Aminophenoxazinones as Inhibitors of Indoleamine 2,3-dioxygenase (IDO).

Synthesis of Exfoliazone and Chandrananimycin A

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ABSTRACT

A range of 2-aminophenoxazin-3-ones has been prepared by oxidative cyclocondensation of 2-aminophenols, including the natural products exfoliazone and chandrananimycin A, both synthesized for the first time. The compounds were evaluated for their ability to inhibit indoleamine 2,3-dioxygenase. Compounds containing additional electron-withdrawing carboxylate groups, such as cinnabarinic acid, showed modest inhibitory activity with a dose response.

INTRODUCTION

Aminophenoxazinones are heterocyclic dyes that occur as core structures in a number of natural products, most notably the actinomycins. These highly colored, potent antibiotics that intercalate with DNA have been widely studied although their clinical application is limited due to their
The aminophenoxazinone core also occurs in a number of structurally simpler bioactive substances such as exfoliazone,\textsuperscript{2,3} the venezuelines,\textsuperscript{4} the bezerramycins,\textsuperscript{5} cinnabarinic acid,\textsuperscript{6} pitucamycin,\textsuperscript{7} the chandrananimycins,\textsuperscript{8} and peristrophine (Figure 1).\textsuperscript{9} However it was the structure of the marine fungal natural product plectosphaeroic acid that caught our attention, not least because of the reported activity as an inhibitor of indoleamine 2,3-dioxygenase.\textsuperscript{10}

\textbf{Figure 1.} Some naturally occurring aminophenoxazinones.
The enzyme indoleamine 2,3-dioxygenase (IDO) is implicated in a different but very attractive approach to cancer therapy, i.e. to recruit the body’s own immune system to reject solid tumours. Therefore some effort has gone into trying to understand how tumours escape the host immune system,\(^\text{11}\) and IDO has been shown to play a major role. Its function, the oxidative cleavage of the essential amino acid tryptophan to \(N\)-formylkynurenine,\(^\text{12}\) suppresses the immune response, and there is now a growing body of evidence to support the hypothesis that inhibition of IDO produces significant anticancer effects.\(^{\text{11,13-16}}\) Hence IDO has emerged as an attractive target,\(^\text{17}\) since it is known to be activated in a number of human cancers, it has a known structure amenable to inhibition by small molecules, and there is little likelihood of off-target action since tryptophan 2,3-dioxygenase (TDO), the only closely related enzyme, is much more localized.

There are a number of known inhibitors of IDO, encompassing many structural types as outlined in a very recent review.\(^\text{18}\) However the most widely studied are based on 1-methyltryptophan (1-MT), a competitive inhibitor with the natural substrate, arylimidazoles such as 4-phenylimidazole,\(^\text{19}\) naphthoquinones such as dehydro-\(\alpha\)-lapachone,\(^\text{20}\) and S-benzylisothioureas\(^\text{21}\) (Figure 2). Hence the reported activity of plectosphaeroic acid, with the activity residing in the phenoxazinone, cinnabarinic acid-like fragment, prompted us to investigate the synthesis and biological evaluation of a range of aminophenoxinones as these would represent a new family of IDO inhibitors.
RESULTS AND DISCUSSION

In nature, aminophenoxazinones are formed by the oxidative cyclocondensation of 2-aminophenols with the enzyme phenoxazinone synthase (PHS), a copper-containing oxidase.\textsuperscript{22} Enzymes, most notably laccase,\textsuperscript{23} can be used to access aminophenoxazinones in the laboratory, but a range of other oxidants can also be employed, including Cu(I)Cl,\textsuperscript{24} K\textsubscript{3}Fe(CN)\textsubscript{6},\textsuperscript{25} NaIO\textsubscript{3},\textsuperscript{26} peroxide,\textsuperscript{27} and benzoquinone.\textsuperscript{28} Initially, we chose to prepare a small range of relatively simple aminophenoxazinones by oxidative self condensation of readily available 2-aminophenols. Thus under the copper catalyzed conditions,\textsuperscript{24} 2-aminophenol itself and 2-amino-3-methylphenol gave the known 2-aminophenoxazinones 1 and 2 in modest yield (Table 1). For the synthesis of cinnabarinic acid 3 from 3-hydroxyanthranilic acid, the most effective oxidant was found to be benzoquinone,\textsuperscript{28} whilst potassium ferricyanide\textsuperscript{25} was used for the oxidative cyclocondensation of methyl 3-hydroxyanthranilate and its 4-methyl substituted homologue into aminophenoxazinones 4 and 5 respectively (Table 1).
Table 1. Oxidative cyclocondensation of 2-aminophenols to give 1,9-disubstituted- or 1,4,6,9-tetrasubstituted-2-aminophenoxazin-3-ones

<table>
<thead>
<tr>
<th>R^{1/9}</th>
<th>R^{4/6}</th>
<th>Method</th>
<th>Product</th>
<th>Yield (%)</th>
</tr>
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<tr>
<td>H</td>
<td>H</td>
<td>CuCl, DMF, rt, 24 h</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Me</td>
<td>H</td>
<td>CuCl, DMF, rt, 24 h</td>
<td>2</td>
<td>55</td>
</tr>
<tr>
<td>CO₂H</td>
<td>H</td>
<td>benzoquinone, EtOH, rt, 1 h</td>
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<td>40</td>
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<td>45</td>
</tr>
<tr>
<td>CO₂Me</td>
<td>Me</td>
<td>K₃Fe(CN)₆, MeOH, pH7 buffer, rt, 16 h</td>
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<td>38</td>
</tr>
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</table>

