ZG-1494α, a Novel Platelet-activating Factor Acetyltransferase Inhibitor from *Penicilium rubrum*, Isolation, Structure Elucidation and Biological Activity

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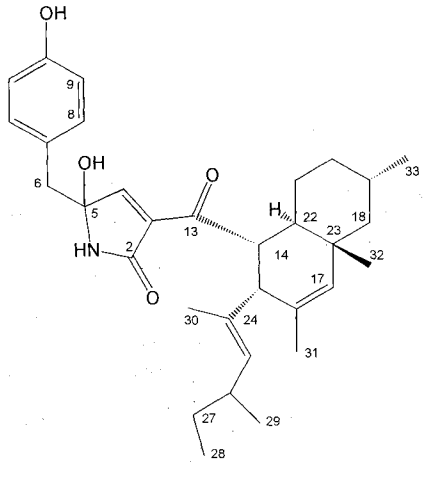
A novel inhibitor of platelet-activating factor (PAF) acetyltransferase, an essential enzyme in the remodeling pathway of platelet-activating factor synthesis, was identified by a high throughput screen of natural product extracts of microbial origin. The compound, ZG-1494α, was isolated from an ethyl acetate extract of a culture broth of *Penicillium rubrum* through bioassay guided fractionation. The structure of ZG-1494α was determined by spectroscopic methods. A key feature of the structure, which is relatively rare among natural products, is the 5-hydroxy-3-pyrrolin-2-one moiety. A ^{13}C-^{13}C INADEQUATE was utilized to unambiguously determine the regiochemistry of this molecule.

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent bioactive phospholipid with a diverse range of both normal and pathophysiological responses which includes: aggregation and degranulation of platelets and neutrophils, bronchoconstriction, systemic hypotension, anaphylaxis, and inflammatory and allergic responses.[1~4] In normal circumstances, PAF is an effective bioactive mediator of a host's response to routine physiological stimuli, such as destruction of foreign substances. However, in certain situations and diseases, PAF may lead to pathologies such as ischemia-reperfusion, necrotizing enterocolitis and asthma, where a response to a foreign body or self-antigen is turned upon the host itself. In this case, an elevated concentration of PAF can initiate tissue injury associated with inflammation.[5] The pathway for synthesis of PAF involved in normal physiological functions differs from the pathway of PAF synthesis in response to inflammatory stimuli.[6] These two routes of PAF synthesis in normal and pathophysiological responses are termed the *de novo* and the remodeling pathways, respectively. Because of the many pathophysiological activities of PAF, there have been numerous efforts to modulate this activity through the identification of PAF inhibitors. A number of PAF receptor antagonists have been identified,[7] but PAF receptor antagonists may inhibit the normal, necessary physiological functions of PAF, along with any pathophysiological activity. A few PAF synthesis inhibitors have also been found,[8,9] but the inhibitors discovered to date have low potency and often inhibit synthesis of a number of physiologically important factors besides PAF, leading to high toxicity. The most appropriate target for therapeutically useful modulators of PAF are compounds capable of inhibiting PAF synthesis selectively through the remodeling pathway. Such compounds should be useful for inhibiting the pathophysiological functions of PAF in clinical inflammatory states.

During the course of a screening program for in-

Fig. 1. Structure of ZG-1494α (**1**).



hibitors of the enzyme responsible for the last step in the remodeling pathway, PAF acetyltransferase, an active compound was discovered, ZG-1494α (**1**), that selectively inhibited this enzyme with an IC_{50} of 40μM. This article describes the isolation, structure elucidation, and the biological activity of this novel compound.

Isolation

Sixteen liters of fermentation broth of *Penicillium rubrum* was adjusted to pH 9.0 and then extracted with ethyl acetate; after evaporation the resultant residue was partitioned between acetonitrile and hexane, and the acetonitrile soluble fraction was evaporated to dryness. The acetonitrile fraction was redissolved into acetonitrile and then chromatographed on a preparative Vydac C-18 column using an acetonitrile/water gradient. Fractions from this column were pooled based upon their PAF acetyltransferase activity to afford approximately 18 mg/liter of pure ZG-1494α.

