Inhibition of Plasminogen Activator Inhibitor-1 Activity by Two Diketopiperazines, XR330 and XR334 Produced by *Streptomyces* sp.

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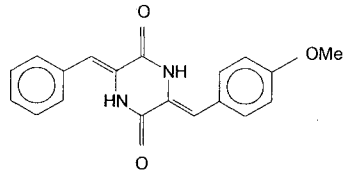
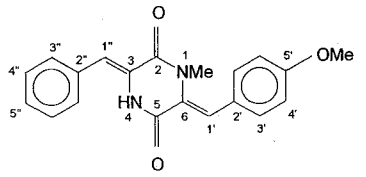
Two diketopiperazines, XR334 (**1**) and the novel compound XR330 (**2**), were isolated from the lyophilised biomass of an unidentified *Streptomyces* sp. Their structures were elucidated on the basis of spectroscopic studies and confirmed by chemical synthesis. Both compounds inhibited plasminogen activator inhibitor-1 activity in an amidolytic assay of tissue plasminogen activator mediated plasmin generation. Compound **1** also enhanced fibrinolysis *ex vivo* and protected against thrombus formation in the rat. These diketopiperazines represent the first low molecular weight inhibitors of plasminogen activator inhibitor-1, a physiological regulator of fibrinolysis

Thrombotic and thromboembolic diseases are major causes of hospitalisation and death in the developed world. A key event in the regulation of thrombus formation and clearance is tissue plasminogen activator (tPA) generation of plasmin[1]. tPA activity is itself regulated primarily by the fast-acting inhibitor plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (serpin) superfamily of protease inhibitors[2]. The PAI-1 cDNA encodes a 402 amino acid protein including a 23 amino acid signal peptide with a predicted, glycosylated mass of 52 kD[3]. PAI-1 inhibition of tPA is mediated through a "bait" residue (Arg 346–Met 347) which mimics the normal substrate[4]. The physiological importance of PAI-1 has been demonstrated in transgenic mice which express high levels of human PAI-1 and suffer severe venous thrombosis[5]. An increase in the plasma concentration of PAI-1 has been proposed as a risk factor in thrombotic disease[6]. During a screening programme for inhibitors of PAI-1 activity we discovered a series of low molecular weight, non-peptidyl inhibitors of PAI-1 from fermentation of an unidentified *Streptomyces* sp. Two examples of this series, XR334 (**1**) and the novel compound XR330 (**2**) were purified from the mycelium[7] (Fig. 1). A series of closely related metabolites has been isolated from *Streptomyces thioluteus* including **1**[8]. Several of these compounds were reported to show weak antibacterial activity. Synthetic analogues of **1** have also been reported previously[9~11]. Related diketopiperazines with putative cytotoxic activity have also been isolated from *Micromonospora neiheunsis*[12]. We report here an important new biologi-

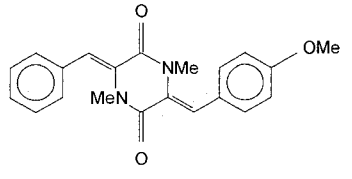
Fig. 1. Structures of XR334, XR330, XR333 and XR335.



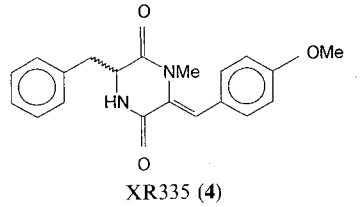
XR334 (**1**)



XR330 (**2**)



XR333 (**3**)



XR335 (**4**)

Structures of **1** and **2** isolated from the culture biomass of *Streptomyces* sp. and the structure confirmed by synthesis. Structures for two additional minor metabolites, **3** and **4**, are proposed.

cal activity for **1** and the related analogue **2**. These diketopiperazines provide the first low molecular weight inhibitors of the physiologically important serpin, PAI-1. In this paper we describe the fermentation, isolation, structure elucidation and synthesis of these compounds together with the preliminary biological evaluation of **1**.

Results

Fermentation

Streptomyces sp. X01/4/100 is an unidentified streptomycete isolated from a soil sample collected in the United Kingdom. It has been deposited under the Budapest Treaty at the National Collection of Industrial and Marine Bacteria Ltd. under the accession number NCIMB 40485.

Table 1. Taxonomic studies of *Streptomyces* sp. X01/4/100.

