Converting Enzyme that is inactive against papain and trypsin. It has a mol wt of 154 and an empirical formula of $C_7H_6O_4$. We propose the name xylaric acid for this compound.

Interleukin-1β (IL-1β) is a major determinant in the etiology of acute and chronic inflammatory disease (1–4). It is synthesized primarily by monocytes as an inactive 31 kDa precursor (pre-IL-1β). Pre-IL-1β is cleaved to yield mature IL-1β, a protein of 17.5 kDa, by Interleukin-1β Converting Enzyme (ICE). ICE is an unique cysteine-containing heterodimeric protease specific for pre-IL-1β (2, 4–7). It consists of two subunits with molecular weights of 20 kDa (p20) and 10 kDa (p10) (4). Although the active-site cysteine is located on the p20 subunit, both subunits are required for catalytic activity (4).

The physiological effects of IL-1β can be modulated by a natural IL-1 receptor antagonist (8–13), soluble IL-1 receptor (14), or antibodies directed towards the IL-1 receptor (15). An alternative approach to regulate the biological activity of IL-1β would be to interfere with the processing of pre-IL-1β by ICE. To identify ICE inhibitors that might be useful as anti-inflammatory agents, microbial and plant extracts were screened for inhibitors of ICE. In this communication, we describe the discovery and isolation of L-741,494 produced by the fungus, *Xylaria*. This compound, γ-pyrone-3-acetic acid, to which we refer as xylaric acid, is a novel, competitive, irreversible inhibitor of ICE.

RESULTS AND DISCUSSION

Thiol alkylating reagents (2), diazomethyl ketones, and peptide aldehydes (4,16), and the gene product of the *crm* A gene found in cowpox virus (17) all inhibit ICE activity. Although these reagents have helped define ICE mechanistically they presently have no therapeutic applicability. The search for novel therapeutic inhibitors of ICE has focused on the screening of traditional sources such as chemical libraries. Non-traditional sources of potentially useful entities include those produced by microorganisms and plants.

To facilitate the search for novel inhibitors of ICE from microorganisms and plants a high-throughput screening assay was developed. The fermentation broth of one culture (Merck Fungus 5809), belonging to the genus *Xylaria*, was specifically active against ICE. This genus includes many species living as endophytes of higher plants or as secondary colonizers of decorticated or dead wood.

Interest in this activity was highlighted by the inability to extract the active

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compound at pH 6.5 with the organic solvents methyl ethyl ketone, ethyl acetate, dimethyl chloride, chloroform, butanol or hexane. However, the active compound was completely miscible in H_2O, MeOH, and aqueous MeOH.

Initial experiments to further characterize the ICE-inhibitory activity were performed on filtered broth. Stability experiments at pH 3.0 and 9.0 yielded an inactive product. A modified agarose-gel-electrophoresis procedure, described by Salvatore *et al.* (18), was used to classify this compound by charge and mobility. Electrophoresis was performed at pH 6.0 and gel sections (1 cm^2) were taken. The ICE-active component was eluted from the gel sections by multiple freeze/thaw and centrifugation cycles. The results of both experiments indicated the presence of an anionic-ICE inhibitory component that was unstable to extremes in pH.

Using this information, the filtered broth was passed through an anion-exchange column equilibrated at pH 6.5. The column was then eluted with 2.5% NaCl. ICE-active fractions were pooled and desalted using hydrophobic adsorption chromatography. Final purification of xylaric acid (L-741,494) was accomplished by reversed-phase hplc. The structure of xylaric acid was determined primarily by nmr and ms.

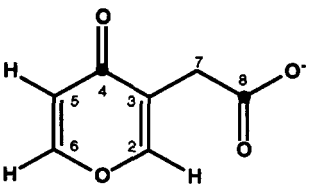
Broad-band decoupled and "gated" coupled ^{13}C-nmr spectra in D_2O indicated 7 signals comprising $1\times CH_2$, $3\times CH=$, $1\times C=$, $1\times COX$ and $1\times CO$ (see Table 1). These data are consistent with the molecular formula, $C_7H_6O_4$, obtained by high-resolution mass spectrometry (calcd *m/z* 154.0266, found *m/z* 154.0268). COSY, HMQC, and HMBC nmr data readily suggested γ-pyrone-3-acetic acid [**1**] as the structure. Long-range ^1H-^1H correlations were observed between H-2 and the protons attached to C-6 and C-7. Ms/ms analysis of the daughter ions of *m/z* 154 clearly indicated loss of H_2O, CO_2, and CO from the molecular ion, consistent with the proposed structure.