Structure Elucidation of ZG-1494α

The physico-chemical properties of ZG-1494α are summarized in Table 1. The molecular formula was determined to be $C_{32}H_{43}NO_4$ based upon HRFAB-MS. UV spectra in ethanol gave maxima at 206, 221, and 266. The carbon NMR of ZG-1494α (**1**) indicated the presence of 30 carbons. Two additional carbons at 39.31 and 14.43, however, could be clearly seen in a HETCOR[10] experiment even though their presence was not observed in either the ^{13}C NMR or DEPT[11] experiments. The ^1H NMR indicated the presence of 43 protons, which allowed the assignment of a molecular formula of $C_{32}H_{43}NO_4$ for the compound. This formula requires 12 rings or sites of unsaturation. Two carbonyl carbons at 196.61 and 167.23, and 12 olefinic carbons at 156.12, 155.99, 135.62, 135.56, 135.45, 132.00, 131.29 (2×), 129.80, 125.30, and 114.50 (2×) accounted for 8 sites of unsaturation which required 4 rings in the molecule. A quarternary carbon at 86.03 indicated the presence of a tertiary oxygen bearing carbon, which was assumed to be an alcohol due to the existence of an HMBC[12] correlation between the C(5)-OH proton at 6.061 which had no HETCOR correlation and this carbon. A ^1H-^1H correlation observed in the double quantum filtered phase sensitive COSY[13,14] between 6.991 and 6.567 (J= 8.0 Hz) indicated the presence of a 1,4 disubstituted benzylic moiety. An HMBC correlation between the C(10)-OH proton at 9.12 which had no HETCOR correlation, as well as an HMBC correlation between the methylene protons at 3.01, H(6a), and 2.88, H(6b), with a quarternary ring carbon at 125.30 C(7), allowed the assignment of a 1,4 disubstituted benzylic phenol group (Fig. 2, A) to these signals. Extensive analysis of the COSY, HETCOR, DEPT, and HMBC data revealed the spin systems shown in part structures B and C (Fig. 2). An isolated methyl group attached to a quarternary carbon could be seen clearly from the proton spectrum. Three additional methylene units, a methine, and a methyl doublet could be seen from the ^1H NMR, DEPT, and HETCOR data, but the exact arrangement of these signals could not be determined due to spectral overlap.

Key HMBC correlations were observed between the tertiary hydroxyl carbon at 86.03 and the N-1 amide proton at 8.557 and the olefinic proton at 7.489 on

Table 1. Physicochemical properties of ZG1494α (**1**).

Physical property	ZG1494α
Appearance	Colorless powder
$[\alpha]_D^{25}$ (c 1.0, ethanol)	−133°
Molecular formula	$C_{32}H_{43}NO_4$
HR-FAB-MS $(M+H)^+$	
Calcd for $C_{32}H_{44}NO_4$	506.3252
Found	506.3270
UV λ_{max}^{EtOH} (nm)	206, 221, 266 nm
IR ν_{max} KBr pellet	3375, 2952, 2920, 2866, 1722, 1700, 1650, 1613, 1597, 1516, 1451, 1375, 1223, 1108, 1054, 848, 832, 680, 625 cm^{-1}
Solubility	
Soluble:	Acn, DMSO, ethyl acetate, MeOH (dec.), $CHCl_3$ (dec.)
Insoluble:	Hexane
Color Reaction	
Positive:	H_2SO_4
Negative:	Ninhydrin

Fig. 2. Selected partial structures of ZG-1494α (**1**).