Character	X01/4/100
Spore chain	Rectiflexibiles
Number of spores in chain	20~50
Spore surface	Smooth
Spore colour	Red
Spore dimensions (widest points)	0.6×1 μm
Fragmentation of mycelium	None
Reverse pigmentation	Yellow/brown
Melanin production	No
Substrate utilisation:	
Adonitol	+
Cellobiose	−
D-Fructose	+
meso-Inositol	−
Inulin	−
Mannitol	+
Raffinose	+
Rhamnose	−
D-Xylose	−
DL-α-Aminobutyric acid	+
L-Histidine	+
L-Hydroxyproline	−
Degradation of:	
Allantoin	+
Arbutin	+
Xanthine	−
Pectin	−
Lecithin	−
Nitrate Reduction	−
Hydrogen sulphide production	+
Tolerance of sodium azide (0.01% w/v)	+
Tolerance of sodium chloride (7% w/v)	+
Tolerance of phenol (0.1% w/v)	+
Growth temperature range	20~35°C
Resistance to neomycin (50 μg/ml)	−
Resistance to rifampicin (50 μg/ml)	−
Antibiosis to *Aspergillus niger* LIV 131	+
Antibiosis to *Bacillus subtilis* NCIMB 3610	+
Antibiosis to *Streptomyces murinus* ISP 5091	+

Taxonomic studies on *Streptomyces* sp. X01/4/100 were carried out according to the recognised methodology of WILLIAMS *et al.*[13] recommended in BERGEY's Manual of Systematic Bacteriology Volume 4. The data obtained from these investigations, summarised in Table 1, do not clearly identify the organism with any of the species groups of the genus Streptomyces defined by WILLIAMS *et al.*[13,14].

A cryovial containing 1ml of a suspension of biomass of *Streptomyces* sp. X01/4/100 in 10% glycerol was retrieved from storage at −135°C and 0.5 ml of this used to inoculate 20 ml of medium S in a 250-ml Erlenmeyer flask. Medium S consisted of D-glucose, malt extract, yeast extract, glycerol, soyabean peptone, NaCl, $CaCO_3$, Junlon PW100 (Honeywill & Stein), Tween 80 (Sigma), pH 7. This culture was incubated at 28°C on an orbital shaker at 240 rpm for 4 days before another 20 ml of medium S was added and the incubation was continued under the same conditions for a further 4 days. At this point the 40 ml culture was used to inoculate 3.5 litres of medium S in a 4.5-litre stirred fermenter which was then incubated at 28°C, 350 rpm for 4 days with an aeration rate of 0.5 volumes of air per volume of fermentation per minute (vvm). The pH of this culture was controlled to between 5.5 and 6.5. The resultant culture was used to inoculate 50 litres of medium P in a 75-litre stirred fermenter. Medium P contained MOPS, proline, sucrose, NaCl, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, trace elements solution, vitamin mixture, pH 7. The trace elements solution consisted of $ZnSO_4 \cdot 7H_2O$, KI, $MnSO_4 \cdot 4H_2O$, H_3BO_3, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, H_2SO_4. The vitamin mixture consisted of thiamine HCl, riboflavin, sodium pantothenate, nicotinic acid, pyridoxine HCl, thioctic acid, folic acid, biotin, cyanocobalamin, potassium *p*-aminobenzoate, vitamin K_1. The fermentation was carried out for 5 days at 28°C with stirring at 250~500 rpm and aeration at 0.5 vvm, the pH was controlled at between 5.75 and 6.5 and antifoam A (Sigma) was used to control foaming.

Effect of Concentration of Medium P on Production of **1** and **2**

Small scale (10 ml) cultures were used to investigate how the concentration of medium and C : N ratio affected the production of these metabolites.

The results shown in Fig. 2 clearly show that reduction in the concentration of medium P had a stimulatory effect on the production of both **1** and **2**. Reduction of the medium concentration to 75% or 50% that of the control

Fig. 2. Effect of medium concentration on the specific production of **1**(a) and **2**(b).

□ 200%, ○ 150%, ▲ 100%, ▽ 75%, ◇ 50%.