TABLE 1. ^1H- and ^{13}C-nmr Data of L-741,494 in D_2O.[a]

Position	δ_H ppm (500 MHz)	δ_C ppm (125 MHz)
2	7.86 (1H, dt, ca. 1, ca. 1)	157.0 (d, 199.5)
3		126.6 (s)
4		182.1 (s)
5	6.27 (1H, d, 5.7)	116.4 (d, 169.5)
6	7.89 (1H, dd, 1.1, 5.7)	158.9 (d, 202)
7	3.02 (2H, d, ca. 1)	34.6 (t, 129.5)
8		179.1 (s)

[a]Referenced to internal dioxane at δ 3.53 (^1H) and 67.4 ppm (^{13}C).

Xylaric acid [**1**] inhibits ICE activity with an IC_{50} (the concentration of compound required to inhibit enzyme activity by 50%) of 33 μM. Xylaric acid was a competitive irreversible inhibitor exhibiting a K_i of 8 μM. The specificity of xylaric acid was examined by testing it against papain (another thiol-containing protease) and trypsin.



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Xylaric acid was inactive against these two enzymes at concentrations up to 300 μM. Structurally, xylaric acid is related to patulin and kojic acid. These two compounds, when tested at concentrations up to 100 μg/ml, inhibited ICE activity by 20% and 16%, respectively, and were not considered active. Furthermore, a sub-structure similarity search was performed and seventy-two structurally related compounds culled from our chemical collection were tested and found to be inactive against ICE. These data affirm the specificity of xylaric acid against ICE. We speculate that xylaric acid inhibits ICE activity by reacting with the active-site cysteine of the enzyme. Presumably, xylaric acid undergoes a Michael addition with cysteine forming a covalent adduct of compound and enzyme. Evidence in favor of this hypothesis is that high levels (>10 mM) of dithiothreitol protect the enzyme against inactivation by xylaric acid.

To examine whether the production of xylaric acid [**1**] was widely distributed within the genus *Xylaria*, twelve other *Xylaria* cultures were fermented and broths were tested against ICE. These broths were inactive against ICE suggesting that the production of xylaric acid is not ubiquitous within the genus and may be limited to MF 5809.

Xylaric acid [**1**] is the first reported "water-soluble" ICE-inhibitory compound isolated from a natural source. In addition, there are no published reports of other fungi belonging to the genus *Xylaria* that produce this or related compounds. To date, the only other ICE-inhibitory compound isolated from natural sources was from the plant *Croton urucurana* (H.B.K.) Baill. (Euphorbiaceae). The compound thaliporphine, in contrast to xylaric acid [**1**], was isolated by extraction with organic solvents such as methylene chloride and methyl ethyl ketone (unpublished results). The discovery of xylaric acid [**1**] is encouraging in that it should promote further screening of natural product sources with the hope of finding more potent novel compounds. Furthermore, the availability of xylaric acid [**1**] and related compounds should permit one to address the question of whether ICE inhibitors would be useful as pharmacological tools in the treatment of IL-β-induced inflammation.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr data were acquired on a Varian Unity 500 nmr spectrometer at ambient temperature in D_2O at 500 and 125 MHz, respectively. HMQC and HMBC nmr spectra were acquired with presaturation of the HOD peak. The HMBC experiment was optimized for 7 Hz. Infrared spectra were obtained from a film on a ZnSe multiple internal reflectance (MIR) crystal, using a Perkin-Elmer Model 1750 Ft-ir spectrometer. High-resolution mass spectra were recorded on Finnigan-MAT Model 212 (electron impact, 90 eV) mass spectrometer. Exact mass measurements were performed by the peak matching method at high resolution using perfluorokerosene (PFK) as internal standard. The low-resolution data and ms/ms data were obtained on a Finnigan TSQ-700.