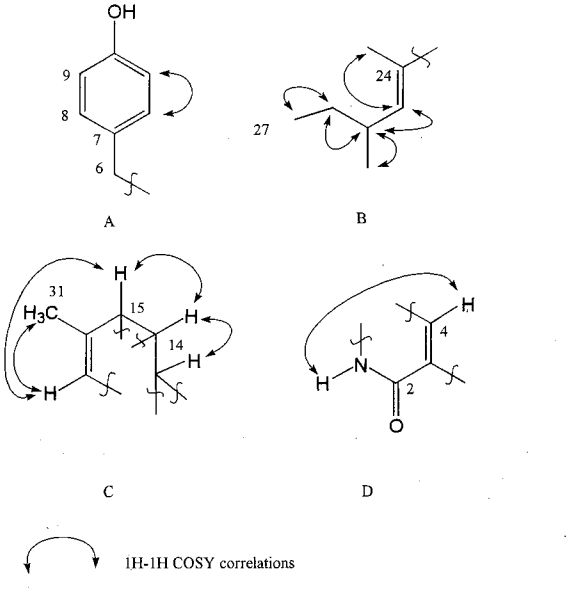


Table 2. ^{1}H, ^{13}C NMR assignments and COSY, ROESY, HMBC, and INADEQUATE correlations of ZG-1494α relative to DMSO-d_6.

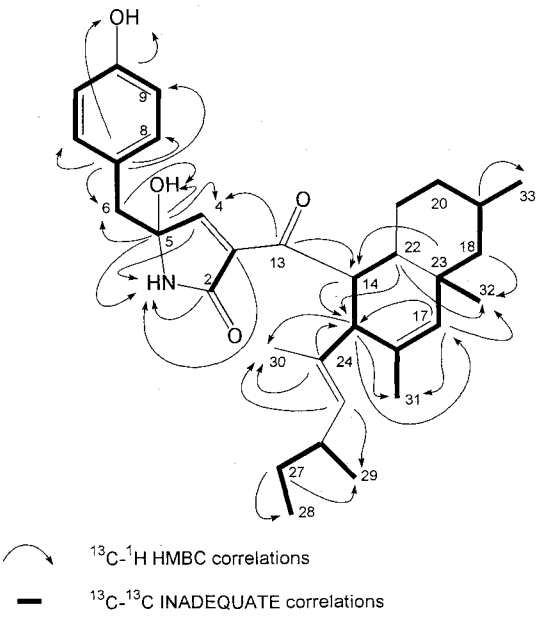
Assignment	^{13}C-(δ, ppm)[a]	^{1}H-(δ, ppm)[b]	^{1}H-^{1}H (COSY)	^{1}H-^{1}H NOE (ROESY)	^{1}H-^{13}C (HMBC)[c]	^{13}C-^{13}C (INADEQUATE)[d]
N(1)-H		8.557 (1H, s)	H(4)	H(6a), H(6b)		
C(2)	167.23				N(1)-H	C(3)
C(3)	135.60				N(1)-H	
C(4)	156.12	7.489 (1H, s)	N(1)-H	H(6a), H(6b)	N(1)-H	C(5)
C(5)-OH	86.03	6.061 (1H, s)			N(1)-H, H(4), H(6a), H(6b)	C(4), C(6)
C(6)-Ha	42.73	3.011 (1H, d, J=13.0 Hz)	H(6b)		C(5)-OH	C(5), C(7)
C(6)-Hb		2.882 (1H, d, J=13.5 Hz)	H(6a)		C(5)-OH	C(5), C(7)
C(7)	125.30				C(10)-OH, H(6a), H(6b), H(8, 12), H(9, 11)	C(8, 12)
C(8)-H/C(12)-H	131.29	6.991 (2H, d, J=8.0 Hz)	H(9, 11)		H(9, 11)	C(9, 11)
C(9)-H/C(11)-H	114.50	6.567 (2H, d, J=8.5 Hz)	H(8, 12)		H(8, 12)	C(8, 12), C(10)
C(10)-OH	155.99	9.115 (1H, s)			H(9, 11), H(8, 12)	C(9, 11)
C(13)	196.61				H(4), H(14)	
C(14)-H	48.45	3.493 (1H, dd, J=7, 12 Hz)	H(15), H(22)	H(4), H(15), H(32)	H(15)	C(15), C(22)
C(15)-H	50.56	2.734 (1H, d, J=7.0 Hz)	H(17), H(14), H(31)		H(31), H(30), H(17)	C(14), C(16), C(24)
C(16)	129.80					C(15), C(17)
C(17)-H	135.45	5.287 (1H, s)	H(15), H(31)	H(32), H(31)	H(15), H(32)	C(16), C(23)
C(18)-Ha	48.00	1.411 (1H, dd?, J=7.5 Hz)			H(32)	C(19), C(23)
C(18)-Hb		0.791 (1H, ?)			H(32)	C(19), C(23)
C(19)-H	26.87	1.643 (1H, ?)			H(33)	C(18), C(20), C(33)
C(20)-Ha	35.54	0.804 (1H, ?)				C(19), C(21)
C(20)-Hb		1.636 (1H, ?)				C(19), C(21)
C(21)-H_2	23.20	1.398 (2H, ?)				C(20)
C(22)-H	39.31	1.596 (1H, ?)			H(32), H(15)	
C(23)	35.08				H(14)	
C(24)	132.00				H(30), H(15)	C(15)
C(25)-H	135.56	4.443 (1H, d, J=8.5 Hz)	H(26), H(30)	H(15)	H(30), H(29)	
C(26)-H	33.52	1.990 (1H, m)	H(25), H(29)		H(29), H(28)	C(27), C(29)
C(27)-Ha	29.76	1.045 (1H, dq?, J=5.0, 7.5 Hz)	H(26), H(27b), H(28)		H(29), H(28)	C(26), C(28)
C(27)-Hb	29.76	1.184 (1H, dq?, J=5.5, 7.0 Hz)	H(27a), H(28)		H(29), H(28)	C(26), C(28)
C(28)-H_3	11.98	0.722 (3H, dd, J=7.0, 7.5 Hz)	H(27a), H(27b)			C(27)
C(29)-H_3	20.23	0.645 (3H, d, J=6.5 Hz)	H(26)			C(26)
C(30)-H_3	14.4	1.251 (3H, s)	H(25)			
C(31)-H_3	21.91	1.427 (3H, s)				C(16)
C(32)-H_3	20.34	0.846 (3H, s)				C(23)
C(33)-H_3	22.72	0.803 (3H, d, J=5.5 Hz)				C(19)