Although the oxidative ‘dimerization’ of two 2-aminophenols as described above is well established,²⁴,²⁵,²⁷,²⁸ to access ‘unsymmetrical’ aminophenoxazinones such as exfoliazone and the chandrananimycins, an oxidative cyclocondensation reaction of two different aminophenols is required, and such reactions are virtually unknown. The main examples employ sodium iodate as oxidant,²⁶ and therefore we attempted to use this protocol in the oxidative coupling of 4-methyl-2-aminophenol and 2-aminophenol. The most satisfactory conditions involved preoxidation of 2-aminophenol (i.e. the aminophenol that is destined to become the A-ring in the aminophenoxazinone) before adding the second aminophenol; this gave the phenoxazinone 6 in a modest 35% yield (Table 2). In a similar manner, 3-methyl-2-aminophenol was coupled with 4-methyl-2-aminophenol to give the 2-amino-1,8-dimethylphenoxazinone 7 in modest yield. The
method was next applied to the synthesis of the naturally occurring aminophenoxazinones chandrananimycin A 11 and exfoliazone 13, which despite their relatively simple structures have never been synthesized previously. Thus reaction of 2-aminophenol with 2-aminoresorcinol under sodium iodate oxidation gave phenoxazinone 8 in poor yield, N-acetylation of which gave chandrananimycin A (Scheme 1), the spectroscopic properties of which were identical to those described for the natural material.8 Likewise reaction of 2-aminophenol with THP-protected 4-hydroxymethyl-2-aminophenol (see Supporting Information) gave phenoxazinone 9, again in poor yield (Table 2). Phenoxazinone 9 was readily deprotected to give the 8-hydroxymethyl-2-aminophenoxazinone 12, or alternatively N-acetylated and then deprotected to deliver exfoliazone 13 itself (Scheme 1), spectroscopically identical to natural material.2 Finally, oxidative coupling of methyl 2-amino-3-hydroxybenzoate with THP-protected 4-hydroxymethyl-2-aminophenol gave phenoxazinone 10, readily deprotected to phenoxazinone 14, a possible precursor to the bezerramycins. Hence a range of 2-aminophenoxazin-3-ones is available, albeit in modest yield, by simple oxidative cyclocondensation of 2-aminophenols.

Table 2. Oxidative cyclocondensation of two different 2-aminophenols to 2-aminophenoxazin-3-ones

<table>
<thead>
<tr>
<th>R^1</th>
<th>R^8</th>
<th>R^9</th>
<th>Product</th>
<th>Yield /%</th>
</tr>
</thead>
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<tr>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>6</td>
<td>35</td>
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With a range of phenoxazinones in hand, attention turned to their evaluation as IDO inhibitors. Human IDO, with histidine tagged N-terminus, was expressed in *E. coli* and purified using Ni$^{2+}$ affinity chromatography. Compounds were evaluated for their ability to inhibit IDO-catalyzed oxidative degradation of *L*-tryptophan to *N*-formylkynurenine, assayed by conversion into kynurenine following trichloroacetic acid cleavage of the *N*-formyl group and subsequent reaction.
with Ehrlich’s reagent to produce a product with strong UV/VIS absorbance at 480 nm. The data that show the amount of enzyme activity remaining at three different inhibitor concentrations are shown in Table 3. The results establish cinnabarinic acid 3 as the most potent IDO inhibitor of the series, and in a separate experiment its IC$_{50}$ was determined as 0.46 µM compared with the previously reported IC$_{50}$ of ~2 µM.$^{10}$ Kinetic analysis using two inhibitor concentrations estimated the $K_i$ at 326 nM demonstrating that cinnabarinic acid 3 is a potent inhibitor of IDO. Cinnabarinic acid was also compared with the known IDO inhibitor 4-phenylimidazole that in our assay had an IC$_{50}$ of 70.3 µM, compared with the reported value of 48 µM.$^{19}$ It is also noted that cinnabarinic acid has comparable potency to dehydro-α-lapachone, a member of the naphthoquinone family of IDO inhibitors (Figure 2), that has an IC$_{50}$ of 0.21 µM.$^{20}$ Interestingly, cinnabarinic acid 3 has been shown to be a product of kynurenine metabolism generated from two molecules of 3-hydroxyanthranilic acid.$^{29}$ Whether feedback inhibition of IDO by cinnabarinic acid is physiologically relevant and plays any role in regulating tryptophan metabolism remains to be seen. In terms of structure activity relationships within the phenoxazinone series, it would appear that the presence of an electron-withdrawing group is beneficial for activity. Other compounds (4, 5, 12) containing an ester substituent at the 1- and/or 9-positions show some inhibitory activity, and like cinnabarinic acid do show a dose response. The remaining compounds lacking an electron-withdrawing group are essentially inactive.
Table 3. Inhibition of rhIDO by 2-aminophenoxazin-3-ones.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R¹</th>
<th>R⁴</th>
<th>R⁶</th>
<th>R⁸</th>
<th>R⁹</th>
<th>% enzyme activity remaining¹</th>
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<td></td>
<td></td>
<td>R⁴</td>
<td>R⁶</td>
<td>R⁸</td>
<td>R⁹</td>
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<td>95</td>
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</table>

¹Results were calculated as the percent activity remaining compared to DMSO treated control.

Results are expressed as the mean of three determinations.

In summary, we have prepared a range of 2-aminophenoxazin-3-ones, albeit in modest yield, by simple oxidative cyclocondensation of 2-aminophenols. The compounds were evaluated for their ability to inhibit IDO, but only compounds containing additional electron-withdrawing groups
had any significant activity. The data suggest that the reported biological activity associated with the natural phenoxazinones exfoliazone and chandrananimycin A$^{3,8}$ is unlikely to be mediated through inhibition of IDO.

**EXPERIMENTAL SECTION**

**General information**

Commercially available reagents were used throughout without purification unless otherwise stated. All anhydrous solvents were used as supplied, except tetrahydrofuran and dichloromethane that were freshly distilled according to standard procedures. Reactions were routinely carried out under an argon atmosphere unless otherwise stated, and all glassware was flame-dried before use. Light petroleum refers to the fraction with bp 40-60 °C. Ether refers to diethyl ether.

Analytical thin layer chromatography was carried out on aluminum backed plates coated with silica gel, and visualized under UV light at 254 and/or 360 nm and/or by chemical staining with basic aqueous potassium permanganate solution. Flash chromatography was carried out using silica gel (pore size 60 Å, 230-400 mesh, 40-63 μm particle size), with the eluent specified. The purity of final compounds was confirmed as >95% by HPLC (see Supporting Information).

Infrared spectra were recorded using an FT-IR spectrometer over the range 4000-600 cm$^{-1}$. NMR spectra were recorded at 400 MHz ($^1$H frequency, 100 MHz $^{13}$C frequency). Chemical shifts are quoted in parts per million (ppm), and are referenced to residual H in the deuterated solvent as the internal standard. Coupling constants, $J$, are quoted in Hz. In the $^{13}$C NMR spectra, signals corresponding to CH, CH$_2$, or CH$_3$ groups are assigned from DEPT. Mass spectra were recorded
on a time-of-flight mass spectrometer using electrospray ionization (ESI), or an EI magnetic sector instrument.