CULTURE SELECTION AND CHARACTERIZATION.—Merck Fungus 5809 (MF 5809) was isolated by a surface-sterilization method (19) as an endophyte from a bark sample of a live oak (*Quercus* sp.) taken from Congree swamp, Columbia, South Carolina.

A typical colony grown on oatmeal agar covered a 9-cm petri dish after 15 days at 25° and 50% relative humidity in a 12-h fluorescent light photoperiod. The growing edge appeared appressed, with an indistinct and scalloped margin. The culture mat, at first white and velvety, was followed by a distinct zone of velvety, olive-black, carbonaceous mycelium, which was the color and texture of the entire colony at maturity. Aerial mycelia, at the inoculation point, were white, cottony, and sparse. Stromata, few and limited to the colony center, were robust, with a villose base and of the "mole" type with dimensions of 1.0–1.5×0.4–0.6 cm. They were olive-black in color with the apical portion white to tan and dissected. No sexual or asexual reproductive structures were observed. The carbonaceous hyphae were olive-green in color and bristle-like with thick walls, and 6.0–7.0 mm wide. The hyphae from the white-underlying mycelium were smooth, hyaline, and 4.0–5.0 mm wide. A clear exudate, formed as droplets on the colony surface and stromata, was present.

The characteristic stromata and general colony morphology of MF 5809 placed it in the genus *Xylaria*. However, the isolate produced no sexual or asexual reproductive structures making species identification

particularly difficult. A synoptic key, developed by Callan and Rogers, to twenty-three species of *Xylaria* based on cultural characters was employed (20). However, the characteristics present in MF 5809 do not fit well into any of the described species. Some key characters of this isolate, such as growth rate or stroma morphology, fit well into one of the species, but all of the characters together do not fit well into any of the reported species. Therefore, this isolate is designated simply as *Xylaria* sp.

FERMENTATION.—A 250-ml unbaffled Erlenmeyer flask with 54 ml of medium A (see Table 2) was inoculated from a soil tube containing the preserved culture (21). This seed flask was incubated in a Kühner model ISF-4-V environmental chamber, under aerobic conditions, at 25° for 3 days at 220 rpm and 85% relative humidity. This seed culture was used as an inoculum for the production fermentation. Twelve flasks, each containing 45 ml of medium B, were inoculated with 2 ml of seed culture. Flasks were incubated at 25° for 21 days at 220 rpm and 85% relative humidity. Fermentations were pooled and processed as described below.

TABLE 2. Fermentation Media Used in the Production of Xylaric Acid [1].

Medium A[a]	(g/liter)	Trace Element Mixture[b]	(g/liter)	Medium B[c]	(g/liter)
		Mixture[b]			
Corn steep liquor	5	$FeSO_4 \cdot 7H_2O$	1.0	Corn meal	50
Tomato paste	40	$MnSO_4 \cdot 4H_2O$	1.0	Dextrose	40
Oat flour	10	$CuCl_2 \cdot 2H_2O$	0.025	Yeast extract	1
Glucose	10	$CaCl_2 \cdot 2H_2O$	0.1		
Agar	4	H_3BO_3	0.056		
Trace element mixture	10 ml	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.010		
		$ZnSO_4 \cdot 7H_2O$	0.2		

[a]Adjusted to pH 6.8.
[b]Adjusted to pH 7.0.
[c]No pH adjustment.

ISOLATION OF XYLARIC ACID [1].—Whole broth (300 ml; pH 6.5) was filtered through a glass Buchner funnel (fine porosity) yielding 250 ml of filtrate. Filtered broth was loaded onto an anion-exchange column (Dow Chemical Dowex 1-1×2; 50 ml resin bed; hydrogen cycle; pH 6.5) using a flow rate of 5 ml/min. The column was washed with 150 ml of distilled H_2O. An ICE-inhibitory fraction eluted with 1 column volume of 2.5% w/v NaCl. This active fraction was loaded onto a reversed-phase column (Mitsubushi Chemical SP207; 50 ml resin bed; pH 6.5) using a flow rate of 5 ml/min. An ICE-inhibitory fraction was eluted with 0.5 to 2.0 column volumes of distilled H_2O. This eluate was lyophilized overnight and the residue was resuspended in 10 ml of distilled H_2O and filtered through a 20-micron Millipore filter. Filtrate (2 ml) was injected onto a reversed-phase-prep. hplc column (Waters/Millipore Delta-pak, C_4 radial-compression column; 15 μm; 25×200 mm with guard column; 500 psi) at a flow rate of 10 ml/min (100% H_2O, pH 6.5). Fractions were collected at 1 min intervals. The remainder of the filtrate was processed as above and all ICE-inhibitory fractions were pooled and lyophilized overnight yielding 7.0 mg of L-741,494. Hrms afforded the molecular formula $C_7H_6O_4$ (calcd *m/z* 154.0266; found *m/z* 154.0268). Ir (ZnSe film) ν max 3382, 1651, 1587, 1385 cm^{-1}. Uv (MeOH) λ max 209 (4.20), 252 (4.12) nm.