[a] 125 MHz. [b] 500 MHz. [c] $^{n}J_{CH}$=8 Hz. [d] J_{CC}=40 Hz.

C(4). A weak COSY correlation between the N(1) amide proton and the C(4) olefinic proton indicated that these two protons were part of the same spin system. Additional HMBC correlations between the amide proton and a carbonyl carbon at 167.23, C(2), as well as a quarternary olefinic carbon at 135.60, C(3), permitted the assignment of a five membered hydroxy pyrollidinone group that contained these signals. The exact regiochemistry of this ring, however, was only determined later through the use of a ^{13}C-^{13}C-INADEQUATE[15] experiment, which fixed this group into the arrangement shown based upon key correlations between the tertiary hydroxyl carbon C(5) and the olefinic methine at C(4), as well as between the amide carbonyl at C(2) and the quarternary olefinic carbon at C(3). An HMBC correlation between the C(5) tertiary hydroxyl carbon at 86.03 and the benzylic methylene protons on C(6) indicated that the benzylic phenol could be placed at this position of the pyrollidinone ring. This placement was later confirmed by the INADEQUATE experiment.

HMBC correlations between the carbonyl carbon at 196.61 and the olefinic proton on C(4) as well as the C(14) methine signal at 3.493 indicated that this carbonyl served as a bridge between these two part structures, C and D. Additional HMBC correlations between the C(15) carbon and the C(30) methyl protons at 1.251 allowed

Fig. 3. HMBC and INADEQUATE correlations for ZG-1494α (**1**)



the juxtaposition of part structure B with part structure C at the C(15) position. Again, the exact regiochemistry of these groups was fixed by the INADEQUATE experiment, particularly by the C(4)-C(5) and C(2)-C(3) correlations, as well as the C(15)-C(24) correlation.