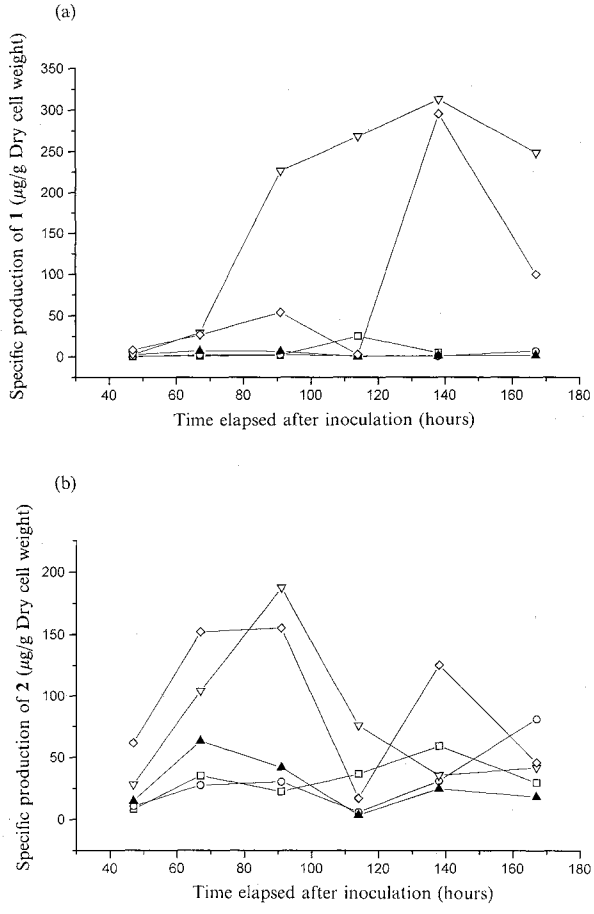
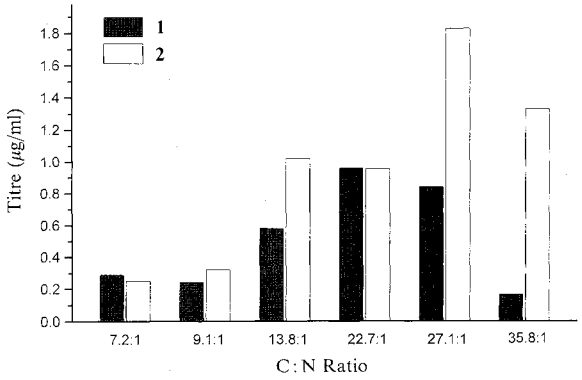


Fig. 3. Production of **1** and **2**.

Maximum measured titres over 110 hours on media with different C:N ratios.



led to 2.96 and 2.4 fold increases respectively in the production of **2** (Fig. 2b), the increase in production in 75% medium resulting in a 2 fold increase in product titre despite a reduced biomass. The effect on specific production of **1** was far more marked with 45.5 and 43-fold increases observed with 75% and 50% medium concentrations respectively (Fig. 2a). This increase in the production of these metabolites may be linked to the greater availability of oxygen per unit biomass in the diluted media. Observations from the large scale fermentations of this organism (data not shown) support this hypothesis. The highest titres were achieved in cultures where the dissolved oxygen tension (DOT) remained above 50%.

Effect of C:N Ratio on Production of **1** and **2**

Fermentations were carried out using variations of 75% medium P with C:N ratios increased or decreased from the original value of 13.8:1. The modifications were made through increasing or decreasing the concentrations of sucrose and glycerol in the medium, concentrations of all other medium components remained constant.

Fig. 3 shows the maximum titre of **1** and **2** obtained in the different media after 110 hours. A reduction in the C:N ratio from 13.8:1 decreased the titres achieved, whereas increasing it to 27:1 increased the titres. The decreased titres at the lower C:N ratios (9.1:1 and 7.2:1) correlated well with a reduction in the production rate of the metabolites (data not shown). In contrast, the higher C:N ratio media did not increase the initial production rate but these conditions appeared to delay cell lysis which resulted in a prolonged production phase (data not shown). The optimum C:N ratio for this system was 27.1:1 as a further increase in the ratio led to a lower production rate and a lower titre at harvest.

Isolation

Compounds **1** and **2** were isolated from the mycelium by solvent extraction and purified by chromatography. Details of the isolation procedure are described in the experimental section.

Physico-chemical Properties and Structure Elucidation

The compounds were characterised by standard physical techniques, including UV, MS, IR and NMR spectra. The physico-chemical properties of **1** and **2** are summarised in Table 2. The ^{13}C and ^{1}H NMR data of **2** are summarised in Table 3.

In the case of compound **2** a molecular formula of $C_{20}H_{18}N_2O_3$ was deduced from accurate mass measurements on the molecular ion. The ^{1}H and ^{1}H-^{1}H COSY NMR spectra included two three-proton singlets corresponding to the presence of two methyl groups attached

Table 2. Physico-chemical properties of **1** and **2**.