2-Amino-3\textit{H}-phenoxazin-3-one 1

To a solution of 2-aminophenol (500 mg, 4.6 mmol) in DMF (15 mL), copper(I) chloride (99 mg, 1.0 mmol) was added, and the resulting mixture was stirred in air for 24 h at room temperature. The reaction mixture was concentrated and the product was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give the title compound 1 as a red solid (293 mg, 50%), mp 258-259 °C (lit.,\textsuperscript{27} mp 256–258 °C; (Found: [M+Na]\textsuperscript{+}, 235.0483 C\textsubscript{12}H\textsubscript{8}N\textsubscript{2}NaO\textsubscript{2} requires 235.0478); \(\lambda_{\text{max}}\) (methanol)/nm 237 (log \(\varepsilon\) 4.98), 432 (log \(\varepsilon\) 4.25); \(v_{\text{max}}\) (CHCl\textsubscript{3})/cm\textsuperscript{-1} 3630, 3515, 3398, 3009, 1710, 1600; \(\delta_{\text{H}}\) (400 MHz; DMSO-\textit{d\textsubscript{6}}) 7.70 (1 H, d, \(J\) 7.8 Hz, H-9), 7.45-7.50 (2 H, m, ArH), 7.38 (1 H, t, \(J\) 7.8 Hz, ArH), 6.83 (2H, br), 6.38 (2H, s, H-1, H-4); \(\delta_{\text{C}}\) (100 MHz; DMSO-\textit{d\textsubscript{6}}) 180.6 (C), 149.3 (C), 148.7 (C), 147.8 (C), 142.4 (C), 134.2 (C), 129.2 (CH), 128.4 (CH), 125.7 (CH), 116.4 (CH), 103.9 (CH), 98.8 (CH).

2-Amino-1,9-dimethyl-3\textit{H}-phenoxazin-3-one 2

To a solution of 2-amino-3-methylphenol (500 mg, 4.1 mmol) in DMF (15 mL), copper(I) chloride (99 mg, 1.0 mmol) was added, and the resulting mixture was stirred in air for 24 h at room temperature. The reaction mixture was concentrated and the product was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give the title compound 2 as a red solid (300 mg, 55%), mp 235-237 °C (lit.,\textsuperscript{27} mp 233 °C; (Found: [M+Na]\textsuperscript{+}, 263.0790. C\textsubscript{14}H\textsubscript{12}N\textsubscript{2}NaO\textsubscript{2} requires 245.9791); \(\lambda_{\text{max}}\) (methanol)/nm 240 (log \(\varepsilon\) 5.05), 422 (log \(\varepsilon\) 4.38); \(v_{\text{max}}\) (CHCl\textsubscript{3})/cm\textsuperscript{-1} 3630, 3514, 3391, 3011, 1710, 1588; \(\delta_{\text{H}}\) (400 MHz; DMSO-\textit{d\textsubscript{6}}) 7.31-7.38 (3 H, m, ArH), 6.41 (2
H, br, NH₂, 6.30 (1 H, s, H-4), 2.64 (3 H, s, Me), 2.27 (3 H, s, Me); δ_C (100 MHz; DMSO-d₆) 180.1 (C), 149.4 (C), 146.2 (C), 142.7 (C), 142.5 (C), 138.2 (C), 129.0 (CH), 125.9 (CH), 113.4 (CH), 108.5 (CH), 102.8 (C), 102.5 (C), 16.8 (CH₃), 9.4 (CH₃).

2-Amino-3-oxo-3H-phenoxazine-1,9-dicarboxylic acid (cinnabarinic acid) 3

Anthranilic acid (100 mg, 0.6 mmol) was dissolved in hot ethanol (60 mL), then after cooling, recrystallized 1,4-benzoquinone (108 mg, 1.0 mmol) was added and the mixture was stirred for 1 h at room temperature. The product was collected by filtration to give the title compound 3 as red solid (36 mg, 40%), mp > 300 °C (lit., mp > 300 °C); (Found: [M+Na]⁺, 323.0279. C₁₄H₈N₂NaO₂ requires 323.0275); λ_max (methanol)/nm 234 (log ε 5.03), 446 (log ε 4.30); v_max (CHCl₃)/cm⁻¹ 3689, 3608, 3413, 3361, 3042, 2976, 1725, 1601 1568, 1241; δ_H (400 MHz; DMSO-d₆) 9.73 (1 H, br. s, NH₂), 8.82 (1 H, br. s, NH₂), 7.96 (1 H, d, J 8.3 Hz, H-8), 7.78 (1 H, d, J 8.3 Hz, H-6), 7.61 (1 H, t, J 8.3 Hz, H-7), 6.62 (1 H, s, H-4); δ_C (100 MHz; DMSO-d₆) 178.5 (C), 169.5 (C), 166.7 (C), 153.0 (C), 150.9 (C), 148.0 (C), 142.9 (C), 129.5 (C), 129.3 (CH), 128.3 (CH), 126.7 (C), 120.6 (CH), 105.3 (CH), 92.2 (C).

Dimethyl 2-amino-3-oxo-3H-phenoxazine-1,9-dicarboxylate 4

(a) 3-Hydroxy-2-nitrobenzoic acid (1.0 g, 5.4 mmol) was dissolved in dry DMF (8.2 mL). Potassium hydrogen carbonate (655 mg, 6.5 mmol) and iodomethane (508 µL, 6.5 mmol) were added, and the resulting mixture was heated at 40 °C for 2 h. The reaction was quenched with water (10 mL), acidified to pH 3 with hydrochloric acid (1 M; 5 mL) and extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried (MgSO₄), filtered and concentrated. The product was purified by flash chromatography (light petroleum/ethyl acetate
8:2) to give methyl 3-hydroxy-2-nitrobenzoate as a yellow solid (600 mg, 75%): mp 113-115 °C (lit.,\textsuperscript{31} mp 112-114 °C); (Found: [M+Na]\textsuperscript{+}, 297.9318. C\textsubscript{8}H\textsubscript{7}NNaO\textsubscript{5} requires 297.9322); $\delta_H$ (400 MHz; CDCl\textsubscript{3}) 10.19 (1 H, s, OH), 7.60 (1 H, t, $J$ 8.8 Hz, H-5), 7.28 (1 H, d, $J$ 8.8 Hz, H-6), 7.10 (1 H, d, $J$ 8.8, Hz, H-4), 3.59 (3 H, s, Me); $\delta_C$ (100 MHz; CDCl\textsubscript{3}) 166.6 (C), 154.5 (C), 136.0 (CH), 131.9 (C), 131.0 (CH), 121.9 (CH), 120.6 (C), 53.4 (CH\textsubscript{3}).