The additional two sites of unsaturation required by the molecule could be satisfied by closing the remaining carbons into a second ring between C(22) and C(23) thereby forming a decalin ring system. Evidence for this was provided by the HMBC correlations between the C(22) carbon and the C(32) methyl proton signal at 0.846, as well as between the C(18) carbon at 48.00 and this same methyl singlet signal. The INADEQUATE correlations between C(23)-C(18), C(18)-C(19), C(19)-C(20), and C(20)-C(21) confirmed this assignment. Finally, the INADEQUATE correlation between C(19) and the methyl carbon at C(33) allowed the unambiguous placement of this methyl at the C(19) position.

Relative stereochemistry for the molecule (Fig. 1) was determined by a combination of a ROESY[16] experiment and measurement of the carbon chemical shifts and proton-proton coupling constants of the decalin ring signals. A coupling constant of 12 Hz between the H(14) and H(22) signals indicated an axial-axial arrangement between these two methine protons. A coupling constant of 7 Hz between the H(14) and H(15) signals placed these two signals in an axial-equatorial configuration. A ROESY correlation between the H(14) proton at 3.493 and the H(32) methyl signal at 0.846 put this methyl group in an axial arrangement. The C(33) methyl group could be assigned as equatorial due to a ^{13}C chemical shift of 22.72 and due to the ^{13}C chemical shift of 26.87 for the C(19) methylene signal.[17] Finally, a strong ROESY correlation between the H(25) olefinic proton at 4.443 and the H(15) methine proton at 2.734 indicates that the C(24)-C(25) double bond is in the *E*-configuration. The remaining two centers of stereochemistry, C-5 and C-26, could not be determined due to free rotation about these centers.

The structure of ZG-1494α contains the unusual 5-hydroxy-3-pyrrolin-2-one moiety which is relatively rare in natural products. Axinellamide, (5-hydroxy-5-((*E*,*E*)-6-methyl-2,4-octadienyl)-3-pyrrolin-2-one), has been isolated from a marine sponge,[18] (−)-5-hydroxy-3-pyrrolin-2-one has been isolated from dwarf peas,[19] and a bile pigment which contains this same heterocycle was isolated from the euphausiid *Meganycriphanes norvegica*.[20] 5-hydroxy-5-methyl-3-pyrrolin-2-one[21] and 5-hydroxy-5-((*E*,*E*)-3,6-octadienyl)-3-pyrrolin-2-one[22] have also been prepared synthetically. A similar compound to 1494α was also reported recently from an unidentified fungal strain.[23]

Results and Discussion

ZG-1494α (**1**) was shown to inhibit PAF acetyltransferase in the remodeling pathway with an IC_{50} of 40 μM. Concurrently, the compound was found to be seven fold less inhibitory to the *de novo* PAF acetyltransferase with an IC_{50} of 304 μM. Subsequent testing of this compound in a panel of fourteen receptor-binding assays designed to indicate antiinflammatory activity showed significant inhibition of binding of [^{3}H] PAF (IC_{50} = 3 μM) from PAF binding sites; inhibition of binding of [^{3}H] pyrilamine (IC_{50} = 3 μM) from histamine H_1 binding sites; and inhibition of binding of [^{3}H] dexamethasone (IC_{50} = 3 μM) from glucocorticoid binding sites. These data indicate that this compound could be a potential lead as an antiinflammatory.

