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Synthesis and Phytotoxicity of Structural Analogues of Thaxtomin Natural Products

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Structural analogues of the phytotoxic thaxtomin natural products have been synthesized by building upon a piperazinedione core and from L-phenylalanine. The compounds were evaluated for their phytotoxic activity against *Arabidopsis thaliana* seedlings and some of the key features for activity have been identified.

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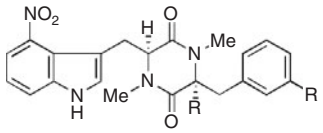
Introduction

The thaxtomins are a class of cyclic dipeptide derived compounds containing a piperazinedione (also commonly referred to as a diketopiperazine) core. They are produced by plant pathogenic *Streptomyces* spp. responsible for common scab disease; one of the most economically significant diseases worldwide for potatoes.[1] Two representative examples, thaxtomin A (**1**) and thaxtomin D (**2**), which differ mainly in hydroxylation on the piperazine ring, are shown in Fig. 1. For the pathogen, thaxtomin production is essential for disease transmission.[2] These compounds are believed to inhibit cellulose deposition[3,4] during cell wall formation and are thought to induce programmed cell death[5] in picomolar concentrations. As part of a study to develop new thaxtomin-tolerant strains of potatoes, we sought to synthesize the natural products, as well as various analogues for testing, to develop a greater understanding about structure activity relationships. A previous model study targeted the synthesis of thaxtomin A, and resulted in the preparation of the de-nitro derivative via alkylation of a *N*,*N*′-dimethylbenzylidine piperazinedione.[6] Herein we report the synthesis and phytotoxic activity of dehydro-structural analogues of the thaxtomins.

Results and Discussion

The synthesis of the analogues was approached by building upon the piperazinedione core of glycine anhydride **3**, as this would allow greater flexibility to introduce different substituents onto either side of the molecule via aldol condensation chemistry. Thus, glycine anhydride (**3**) was activated by reaction with acetic anhydride[7] to give diacetyl derivate **4**, which was then condensed with benzaldehyde. We found the use of a modified method[8] using potassium carbonate in DMF preferential to triethyl amine/DMF[9] or *t*-BuOK/*t*-BuOH[10] systems to introduce the phenyl substituent; and while cesium carbonate has been reported,[8] it gave no significant improvement.



R = OH, thaxtomin A (**1**)
R = H, thaxtomin D (**2**)

Fig. 1. Representative thaxtomin phytotoxins.

Aqueous workup gave the mono acetyl derivative **5**. Subequent hydrogenation[9] in a non-nucleophilic solvent (either THF or ethyl acetate) with acetic acid to avoid catalyst poisoning, gave **7a** and **7b**, with the latter arising from migration of the acetyl group in solution. The compounds could be readily separated by chromatography and differ in the ^{1}H NMR for the methylene adjacent the phenyl group. In **7a** these protons resonate as two clearly defined doublet of doublets; while in the previously unreported **7b**, these protons appear as a multiplet. The assignment of **7a** is in excellent agreement with that previously reported,[10] although the melting point obtained was 15° higher than literature. Further evidence for **7b** being a structural isomer of **7a** was obtained by conversion of the individual compounds to the *N*,*N*′-diacetylpiperazinedione **8** by reaction with acetic anhydride. The hydrogenation of **5** required a non-nucleophilic solvent as ring opening to give acetamide **6** was observed when methanol was used (Scheme 1).

From compound **8** a second aldol condensation, using cesium carbonate/DMF with the Boc-protected indole-3-carbaldehyde[11] as a model, gave **9** which contains the carbon skeleton of the thaxtomin but lacks the nitro group on the indole. The *Z* geometry was confirmed for the alkene by X-ray crystal structure determination of compound **9** (Fig. 2). The acetyl and Boc-protecting groups could be removed by reaction with an excess of hydrazine hydrate[6] to give thaxtomin-like derivative **10**.