(b) To a solution of methyl 3-hydroxy-2-nitrobenzoate (800 mg, 4.0 mmol) in methanol (60 mL) was added palladium on carbon (10% w/w; 80 mg) and the suspension was stirred under an atmosphere of hydrogen for 2 h at room temperature. The reaction mixture was filtered through a pad of Celite and concentrated \textit{in vacuo} to give methyl 2-amino-3-hydroxybenzoate as a colorless solid (668 mg, 98%): mp 94-95 °C (lit.,\textsuperscript{32} mp 94-97 °C); (Found: [M+Na]\textsuperscript{+}, 190.0481. C\textsubscript{8}H\textsubscript{9}NO\textsubscript{3} requires 190.0475); $\delta_H$ (400 MHz; CDCl\textsubscript{3}) 7.52 (1 H, d, $J$ 8.0 Hz, H-6), 6.85 (1 H, d, $J$ 8.0 Hz, H-4), 6.53 (1 H, t, $J$ 8.0 Hz, H-5), 2.97 (3 H, s, CH\textsubscript{3}); $\delta_C$ (100 MHz; CDCl\textsubscript{3}) 166.6 (C), 142.8 (C), 140.6 (C), 123.4 (CH), 117.9 (CH), 114.9 (CH), 111.4 (C), 51.4 (CH\textsubscript{3}).

(c) Methyl 2-amino-3-hydroxybenzoate (500 mg, 2.6 mmol) in MeOH (40 mL) was added to a solution of potassium ferricyanide (2.31 g, 7.01 mmol) in sodium phosphate buffer (pH 7.0, 50 mL). The pH of the reaction mixture decreased to around 5 and was quickly adjusted to 7 by addition of aqueous sodium hydroxide (7.5 M). The mixture was stirred for 16 h at room temperature, then extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried (MgSO\textsubscript{4}), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate, 7:3) to give the \textit{title compound} 4 as a brown solid (191 mg, 45%), mp 229-231 °C (lit.,\textsuperscript{25} mp 225-226 °C); (Found: [M+Na]\textsuperscript{+}, 329.0768. C\textsubscript{16}H\textsubscript{12}N\textsubscript{2}NaO\textsubscript{6} requires 329.0767); $\lambda_{\text{max}}$ (methanol)/nm 240 (log $\varepsilon$ 5.34), 430 (log $\varepsilon$ 4.23); $\nu_{\text{max}}$ (CHCl\textsubscript{3})/cm\textsuperscript{-1} 3631, 3445, 3323, 3011, 2953 1711, 1648 1580; $\delta_H$ (400 MHz; DMSO-$d_6$) 7.83 (2 H, br, NH\textsubscript{2}), 7.68 (1 H, d,
$J$ 8.0 Hz, H-8), 7.55-760 (2 H, m, ArH), 6,50 (1 H, s, H-4), 3.90 (3 H, s, CH$_3$), 3.84 (3 H, s, CH$_3$); δ$_C$ (100 MHz; DMSO-$d_6$) 178.9 (C), 167.7 (C), 167.6 (C), 149.2 (C), 148.4 (C), 146.2 (C), 141.8 (C), 132.9 (C), 131.1 (CH), 129.4 (CH), 125.0 (C), 118.6 (CH), 104.3 (CH), 99.8 (C), 52.8 (CH$_3$), 52.0 (CH$_3$).

**Dimethyl 2-amino-4,6-dimethyl-3-oxo-3H-phenoxazine-1,9-dicarboxylate 5**

(a) To a solution of 3-hydroxy-4-methyl-2-nitrobenzoic acid (1 g, 5.4 mmol) in dry DMF (8.2 mL), were added potassium hydrogen carbonate (655 mg, 6.5 mmol) and iodomethane (508 µL, 6.5 mmol) and the resulting mixture was heated to 40 °C for 2 h. The reaction was quenched with water (10 mL), acidified with HCl (1 M; 3 mL) and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried (MgSO$_4$), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 7:3) to give methyl 3-hydroxy-2-nitro-4-methylbenzoate as a yellow solid (790 mg, 70%), mp 115-117 °C (lit.,$^{33}$ mp 116-117 °C); (Found: [M+Na]$^+$, 234.0416. C$_9$H$_7$NNaO$_5$ requires 234.0409); δ$_H$ (400 MHz; CDCl$_3$) 10.42 (1 H, s, OH), 7.45 (1 H, d, $J$ 8.2 Hz, H-5), 7.02 (1 H, d, $J$ 8.2 Hz, H-6), 3.94 (3 H, s, Me), 2.38 (3 H, s, Me); δ$_C$ (100 MHz, CDCl$_3$) 166.8 (C), 152.9 (C), 136.2 (C), 132.1 (CH), 129.4 (C) 128.2 (CH), 119.8 (C), 53.2 (CH$_3$), 16.2 (CH$_3$).

(b) To a solution of methyl 3-hydroxy-2-nitro-4-methylbenzoate (500 mg, 2.4 mmol) in methanol (50 mL) was added palladium on carbon (10% w/w, 80 mg) and the suspension was stirred under an atmosphere of hydrogen for 2 h at room temperature. The reaction mixture was filtered through a pad of Celite and concentrated *in vacuo* to give methyl 2-amino-3-hydroxy-4-methylbenzoate as a colorless solid (434 mg, 98%) that it was used in the next step without further purification.
A solution of methyl 2-amino-3-hydroxy-4-methylbenzoate (320 mg, 1.2 mmol) in MeOH (30 mL) was added to a solution of potassium ferricyanide (2.31 g, 7.01 mmol), in sodium phosphate buffer (pH 7.0, 50 mL). The pH of the reaction mixture went down initially to about 5 and was quickly adjusted to 7 by addition of aqueous NaOH (7.5 M). The mixture was stirred for 16 h at room temperature, then extracted with ethyl acetate (3 × 50 mL), the combined organic layers were dried (MgSO₄), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate, 7:3) to give the title compound 5 as a brown solid (96 mg, 38%) mp 214-215 °C (lit., mp 195-198 °C); (Found: [M+H]+, 357.1071. C₁₈H₁₇N₂O₆ requires 357.1074); λmax (methanol)/nm 240 (log ε 5.21), 434 (log ε 4.14); νmax (CHCl₃)/cm⁻¹ 3631, 3446, 3323, 3005, 2952, 1710, 1648 1579; δH (400 MHz; CDCl₃) 7.66 (1 H, d, J 8.1 Hz, ArH) 7.35 (1 H, d, J 8.1 Hz, ArH), 4.04 (3 H, s, CH₃), 4.03 (3 H, s, CH₃), 2.56 (3 H, s, CH₃), 2.27 (3 H, s, CH₃), δC (100 MHz; CDCl₃) 178.2 (C), 169.1 (C), 166.9 (C), 150.4 (C), 145.8 (C), 145.3 (C), 140.3 (C), 131.3 (C), 129.9 (CH), 128.9 (C), 128.5 (C), 125.5 (CH), 113.3 (C), 97.1 (C), 52.4 (CH₃), 51.8 (CH₃), 15.0 (CH₃), 7.8 (CH₃).