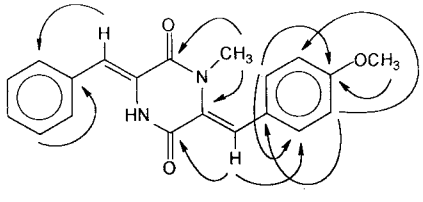
	1	**2**
Appearance	White/yellow powder	White/yellow powder
Molecular weight	320	334
Molecular formula	$C_{19}H_{16}N_2O_3$	$C_{20}H_{18}N_2O_3$
HREI-MS (*m/z*)		
Calcd:	320.1161	334.1317
Found:	320.1164	334.1320
UV MeOH λ_{max} nm	330, end absorbance	350, end absorbance
IR ν_{max} (KBr) cm^{-1}	3200, 3050, 1735, 1720, 1400, 1230, 1010, 730	3200, 3050, 1720, 1620, 1390, 1350, 750

Table 3. ^{13}C and ^{1}H NMR data of **2** in $CDCl_3$ (30°C).

Position	δ_C[a]	δ_H[b]
2	159.63	
3	125.99	
4		8.05 (1H, br s)
5	159.88[c]	
6	128.95	
1′	121.30	7.25 (1H, s)
2′	125.93	
3′	131.05	7.24 (2H, d, J=8.8 Hz)
4′	113.79	6.93 (2H, d, J=8.6 Hz)
5′	159.80[c]	
1″	116.95	7.07 (1H, s)
2″	132.94	
3″	128.45	7.42 (2H, m)
4″	129.35	7.42 (2H, m)
5″	128.71	7.36 (1H, m)
N-CH_3	36.54	3.05 (3H, s)
O-CH_3	55.27	3.83 (3H, s)

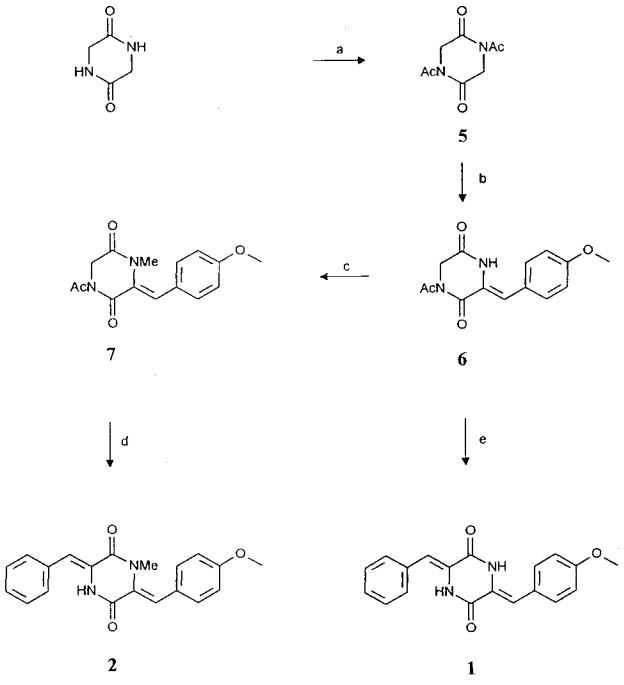
[a] Chemical shifts are shown referenced to $CDCl_3$ as 77.00 ppm.
[b] Chemical shifts are shown referenced to $CDCl_3$ as 7.25 ppm.
[c] Assignments may be exchanged.

Fig. 4. Selected long range ^{1}H-^{13}C couplings observed for **2** in a COLOC experiment.



to oxygen and nitrogen respectively, a broad one proton singlet at 8.05 ppm and revealed the presence of eleven further protons corresponding to a benzylidene and a *para*-substituted benzylidene group. The ^{13}C NMR sprectrum supported the above conclusions and further indicated the presence of two quaternary carbons carbons resonating at 159.63 and 159.88 ppm. The

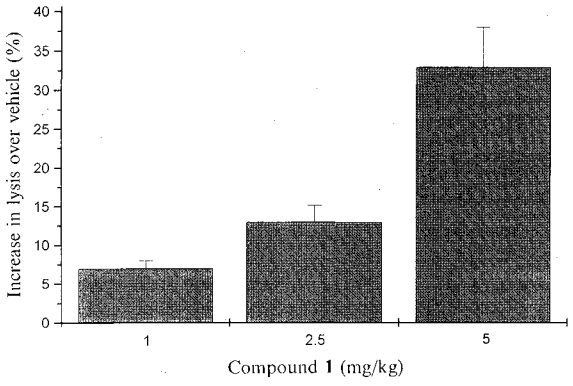
Fig. 5. Synthesis of **1** and **2**.