INTERLEUKIN-1β CONVERTING ENZYME.—The enzyme used in these studies was purified approximately 100-fold from THP.1 cells using DEAE ion-exchange chromatography as described by Thornberry *et al.* (4) and was provided by Dr. Douglas K. Miller (Department of Inflammation and Immunology, Merck Research Laboratories).

SYNTHESIS OF THE PEPTIDE SUBSTRATE FOR ICE.—The peptide substrate (acetyl-tyrosine-valine-alanine-aspartate-7-amino-4-methylcoumarin, YVAD-AMC) was prepared as described by Thornberry *et al.* (4) and was provided by Dr. Kevin T. Chapman (Department of Medicinal Chemistry, Merck Research Laboratories).

ASSAY OF ICE ACTIVITY.—ICE activity was monitored essentially as described by Thornberry *et al.* (4) with the following modifications. Assays were performed using Dynatech Microfluor 96-well microtiter plates. Fermentation extracts (or purified compound) were incubated with 15 μM YVAD-AMC and 0.1 enzyme units in a final volume of 100 μl containing 100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethane sulfonic acid) (HEPES), pH 7.5, 10 mM dithiothreitol (DTT), 10% sucrose, 0.1% (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS), and 0.1% BSA at 25°. The release of AMC was

monitored (using excitation and emission filters of 360 and 460 nm, respectively) over time using a Cambridge Model 7620 fluorimeter capable of reading microtiter plates.

ASSAY TO DETECT REVERSIBILITY.—To determine whether xylaric acid [**1**] was a reversible inhibitor the following experiment was performed. Ten microliters of solvent or xylaric acid [**1**] at 500-fold the IC_{100} concentration was pre-incubated with 5 units of ICE enzyme plus 85 μl of 100 mM HEPES, pH 6.5, 10 mM DTT, 10% sucrose, 0.1% CHAPS and 0.1% BSA at room temperature for 15 min. A 2-μl aliquot was transferred to a 96-well plate and tested for ICE activity as described above. The remainder of the pre-incubation mix was concentrated to 20 μl by ultra-filtration through a Millipore 10,000 mol wt cut-off filter unit and diluted back to 200 μl with the above buffer. A 4-μl aliquot of the diluted first spin was then transferred to a 96-well plate and tested for ICE activity as described above. The remainder of the diluted first spin supernatant was again reduced to 20 μl, re-diluted to 200 μl and another 4-μl aliquot was taken to test for ICE activity. The remainder of the diluted second spin supernatant was again reduced to 20 μl and a 0.4-μl aliquot of this final spin concentrate was taken to test for ICE activity.

ASSAY OF PAPAIN ACTIVITY.—Papain activity was measured as described by Kanaoka *et al.* (22). L-741,494 was incubated with papain (50 ng, Worthington Biochemicals) in 50 mM Tris.HCl buffer, pH 7.5, 5 mM L-cysteine-HCl, 2 mM EDTA, and benzoyl-L-arginine-AMC (1 mM, Sigma) at 25°. The release of AMC was monitored over time as described above.

ASSAY OF TRYPSIN ACTIVITY.—Trypsin activity was measured as described by Kanaoka *et al.* (22). L-741,494 was incubated with trypsin (10 ng, Worthington Biochemicals) in 50 mM Tris.HCl buffer, pH 8.0, 5 mM $CaCl_2$, and benzoyl-L-arginine-AMC (100 μm) at 25°. The release of AMC was monitored over time as described above.

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