2-Amino-8-methyl-3H-phenoxazin-3-one 6

To a solution of 2-aminophenol (109 mg, 1.0 mmol) in acetone (10 mL) was added a solution of NaIO₃ (295 mg, 1.5 mmol) in water (26 mL), and the mixture was stirred for 10 min at room temperature. Then a solution 4-methyl-2-aminophenol (184 mg, 1.5 mmol) in acetone (10 mL) was added. The suspension was stirred for 20 h at room temperature, then extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried (MgSO₄), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give the title compound 6 as a brown solid (79 mg, 35%), mp 222-224 °C; (Found:
[M+H]^+, 227.0820. C_{13}H_{11}N_{2}O_{2} requires 227.0815; \lambda_{\text{max}} \text{ (methanol)/nm 238 (log } \varepsilon \text{ 4.96), 436 (log } \varepsilon \text{ 4.20); } v_{\text{max}} \text{ (CHCl}_3)/\text{cm}^{-1} \text{ 3631, 3515, 3393, 3011, 1710, 1600; } \delta_H \text{ (400 MHz; DMSO-}d_6) \text{ 7.72 (1 H, d, } J 8.0 \text{ Hz, H-6), 7.40-7.50 (2 H, m, ArH), 6.79 (2 H, br, NH}_2\text{), 6.37 (2 H, s, H-1, H-4), 2.09 (3 H, s, CH}_3\text{); } \delta_C \text{ (100 MHz; DMSO-}d_6) \text{ 180.1 (C), 149.3 (C), 148.7 (C), 147.8 (C), 142.4 (C), 134.2 (C), 129.2 (CH), 128.4 (CH), 125.7 (C), 116.4 (C), 103.9 (CH), 98.8 (C), 31.1 (CH}_3\text{).}

**2-Amino-1,8-dimethyl-3H-phenoxazin-3-one 7**

To a solution of 3-methyl-2-aminophenol (123 mg, 1.0 mmol) in acetone (10 mL) was added a solution of NaIO\textsubscript{3} (295 mg, 1.5 mmol) in water (26 mL), and the mixture was stirred for 10 min at room temperature. Then a solution of 2-amino-4-methylphenol (184 mg, 1.5 mmol) in acetone (10 mL) was added. The reaction mixture was stirred for 20 h at room temperature, then extracted with ethyl acetate (3 \times 15 mL), the combined organic layers were dried (MgSO\textsubscript{4}), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give the *title compound 7* as a brown solid (96 mg, 40%); mp 226-227 °C; (Found: [M+Na]^+, 263.0780. C_{14}H_{12}N_{2}NaO_{2} requires 263.0765); \lambda_{\text{max}} \text{ (methanol)/nm 242 (log } \varepsilon \text{ 4.55), 435 (log } \varepsilon \text{ 4.13); } v_{\text{max}} \text{ (CHCl}_3)/\text{cm}^{-1} \text{ 3515, 3391, 3011, 1710, 1594, 1577; } \delta_H \text{ (400 MHz; DMSO-}d_6) \text{ 7.60 (1 H, s, H-9), 7.41 (1 H, d, } J 8.1 \text{ Hz, H-6), 7.30 (1 H, d, } J 8.1 \text{ Hz, H-7), 6.42 (2 H, br, NH}_2\text{), 6.28 (1 H, s, H-4), 2.41 (3 H, s, CH}_3\text{), 2.23 (3 H, s, CH}_3\text{); } \delta_C \text{ (100 MHz; DMSO-}d_6) \text{ 180.0 (C), 149.5 (C), 147.6 (C), 144.2 (C), 140.4 (C), 135.0 (C), 133.5 (CH), 130.4 (CH), 128.5 (C), 115.8 (CH), 105.7 (CH), 102.6 (C), 20.8 (CH}_3\text{), 10.3 (CH}_3\text{).}

**2-Amino-9-hydroxy-3H-phenoxazin-3-one 8**
(a) To a solution of 2-nitroresorcinol (310 mg, 2 mmol) in methanol (30 mL) was added palladium on carbon (10% w/w, 31 mg) and the suspension was stirred under an atmosphere of hydrogen for 2 h at room temperature. The reaction mixture was filtered through a pad of Celite and concentrated in vacuo to give 2-aminoresorcinol as dark brown solid (245 mg, 98%) mp 158-160 °C (lit., mp 152.5 °C); (Found: [M+Na]+, 148.0369. C₆H₇NNaO₂ requires 148.0369); δH (400 MHz; DMSO-d₆) 8.82 (2 H, br, OH), 6.19-6.29 (3 H, m, ArH), 3.85 (2 H, br, NH₂); δC (100 MHz; DMSO-d₆) 145.3 (C), 124.3 (C), 116.2 (CH), 107.0 (CH).

(b) To a solution of 2-aminophenol (145 mg, 1.33 mmol) in acetone (10 mL) was added sodium iodate (262 mg, 1.3 mmol) in water (17 mL), and the mixture was stirred for 10 min at room temperature. Then 2-aminoresorcinol (200 mg, 1.6 mmol) in acetone (5 mL) was then added. The resulting mixture was stirred for 20 h at room temperature, extracted with ethyl acetate (3 × 10 mL), the combined organic layers were dried (MgSO₄), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) gave the title compound 8 as brown solid (75 mg, 25%), mp > 300 °C; (Found: [M+H]+, 229.0607. C₁₂H₉N₂O₃ requires 229.0608); λmax (methanol)/nm 272 (log ε 3.89), 430 (log ε 4.11); νmax (CHCl₃)/cm⁻¹ 3592, 3457, 3393, 3331, 3011, 1590; δH (400 MHz; DMSO-d₆) 10.2 (1 H, br, OH), 7.29 (1 H, t, J 8.0 Hz, H-7), 6.92 (1 H, d, J 8.0 Hz, H-8), 6.84 (1 H, d, J 8.0 Hz, H-6), 6.68 (2 H, br, NH₂), 6.45 (1 H, s, H-1), 6.34 (1 H, s, H-4); δC (100 MHz; DMSO-d₆) 180.5 (C), 154.2 (C), 149.2 (C), 147.3 (C), 146.2 (C), 143.2 (C), 129.6 (C), 124.2 (CH), 111.3 (CH), 106.4 (CH), 103.7 (CH), 99.3 (CH).