(a) Ac_2O, 130°C, 6 hours; 85%.
(b) KOtBu, 4-methoxybenzaldehyde, tBuOH, THF, 0°C to room temperature, overnight; 69%.
(c) Na_2CO_3, MeI, DMF, room temperature, 4 days; 88%.
(d) Cs_2CO_3, benzaldehyde, DMF, 80°C; 2 hours; 54%.
(e) Cs_2CO_3, benzaldehyde, DMF, 90°C, 2 hours; 67%.

structure of compound **2** was elucidated by interpretation of spectral data, including the results of DEPT, ^{13}C-^{1}H correlation and COLOC (COrrelation spectroscopy *via* LOng range Coupling) experiments. Key correlations observed in the COLOC experiment are illustrated in Fig. 4. The structure of compound **1** was determined in similar fashion. The stereochemistry of **1** and **2** was proposed to be 3*Z*, 6*Z* based on the geometric isomer produced by *S. thioluteus* and biosynthetic grounds. The proposed structures of **1** and **2** were confirmed by synthesis.

Fig. 6. Effect of different doses of **1** on fibrinolysis *ex vivo* in the rat dilute blood clot lysis time assay.



Vehicle (10% Solutol in saline) or **1** (1, 2.5 and 5 mg/kg) was administered by single bolus injection *via* the tail vein. Data show maximum increase in lysis over vehicle control expressed as a percentage. Results indicate mean and standard error of 3 experiments.

Synthesis of 1 and 2

The synthesis of compounds **1** and **2** is outlined in Fig. 5. Details of the synthesis are described in the experimental section.

Biological Activity

Chromogenic Assays

Compounds **1** and **2** inhibited PAI-1 in an *in vitro* tPA-mediated plasmin generation assay (S2251) with IC_{50} values of $51 \pm 7\,\mu M$ (mean $\pm$ SE) and *ca.* $30\,\mu M$, respectively. Compound **1** was chosen to profile further and was shown to also inhibit PAI-1 in a urokinase amidolytic assay (S2444) with an IC_{50} value of $80 \pm 4\,\mu M$. Various controls were included to exclude the possibility that the diketopiperazine interfered with the substrates or acted directly on the plasminogen activators. Details of these assays are described in the experimental section.

Rat Dilute Blood Clot Lysis Time Assay

The effect of **1** on *ex vivo* fibrinolysis in the rat is shown in Fig. 6.

Discussion

The diketopiperazines described in this paper provide a chemical template for the design of low molecular weight inhibitors of PAI-1. The two related metabolites, including the novel compound **2**, were isolated from the lyophilised biomass of an unidentified *Streptomyces* sp. It is probable that this family of diketopiperazine adducts are derived from phenylalanine and *O*-methyltyrosine. The structures of **1** and **2** were confirmed by total synthesis.

As part of a programme to identify additional active metabolites we assessed the effect of medium component concentration and C : N ratio on production of **1** and **2**. Dilution of the growth medium resulted in increased production of both compounds but the increase was greater for **1**. It is probable that increased oxygen availability contributed to the enhanced production. A marked improvement in titre for both **1** and **2** was achieved by increasing the C : N ratio from 13.8 : 1 to 27.1 : 1. Additional related metabolites were isolated from the biomass. The proposed structures (XR333, **3**, and XR335, **4**) are shown in Fig. 1 but there was insufficient material for full structural confirmation.