Experimental

General Procedures

All solvents were HPLC grade from EM Science; deuterated NMR solvents were from Isotec, Inc. Media components were from Sigma, except for yeast extract which was from Difco. Preparative HPLC was carried out on a Rainin Dynamax system. A Rainin UV-D II dual wavelength detector set at 220 and 270 nm was used to monitor the chromatography, and fractions were collected with a Rainin Dynamax FC-2 fraction col-

lector. Analytical HPLC was carried out on a Hewlett-Packard HP1090 liquid chromatograph. UV spectra were recorded on a DU 640 spectrophotometer. IR spectra were recorded on a Perkin Elmer 1600 FTIR. Optical rotations were obtained on a Perkin Elmer Model 241 Polarimeter. NMR experiments were run on a Varian Unity-Plus 500 MHz. Mass spectral analysis was performed on a VG-70 SEQ Tandem Hybrid (EBqQ) magnetic sector mass spectrometer, and run in the fast atom bombardment mode (FAB). High resolution mass spectrometry was accomplished using PEG 300 for exact mass calibration.

Bioassay of PAF Acetyltransferase Activity

Briefly, to assay the remodeling pathway acetyltransferase, 400 ml of 500 mg/ml bovine serum albumin (fatty acid free; Sigma) in PBS (120 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4, supplemented with 10 μM $CaCl_2$) was added to each well of a 96-well, flat-bottom microtiter plate. The plate was incubated for 15 minutes at ambient temperature, then the wells were aspirated and 50 μl of PBS was added to each well. Duplicate test samples in DMSO ($\leq$5% (v/v)) were added to each well. HL-60 cells (a stable, transformed hematopoietic cell line) were combined with PBS containing 0.05% TWEEN-20 and mixed vigorously at 500 rpm for 30 seconds. This treatment permeabilized the HL-60 cells. An aliquot (50 μl) of HL-60 lysate so formed was added to each well of the plate, and the plate was incubated for 5 minutes at ambient temperature with gentle shaking. PAF acetyltransferase substrates were added to each well, such that 25 μl of the following solution was added per well: Lyso-PAF (160 mM; Calbiochem, La Jolla, CA), acetyl-CoA (80~400 mM; Calbiochem), and ^{3}H-acetyl-CoA (25 μCi/ml; Amersham). The plate was gently mixed for 45 seconds and then incubated at 37°C for 20 minutes. Newly synthesized PAF was extracted by adding 1.25 mg of AMBERCHROM CG-71 resin (Supelco, Bellefonte, PA) in 25 μl of distilled water to each well. The plate was gently shaken for 1 minute, following which the resin was collected on a 102 mm x 258 mm glass-fiber filter using a MICRO-CELL HARVESTER (Skatron, Waltham, MA). The filter was washed with 500 ml water to remove unincorporated ^{3}H-acetyl-CoA. Following the wash, the filter was dried in a microwave oven for 5~10 minutes.

After the filter was dried, it was soaked in 10 ml of Beta Plate scintillation fluid (LKB, Loughborough, England), and bound counts were determined in a TOP COUNT scintillation counter (Pharmacia). Percentage inhibition was determined by dividing the average of 2 or 3 replicate wells by the average of 3 control wells (5% DMSO alone), and multiplying by 100.

The *de novo* pathway of PAF synthesis was assayed in the same manner, except: (1) PBS buffer was supplemented with 5 mM EGTA, rather than 10 μM $CaCl_2$; and (2) newly synthesized 1-*O*-alkyl-2-*sn*-acetyl-3-glycero-phosphate, rather than newly synthesized PAF, was extracted with AMBERCHROM CG-71 resin. ZG-1494α was tested for inhibition of remodeling PAF-acetyltransferase at 1.5 to 380 μM.

Assays of Binding Activity

Binding assays for PAF, histamine, and glucocortocoid receptor binding were performed by Panlabs Incorporated, Bothell, Washington, according to the protocols described for these assays in their "Current Test Protocols of Pharmacology Services" catalogue.