*N-(9-Hydroxy-3-oxo-3H-phenoxazin-2-yl)acetamide (chandrananimycin A) 11*

To a solution of 2-amino-9-hydroxy-3H-phenoxazin-3-one (60 mg, 0.22 mmol) in dichloromethane (5 mL), were added acetic anhydride (218 µl, 2.30 mmol) and 4-
dimethylaminopyridine (5.6 mg, 0.04 mmol), and the resulting mixture was stirred 16 h at room temperature. Then the mixture was poured into water (10 mL), and extracted with dichloromethane (3 × 10 mL). The combined organic layers were dried (MgSO₄), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give the title compound 11 as brown solid (23 mg, 39%), mp > 300 °C (lit., mp not given); (Found: [M+Na]⁺, 293.0535. C₁₄H₁₀N₂NaO₄ requires 293.0533); λₘₐₓ (methanol)/nm 268 (log ε 3.94), 422 (log ε 4.32); νₘₐₓ (CHCl₃)/cm⁻¹ 3469, 3355, 3011, 1701, 1648, 1503; δₜₜ (400 MHz; DMSO-d₆) 10.6 (1 H, br NH), 9.67 (1 H, br, OH), 8.38 (1 H, s, H-1), 7.45 (1 H, t, J 8.0 Hz, H-7), 6.96 (1 H, d, J 8.0 Hz, H-8), 6.89 (1 H, d, J 8.0 Hz, H-6), 6.45 (1 H, s, H-4), 2.24 (3H, s, Me); δₜₜ (100 MHz; DMSO-d₆) 179.8 (C), 171.1 (C), 155.6 (C), 149.3 (C), 146.1 (C), 144.1 (C), 137.1 (C), 132.9 (CH), 124.4 (C), 114.3 (CH), 112.4 (CH), 106.7 (CH), 104.0 (CH), 25.2 (CH₃).

2-Amino-8-[(tetrahydropyran-2-yloxy)methyl]-3H-phenoxazin-3-one 9

(a) Sodium borohydride (76 mg, 2 mmol) was added in small portions to a solutions of 4-hydroxy-3-nitrobenzaldehyde (130 mg, 0.78 mmol) in methanol (2 mL) at 0 °C. The reaction was maintained at 0 °C for 1 h and then diluted with chloroform (20 mL) and poured into HCl (1 M; 10 mL). the organic lauer was separated and washed with water (10 mL). The organic extract was dried (MgSO₄), filtered and concentrated to give 4-hydroxymethyl-2-nitrophenol as a yellow solid (790 mg, 80%), mp 97-99 °C (lit., mp 94-98 °C); (Found: [M+Na]⁺, 192.0266. C₇H₇NNaO₄ requires 192.0267); δₜₜ (400 MHz; CDCl₃) 10.58 (1H, s, OH), 8.14 (1 H, s, H-3), 7.63 (1 H, d, J 8.0 Hz, H-5), 7.19 (1 H, d, J 8.0 Hz, H-6), 4.72 (2 H, s, CH₂); δₜₜ (100 MHz; CDCl₃) 154.5 (C), 136.3 (CH), 133.2 (C), 123.0 (CH), 120.2 (C), 63.7 (CH₂); one C unobserved.
(b) To a solution of 4-hydroxymethyl-2-nitrophenol (500 mg, 3.0 mmol) in dry dichloromethane (30 mL) under argon atmosphere, were added 3,4-dihydro-2H-pyran (285 µL, 3.0 mmol) and pyridinium p-toluenesulfonate (80 mg, 0.3 mmol). The resulting mixture was stirred at room temperature for 16 h. Then the mixture was poured into water (30 mL) and extracted with dichloromethane (3 × 20 mL), the combined organic layers were dried (MgSO₄), filtered and concentrated. The product was purified by flash chromatography (light petroleum /ethyl acetate 8:2) to give 2-nitro-4-[(tetrahydropyran-2-yloxy)methyl]phenol as a colorless oil (673 mg, 88%); (Found: [M+Na]⁺, 276.0833. C₁₂H₁₅NNaO₅ requires 276.0842); δ_H (400 MHz; DMSO-d₆) 10.94 (1 H, s, OH), 7.85 (1 H, s, H-3), 7.53 (1 H, d, J 8.1 Hz, H-5), 7.13 (1 H, d, J 8.1 Hz, H-6), 4.62-4.69 (2 H, m, CH₂), 4.43 (1 H, d, J 8.0 Hz, CH), 3.75-3.81 (1 H, m, CH₂), 3.45-3.51 (1 H, m, CH₂), 1.4-1.8 (6 H, m); δ_C (100 MHz; DMSO-d₆) 151.9 (C), 136.8 (C), 135.2 (C), 130.1 (CH), 124.5 (CH), 119.5 (CH), 97.8 (CH), 67.2 (CH₂), 61.8 (CH₂), 30.6 (CH₂), 25.4 (CH₂), 19.5 (CH₂).