There is a growing body of evidence that elevated PAI-1 activity is a risk factor for thromboembolic disease as a result of decreased fibrinolytic activity[15]. The importance of PAI-1 is supported by recent studies utilising antibodies as PAI-1 inhibitors. In a rabbit jugular vein model an anti-PAI-1 monoclonal antibody was shown both to promote thrombolysis and to limit thrombus growth[16]. In a second study a fragment of a polyclonal antibody against PAI-1 was reported to increase fibrinolysis[17]. Compounds **1** and **2** inhibited PAI-1 activity in an amidolytic assay of tPA-mediated plasmin generation. These results were not due to a direct effect on tPA as the compounds did not enhance tPA activity in the absence of PAI-1. Although both compounds were poorly soluble in aqueous media **1** was selected for further evaluation as a higher concentration could be achieved. In a second amidolytic assay **1** also reversed the inhibitory effects of PAI-1 on urokinase and these data implied that the diketopiperazine was modulating the PAI-1: serine protease interaction possibly through interaction with the PAI-1 protein. In the light of the limited solubility of **1** the estimated IC_{50} values may be an underestimate due to precipitation of the compound in the *in vitro* assay buffer. Importantly **1** also enhanced fibrinolysis in an *ex vivo* assay of clot lysis in whole rat blood following intravenous bolus administration demonstrating that activity was retained in a more complex physiological medium[18]. Compound **1** dose dependently increased clot lysis in blood from animals treated with the diketopiperazine compared to the vehicle group (Fig. 6). At 5 mg/kg **1** increased clot lysis by $32.0 \pm 5.1\%$ ($n=25$, $P<0.01$). Finally an infusion of **1** effectively doubled the time to thrombus formation in the rat electrically stimulated carotid artery thrombosis model[18]. An infusion of **1** (1 mg/kg/minute, 20 minutes) significantly increased the time to vessel occlusion from 14.4 ± 0.9 minutes (vehicle, 10% Solutol in 0.9% saline, $n=43$) to 32.0 ± 3.2 minutes ($n=19$, $P<0.01$). Compound **1** had comparable activity in this model to an infusion of the reference compound aspirin, which inhibits platelet aggregation. Importantly **1** at high concentration ($100\,\mu M$) had no effect on human platelet aggregation stimulated by collagen, ADP and sodium arachidonate (data not shown). Thus **1** appears to

prolong the time to thrombus formation by a mechanism distinct from that of aspirin. Details of the pharmacological evaluation of **1** will be the subject of a further communication[18].

As part of a screening programme we have identified a series of low molecular weight inhibitors of PAI-1 including compound **1**. This diketopiperazine inhibited the PAI-1/tPA interaction *in vitro*, enhanced fibrinolysis *ex vivo* and protected against thrombus formation in the rat. These data demonstrate that a low molecular weight compound can modulate PAI-1 activity and these diketopiperazine compounds have provided an important chemical template for synthetic modification and optimisation. Ultimately, inhibitors of PAI-1 may have utility in the treatment of thromboembolic diseases, including deep vein thrombosis and unstable angina.

Experimental

General Procedures

NMR spectra were recorded on a Bruker AC400 spectrometer at 400 MHz. Chemical shifts were measured in ppm on the δ scale, downfield from tetramethylsilane as internal standard. Infra-red spectra were recorded on a Nicolet 5PC spectrophotometer. Mass spectra were obtained by desorption chemical ionisation (NH_3) or EI on a VG Trio 3 spectrometer. Accurate mass measurements were recorded on a VG Autospec spectrometer. Analytical TLC was carried out on Merck aluminium sheet silica gel 60 F_{254} plates. Anhydrous dimethylformamide was purchased from Aldrich and stored under nitrogen.

Isolation of **1** and **2**

A 50 litre fermentation was centrifuged and the supernatant discarded. The resulting mycelium (*ca.* 10 kg wet weight) was mixed briefly with methanol to remove any excess supernatant and then extracted with dichloromethane-methanol mixture (1:1) followed by dichloromethane. The extracts were pooled and evaporated under reduced pressure to dryness (*ca.* 10 g).

A column (internal diameter 3.5 cm, depth 25 cm) was slurry packed with silica gel (Baker, 40 to 60 μm) in hexane. A solution of the crude extract in dichloromethane was dispersed in silica gel, the solvent removed and the remaining material applied to the column and eluted using a stepwise hexane/dichloromethane/ethyl acetate/methanol gradient increasing from 0% hexane to 100% methanol; 50 × 50 ml fractions were collected.

The fractions were concentrated to dryness, redissolved in 1% dichloromethane/methanol (volume varied from 5 to 15 ml) and monitored by high performance liquid chromatography and for PAI-inhibitory activity and those fractions containing the diketopiperazines were combined and concentrated to dryness. The residue was purified further by preparative HPLC using an octadecyl silica cartridge (Waters, 25 × 100 mm Nova Pak HR C_{18} 6 μm) eluting at 12 ml/minute with a solvent gradient programme commencing at 90% water/10% acetonitrile and terminating at 100% acetonitrile after 20 minutes. The eluant was monitored at 350 nm and a peak eluting at ca 15 minutes was collected and evaporated at reduced pressure to yield a crude fraction containing the diketopiperazines (*ca.* 100 mg).