For the PAF binding assay, platelets were prepared from fresh rabbit blood collected from carotid arteries 50 mg of platelet prep was incubated with 0.4 nM [^{3}H]PAF for 60 minutes at 25°C. Non-specific binding was determined in the presence of 1 μM native PAF. Bound [^{3}H]PAF was separated from free radioligand by rapid filtration through glass fiber filtermats and subsequently washed 3 times. Filtermats were then counted in an LKB Betaplate to determine specifically bound [^{3}H]PAF. Compounds were screened at 3 different concentrations.[24]

For the histamine (H_1) binding assay, membranes were prepared from whole brains of guinea pigs. A 10 mg sample of the membrane preparation was incubated with 3 nM [^{3}H]pyrilamine for 60 minutes at 25°C. Non-specific binding was determined in the presence of 1 μM pyrilamine. Bound [^{3}H]pyrilamine was separated from free radioligand and specifically bound [^{3}H]pyrilamine was determined as in the assay mentioned above for [^{3}H]PAF.[25]

For the glucocorticoid binding assay, jurkat cells were obtained by centrifugation at 1,500 rpm for 10 minutes. Resuspended cells were incubated with [^{3}H]-dexamethasone for 2 hours at 25°C. Bound [^{3}H]dexamethasone was separated from free radioligand and specifically bound [^{3}H]dexamethasone was determined as in the assay mentioned above for [^{3}H]PAF.[26]

Taxonomy of the Producing Organism

Strain NN005289/A03191 was originally isolated from red pepper in Denmark. The culture was taxonomically characterized as *Penicillium rubrum* at Centraalbureau voor Schimmelcultures, Oosterstraat 1, 3742 SK Baarn, The Netherlands. This fungus is maintained in the Culture and Metabolites Collection of Novo Nordisk A/S, Novo Alle, 2880 Bagsvaerd, Denmark as culture number NN005289/A03191. A viable culture of this microorganism was deposited with the Centraalbureau voor Schimmiel cultures, Oosterstraat 1, 3742 SK Baarn, The Netherlands on March 28, 1995. It has been deposited under the Budapest Treaty and assigned the strain designation CBS 238.95.

Fermentation

Three ml of a spore/mycelium suspension of NN005289/A03191 were used to inoculate 160 250 ml baffled shake flasks containing 100 ml of medium (sucrose, 30 g/liter; yeast extract, 5 g/liter; $NaNO_3$,

3.0 g/liter; KH_2PO_4, 1.0 g/liter; $MgSO_4 \cdot 7H_2O$, 0.5 g/liter; KCl, 0.5 g/liter; $FeSO_4 \cdot 7H_2O$ at 1%, 1.0 ml/liter). The flasks were incubated for 12 days at 26°C at 200 RPM rotation.

Isolation

All steps in the isolation of ZG-1494α were guided by bioassay. The flasks were combined to give a total of 16 liters. The entire culture (medium plus cells) was adjusted to pH 9.0, then combined with ethyl acetate in a 1 : 1 ratio and vigorously stirred for 16~18 hours at room temperature. The ethyl acetate/broth mixture was centrifuged at 1500 g for 10 minutes, the organic layer was removed by aspiration and evaporated to dryness using a rotary evaporator under low heat (30°C). The dried organic material was dissolved into 500 ml of acetonitrile, and then partitioned with 500 ml hexane in a 2 liters separatory funnel. The hexane layer was removed, and the acetonitrile fraction was repartitioned with 500 ml hexane. The acetonitrile layer was filtered through a sintered glass funnel, and then evaporated to dryness using a rotary evaporator under low heat, yielding 2.31 g of dried organic material. The acetonitrile soluble material was redissolved into acetonitrile and injected onto a preparatory HPLC column (22 × 250 mm, 10 μm, 300 Å, Vydac), 35 mg at a time. The HPLC column was eluted using a gradient of 50% acetonitrile in water to 100% acetonitrile in 60 minutes with a flow rate of 20 ml/minute UV detection was employed using a dual wavelength detector set at 220 and 270 nm. Fractions were collected every minute by an automatic fraction collector, and 0.5 ml of each of these fractions was removed and evaporated to dryness. The dried fractions were redissolved in DMSO for bioassay. The activity correlated with a pure peak that eluted at 21.5 minutes using the system mentioned above. Once activity was confirmed in this peak, then subsequent purifications were monitored by UV detection. 288 mg of pure compound was isolated from 16 liters of broth.

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