(c) To a solution of 2-nitro-4-[(tetrahydropyran-2-yloxy)methyl]phenol (600 mg, 2.3 mmol) in methanol (60 mL) was added palladium on carbon (10% w/w, 80 mg) and the suspension was stirred under an atmosphere of hydrogen for 2 h at room temperature. The reaction mixture was filtered through a pad of Celite and concentrated in vacuo to give 2-amino-4-[(tetrahydropyran-2-yloxy)methyl]phenol as dark brown oil (502 mg, 98%) used without further purification; (Found: [M+Na]⁺, 246.1081. C₁₂H₁₇NNaO₃ requires 246.1101); δ_H (400 MHz; DMSO-d₆) 8.88 (1 H, s, OH), 6.58-6.60 (2 H, m, ArH), 6.36 (1 H, d, J 6.0 Hz, H-5), 4.61 (1 H, m, CH), 4.50 (2 H, br, NH₂), 4.45 (1 H, d, J 11.2 Hz, CHH), 4.20 (1 H, d, J 11.2 Hz, CHH), 3.75-3.81 (1 H, m, CHH), 3.45-3.51 (1 H, m, CHH), 1.4-1.8 (6 H, m, CH₂); δ_C (100 MHz; DMSO-d₆) 143.9 (C), 136.8 (C), 129.4 (C), 116.8 (CH), 114.9 (CH), 114.3 (CH), 97.1 (CH), 68.9 (CH₂), 61.6 (CH₂), 30.7 (CH₂), 25.5 (CH₂), 19.6 (CH₂).
(d) To a solution of 2-aminophenol (109 mg, 1.0 mmol) in acetone (10 mL) was added sodium iodate (295 mg, 1.5 mmol) in water (26 mL), and the mixture was stirred for 10 min at room temperature. Then a solution of 2-amino-4-[(tetrahydropyran-2-yloxy)methyl]phenol (334 mg, 1.5 mmol) in acetone (10 mL) was added. The suspension was stirred for 20 h at room temperature, extracted with ethyl acetate (3 × 15 mL), the combined organic layers were dried (MgSO$_4$), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) gave the title compound 9 as brown solid (146 mg, 45%), mp 198-200 °C; (Found: [M+Na]$^+$, 349.1162. C$_{18}$H$_{18}$N$_2$NaO$_4$ requires 349.1159); $\nu_{\text{max}}$ (CHCl$_3$)/cm$^{-1}$ 3629, 3086, 2950, 1645, 1578, 1442, 1192; $\delta$$_H$ (400 MHz; DMSO-d$_6$) 7.80 (1 H, s, H-9), 7.47 (1 H, d $J$ 8.1 Hz, H-6), 7.39 (1 H, d $J$ 8.1 Hz, H-7), 6.50 (1 H, s, H-1), 6.43 (1 H, s, H-4), 5.15 (2 H, br, NH$_2$), 4.91 (1 H, d $J$ 12.0 Hz), 4.79 (1 H, m), 4.63 (1 H, d $J$ 12.0 Hz, CH$_2$), 3.95 (1 H, m, CH), 3.62 (1 H, m, CH$_2$), 1.90-1.60 (6 H, m); $\delta$$_C$ (100 MHz; DMSO-d$_6$) 180.3 (C), 149.4 (C), 148.7 (C), 145.7 (C), 142.0 (C), 135.9 (C), 133.7 (C), 129.0 (C), 127.5 (CH), 115.9 (CH), 104.1 (CH), 100.8 (CH), 97.9 (CH), 67.9 (CH$_2$), 62.1 (CH$_2$), 30.5 (CH$_2$), 25.4 (CH$_2$), 19.2 (CH$_2$).

**2-Amino-8-hydroxymethyl-3H-phenoxazin-3-one 12**

A solution of 2-amino-8-[(tetrahydropyran-2-yloxy)methyl]-3H-phenoxazin-3-one (90 mg, 0.3 mmol) in a solution of ethanol-THF (1:1, 25 mL) was treated with hydrochloric acid (1 M; 6 mL). The resulting solution was stirred for 6 h at room temperature. Water (15 mL) was added and the reaction mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were dried (MgSO$_4$), filtered and concentrated. The residue was purified by flash chromatography (dichloromethane/ethyl acetate 8:2) to give the title compound 12 as a brown solid (39 mg, 65%), mp 235-240 °C; (Found: [M+Na]$^+$, 265.0575. C$_{13}$H$_{10}$N$_2$NaO$_3$. requires
N-(3-Oxo-8-hydroxymethyl-3H-phenoxazin-2-yl)acetamide (exfoliazone) 13

(a) To a solution of 2-amino-8-[(tetrahydropyran-2-yl)oxy]methyl]-3H-phenoxazin-3-one (150 mg, 0.5 mmol) in dichloromethane (5 mL), were added acetic anhydride (218 µl, 2.3 mmol) and 4-dimethylaminopyridine (5.6 mg, 0.04 mmol). The resulting mixture was stirred 16 h at room temperature. Then the mixture was washed with water (5 mL), dried (MgSO₄), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give N-(3-oxo-8-[(tetrahydropyran-2-yl)oxy]methyl]-3H-phenoxazin-2-yl)acetamide as a brown solid (74 mg, 43%), mp 215-220 °C; (Found: [M+Na]⁺, 391.1261. C₂₀H₂₀N₂NaO₅ requires 391.1264); νmax (CHCl₃)/cm⁻¹ 3393, 3042, 1596, 1575, 1186; δH (400 MHz; DMSO-d₆) 7.64 (1 H, s, H-9), 7.47 (1 H, d J 8.0 Hz, H-6), 7.41 (1 H, d J 8.0 Hz, H-7), 6.80 (1 H, br, NH₂), 6.36 (2 H, s, ArH), 5.45 (1 H, br, OH), 4.58 (2 H, s, CH₂); δC (100 MHz; DMSO-d₆) 180.6 (C), 149.4 (C), 148.6 (C), 147.8 (C), 141.1 (C), 140.3 (C), 133.9 (C), 127.6 (CH), 125.8 (CH), 116.0 (CH), 103.7 (CH), 98.8 (CH), 62.6 (CH₂).
(b) A solution of N-(3-oxo-8-[(tetrahydropyran-2-yloxy)methyl]-3H-phenoxazin-2-yl)acetamide (55 mg, 0.2 mmol) in THF/ethanol (6 mL, 1:1) and hydrochloric acid (1 M; 2 mL) was added. The resulting solution was stirred 6 h at room temperature. Then water (10 mL) was added and the reaction mixture was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried (MgSO₄), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give the title compound 13 as a brown solid (15 mg 37%), mp 288-290 °C (lit., mp 294-298 °C); (Found: [M+Na]+, 307.0694. C₁₅H₁₂N₂NaO₄ requires 307.0689); λ<sub>max</sub> (methanol)/nm 239 (log ε 7.69), 405 (log ε 4.32); ν<sub>max</sub> (CHCl₃)/cm⁻¹ 3643, 3006, 2961, 1601, 1432, 1157; δ<sub>H</sub> (400 MHz; DMSO-d₆) 9.72 (1 H, br, NH), 8.29 (1 H, s, H-9), 7.78 (1 H, s, H-1), 7.57 (1 H, d, J 8.2 Hz, H-6), 7.54 (1 H, d, J 8.2 Hz, H-7), 6.49 (1 H, s, H-4), 5.43 (1 H, t, J 5.8 Hz, OH), 4.62 (2 H, d, J 5.8 Hz, CH₂), 2.25 (3 H, s, CH₃); δ<sub>C</sub> (100 MHz; DMSO-d₆) 179.8 (C), 171.2 (C), 149.4 (C), 149.1 (C), 142.1 (C), 140.8 (C), 138.2 (C), 133.6 (C), 130.6 (CH), 127.1 (CH), 116.2 (CH), 113.7 (CH), 104.1 (CH), 62.3 (CH₂), 24.8 (CH₃).