The crude fraction was chromatographed further by semi-preparative HPLC using an octadecyl silica cartridge (Waters, 8 × 100 mm C_{18} Nova Pak 4 μm). The fractions containing the diketopiperazines were concentrated to dryness to yield a white/yellow powder (*ca.* 5 mg).

tPA-Mediated Plasmin Generation Assay (S2251)

This assay is a modification of the method of NILSSON *et al.*[19] and utilises the COASET tPA kit (Chromogenix, Mölndal, Sweden). Briefly, the assay is as follows. Human PAI-1 was standardised by titration against tPA. A concentration of PAI-1 was chosen that just inhibited tPA activity. The compounds were incubated with PAI-1 for 10 minutes prior to addition to the tPA assay system. Inhibition of PAI-1 activity resulted in tPA-mediated cleavage of plasminogen to plasmin. The plasmin released then cleaved the chromogenic substrate S2251 (H-D-Val-Leu-Lys-pNA) (Chromogenix, Mölndal, Sweden) to produce *p*-nitroaniline which was detected spectrophotometrically at 405 nM.

Urokinase Assay (S2444)

Human PAI-1 was standardised by titration against urokinase (human kidney cell, Sigma Chemical Co., UK) and the assay performed essentially by the method outlined above. Inhibition of PAI-1 activity resulted in cleavage of the chromogenic substrate S2444 (Glu-Glu-Arg-pNA·HCl) by urokinase and release of *p*-nitroaniline.

Rat Dilute Blood Clot Lysis Time (DBCLT)

The *ex vivo* DBCLT assay was performed as described[18]. Either vehicle (10% Solutol in 0.9% saline) or **1** (1, 2.5 or 5 mg/kg) was administered by single bolus injection *via* the tail vein to male Wistar rats. A blood sample was collected after 2.5 minutes, diluted 1:4 then ^{125}I-fibrinogen added and the sample clotted by the addition of thrombin. Lysis was measured by the release of ^{125}I-fibrin fragments into the supernatant.

Synthesis

1,4-Diacetyl-2,5-piperazinedione (**5**)

2,5-Piperazinedione (200 g, 1.75 mol) was heated at 130°C in acetic anhydride (800 ml). After 6 hours the mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was recrystallised from a hexane-ethyl acetate mixture to give 294 g (85%) of a buff coloured solid. ^{1}H NMR ($CDCl_3$): δ 2.59 (3H, s, $COCH_3$); 4.58 (2H, s, CH_2). MS (DCI NH_3) *m/z*: 216 ($[MNH_4]^+$, 100%). IR (KBr diffuse

reflectance) ν_{max}: 740, 920, 1250, 1730, 3000. Microanalysis: calculated C 48.49%, H 5.09%, N 14.14%; found C 48.52%, H 5.11%, N 14.12%.

(3*Z*)-1-Acetyl-3-(4-methoxybenzylidene)-2,5-piperazinedione (**6**)

A solution of 1,4-diacetyl-2,5-piperazinedione (**5**) (10 g, 50.5 mmol) and 4-methoxybenzaldehyde (6.14 ml, 50.5 mmol) in dry tetrahydrofuran (100 ml) was cooled to 0°C. A solution of potassium *t*-butoxide (5.67 g, 50.5 mmol) in *t*-butanol (100 ml) was then added dropwise over 30 minutes. After the addition was complete the mixture was allowed to warm to room temperature overnight. The mixture was diluted with ethyl acetate (400 ml) and washed with water (2 × 200 ml) and saturated brine (2 × 200 ml). The white precipitate which formed in the organic phase during this process was filtered off and dried to give 9.55 g (69%) of a white solid. ^{1}H NMR ($CDCl_3$): δ 2.66 (3H, s, $COCH_3$); 3.85 (3H, s, OCH_3); 4.53 (2H, s, CH_2); 6.98 (2H, d, J=8 Hz, Ar); 7.14 (1H, s, vinylic); 7.47 (2H, d, J=8 Hz, Ar); 7.90 (1H, br s, N*H*). MS (DCI NH_3) *m/z*: 275 (MH^+, 100%). IR (KBr diffuse reflectance) ν_{max}: 740, 1230, 1380, 1740, 3000, 3220. Microanalysis: calculated C 61.31%, H 5.14%, N 10.21%; found C 61.29%, H 5.13%, N 10.20%.

(3*Z*)-1-Acetyl-3-(4-methoxybenzylidene)-4-methyl-2,5-piperazinedione (**7**)

A mixture of (3*Z*)-1-acetyl-3-(4-methoxybenzylidene)-2,5-piperazinedione (**6**), methyl iodide (1.71 ml, 27.4 mmol) and sodium carbonate (1.93 g, 18.25 mmol) in dry dimethylformamide (100 ml) was stirred at room temperature for 4 days. After 4 days TLC (ethyl acetate: hexane 1 : 1) indicated that no starting material remained. The mixture was diluted with ethyl acetate (750 ml) and washed with saturated brine (4 × 300 ml). The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was purified by flash chromatography (silica, eluting with ethyl acetate: hexane 1 : 2, then with ethyl acetate : hexane 1 : 1) to give 4.62 g (88%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 2.64 (3H, s, $COCH_3$); 2.96 (3H, s, N-CH_3); 3.85 (3H, s, OCH_3); 4.52 (2H, s, CH_2); 6.90 (2H, d, J=8 Hz, Ar); 7.25 (3H, m, Ar and vinylic). MS (DCI NH_3) *m/z*: 306 ($[MNH_4]^+$, 32%); 289 (MH^+, 100%). IR (KBr diffuse reflectance) ν_{max}: 730, 910, 1730, 1735, 3000. Microanalysis: calculated C 62.49%, H 5.59%, N 9.72%; found C 62.48%, H 5.58%, N 9.68%.

(3*Z*,6*Z*)-3-Benzylidene-6-(4-methoxybenzylidene)-1-methyl-2,5-piperazinedione (**2**)

A mixture of (3*Z*)-1-acetyl-3-(4-methoxybenzylidene)-4-methyl-2,5-piperazinedione (**7**) (4.47 g, 15.5 mmol) caesium carbonate (6.03 g, 18.5 mmol) and benzaldehyde (1.58 ml, 15.5 mmol) in dry DMF (90 ml) was heated at 80°C. After 2 hours TLC dichloromethane : methanol 50 : 1) showed no starting material remained. The mixture was cooled to room temperature and poured into ethyl acetate (500 ml). The organic solution was washed with saturated brine (3 × 200 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was triturated with methanol to give 2.80 g (54%) of a pale yellow solid. A sample was recrystallised from ethanol for analysis. ^{1}H NMR ($CDCl_3$) δ 3.03 (3H, s, N-CH_3); 3.85 (3H, s, OCH_3); 6.95 (2H, d, J=8 Hz, Ar); 7.09 (1H, s, vinylic); 7.22 (1H, s, vinylic); 7.30 ~ 7.50 (7H, m, Ar); 8.01 (1H, br s, N*H*). MS (DCI NH_3) *m/z*: 335 (MH^+, 100%). IR (KBr diffuse reflectance) ν_{max}: 740, 910, 1390, 1720, 3000, 3400. Microanalysis: calculated C 71.84%, H 5.43%, N 8.38%; found C 71.81%, H 5.31%, N 8.31%.

(3*Z*,6*Z*)-3-(4-Methoxybenzylidene)-6-benzylidene-2,5-piperazinedione (**1**)

A mixture of (3*Z*)-1-acetyl-3-(4-methoxybenzylidene)-2,5-piperazinedione (**6**) (5.0 g, 18.25 mmol) caesium carbonate (5.95 g, 18.25 mmol) and benzaldehyde (1.85 ml, 18.25 mmol) in dimethylformamide (50 ml) was heated at 90°C. After 2 hours the mixture was cooled to room temperature, diluted with ethyl acetate (100 ml) and poured into water (100 ml). The resultant precipitate was collected by filtration, washed with water followed by methanol and diethyl ether and recrystallised from acetic acid to give 4.0 g (67%) of a yellow solid. ^{1}H NMR (d_6-DMSO): δ 3.78 (3H, s, OCH_3); 6.74 (1H, s, vinylic); 6.75 (1H, s, vinylic); 6.98 (2H, d, J=8 Hz, Ar); 7.32 (1H, m, Ar); 7.40 (2H, m, Ar); 7.54 (4H, m, Ar); 10.16 (1H, br s, N*H*). MS (DCI NH_3) *m/z*: 321 (MH^+, 36%); 160 (100%). IR (KBr diffuse reflectance) ν_{max}: 730, 1010, 1230, 1405, 1720, 1735, 3000, 3200. Microanalysis: calculated C 71.24%, H 5.03%, N 8.74%, found C 71.27%, H 5.01%, N 8.72%.

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