**Methyl 2-amino-3-oxo-8-[(tetrahydropyran-2-yloxy)methyl]-3H-phenoxazine-1-carboxylate 10**

To a solution of methyl 2-amino-3-hydroxybenzoate (550 mg, 4.1 mmol) in acetone (26 mL) was added sodium iodate (685 mg, 4.5 mmol) in water (47 mL), and the resulting mixture was stirred for 10 min at room temperature. Then a solution of 2-amino-4-[(tetrahydropyran-2-yloxy)methyl]phenol (771 mg, 6.0 mmol) in acetone (15 mL) was added. The suspension was stirred for 20 h at room temperature, then extracted with ethyl acetate (3 × 25 mL). The combined organic layers were dried (MgSO₄) and concentrated. The residue was purified by
flash chromatography (light petroleum/ethyl acetate 8:2) to give the title compound 10 as brown solid (387 mg, 29%), mp 223-227 °C; (Found: [M+Na]⁺, 407.1214. C₂₀H₂₀N₂NaO₆ requires 407.1214); ν_max (CHCl₃)/cm⁻¹ 3686, 3006, 2974, 1578, 1473, 1192; δ_H (400 MHz; CDCl₃) 7.87 (1 H, s, H-9), 7.51 (1 H, d, J 8, H-6), 7.38 (1 H, d, J 8.1 Hz, H-7), 6.50 (1 H, s, H-4), 4.89 (1 H, d, J 8.2 Hz, CH₂), 4.77 (1 H, t, J 8 Hz, CH₂), 4.62 (1 H, m, CH), 4.04 (3 H, s, Me), 3.94-3.96 (1 H, m, CH₂), 3.5-3.53 (1 H, m, CH₂), 1.52-1.93 (6H, m); δ_C (100 MHz; DMSO-d₆) 178.8 (C), 167.2 (C), 149.5 (C), 147.4 (C), 145.9 (C), 141.4 (C), 136.4 (CH), 133.3 (CH), 129.4 (C), 127.5 (C), 116.2 (CH), 104.1 (CH), 100.3 (C), 97.9 (CH), 67.9 (CH₂), 62.7 (CH₂), 52.3 (CH₃), 30.6 (CH₂), 25.4 (CH₂), 19.5 (CH₂).

Methyl 2-amino-8-(hydroxymethyl)-3-oxo-3H-phenoxazine-1-carboxylate 14

To a solution of methyl 2-amino-3-oxo-8-[(tetrahydropyran-2-yloxy)methyl]-3H-phenoxazine-1-carboxylate (48 mg, 0.2 mmol) in a solution of ethanol-THF 1:1 (10 mL), hydrochloric acid (1 M; 1 mL) was added. The resulting solution was stirred 6 h at room temperature. Then water (10 mL) was added and the reaction mixture was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried (MgSO₄), filtered and concentrated. The residue was purified by flash chromatography (light petroleum/ethyl acetate 8:2) to give the title compound 14 as a brown solid (15 mg, 50%), mp 285-293 °C; (Found: [M+Na]⁺, 323.0644. C₁₅H₁₂N₂NaO₅ requires 323.0638); λ_max (methanol)/nm 235 (log ε 5.63), 433 (log ε 4.33); ν_max (CHCl₃)/cm⁻¹ 3697, 3603, 3426, 3397, 2952, 1710, 1648; δ_H (400 MHz; DMSO-d₆) 7.67 (1 H, s, H-9), 7.62 (2 H, br, NH₂), 7.44-7.52 (2 H, m, ArH), 6.49 (1 H, s, H-4), 5.38 (1 H, t, J 7.8 Hz, OH), 4.61 (2 H, d, J 7.8 Hz, CH₂), 3.87 (3 H, s, Me); δ_C (100 MHz; DMSO-d₆) 178.7 (C), 167.3 (C), 149.5 (C),
IDO Assay

Human N-terminus 6X-histidine IDO was expressed in *E. coli* and purified using Ni$^{2+}$ affinity chromatography as described. Purified rhIDO had an enzymatic activity of 1 µmol kynurenine formed /min/mg and was stored in 25 mM Tris-HCl, pH 7.4 containing 250 mM sucrose at -80 °C.

The oxidative cleavage of *L*-tryptophan catalyzed by rhIDO was measured as described previously by Takikawa *et al.*, and modified by Austin *et al.* Reactions (0.25 mL) were performed in 50 mM potassium phosphate buffer pH 7.4, containing 20 mM ascorbic acid, 10 µM methylene blue, 0.4 mg/mL catalase and 4 µg/mL rhIDO. DMSO or inhibitor dissolved in DMSO was added for 10 min then reactions were initiated by the addition of 400 µM *L*-tryptophan. After 30 min at 37 °C, reactions were terminated by the addition of 100 µl of 30% (w/v) trichloroacetic acid. Samples were heated to 65 °C for 15 min and then centrifuged at 13k rpm for 5 min. Supernatant (100 µl) was transferred to a 96-well plate and 100 µl of 4-dimethylaminobenzaldehyde (Ehrlich’s reagent, 2% in acetic acid) was added to each well. After 2 min, the absorbance was determined using a microplate reader at 490 nm. Results were quantified against a standard curve generated using authentic kynurenine. Final results were calculated as percent of DMSO treated controls.

Kinetic studies were performed with compound 3 as previously described, using tryptophan concentrations between 10 and 240 µM and inhibitor concentrations of 0.25 and 0.5 µM. IC$_{50}$
values were determined as described above in triplicate using 10-12 concentrations of inhibitors (cinnabarinic acid 3, 0.05-25 µM; 4-phenylimidazole, 0.6-1250 µM).

ASSOCIATED CONTENT

Supporting Information. Copies of HPLC data, and ^1^H and ^13^C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

IDO, indoleamine-2,3-dioxygenase; rh, recombinant human.

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![Chemical Structure of IDO Inhibitors](image-url)