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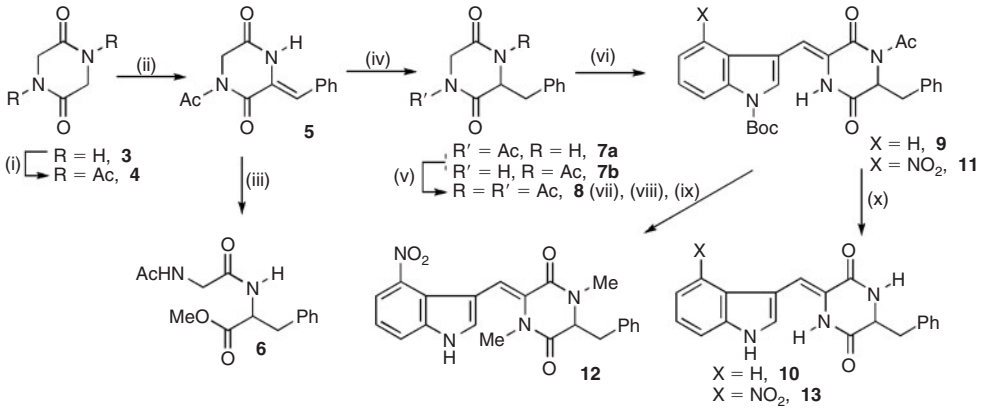


Scheme 1. Reagents and conditions: (i) ref. [2]; (ii) (a) PhCHO, K_2CO_3, DMF; (b) H_2O; (iii) H_2, 10% Pd/C, MeOH; (iv) H_2, 10% Pd/C, THF, acetic acid; (v) Ac_2O, Py, DMAP, CH_2Cl_2; (vi) (a) *N*-Boc-4-X-indole-3-carbaldehyde, Cs_2CO_3, DMF; (b) H_2O; (vii) 1 equiv. $NH_2NH_2{\cdot}H_2O$, DMF; (viii) MeI, K_2CO_3, DMF; (ix) and (x) excess $NH_2NH_2{\cdot}H_2O$, DMF.

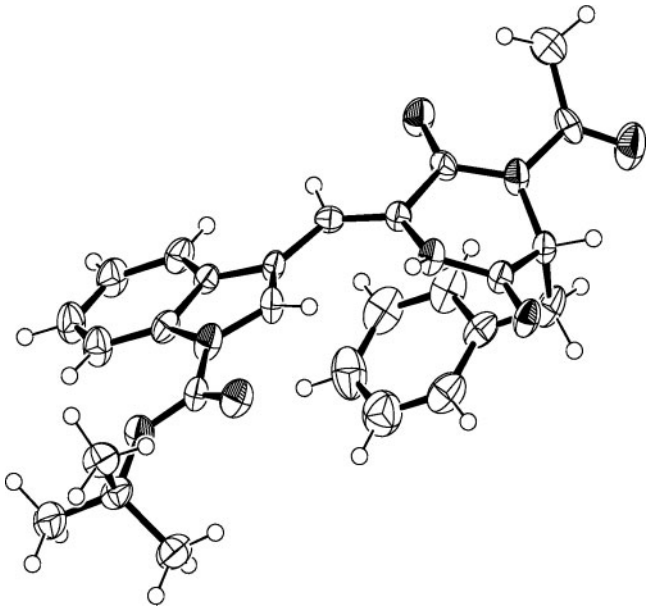


Fig. 2. X-ray crystal structure of **9**; Molecules form centrosymmetric dimers through H-bonding involving the amide N-H and the adjacent carbonyl oxygen centre.

With the model study completed we then reacted **8** with the *N*-Boc-4-nitroindole-3-carbaldehyde, prepared in four steps from 2-methyl-3-nitroaniline using a modification of the Bergman 4-nitroindole synthesis,[12] followed by a Vilsmeier–Haack formylation and *N*-Boc protection (see Accessory Publication). The condensation gave compound **11**, which was reacted with one equivalent of hydrazine hydrate to selectively remove the acetyl group. Reaction with methyliodide and potassium carbonate in DMF, methylated the two nitrogen atoms of the piperazinedione ring, and subsequent removal of the remaining protecting group with excess hydrazine hydrate gave the desired compound **12**. If compound **11** is reacted with excess hydrazine hydrate both the acetyl and Boc-groups are removed to give **13**. Compounds **12** and **13** are structural analogues of thaxtomin D and des-*N*-methyl-thaxtomin C, compounds that differ only by the presence of an alkene between the indole and central piperazinedione rings. It should be noted that reduction of the alkene would give the two natural products, which have not been previously prepared, however attempts to do this selectively in the presence of the nitro group, by reaction with magnesium in methanol, borohydrides, or triethylsilane, were unsuccessful.

After initial phytotoxicity testing, vide infra, we sought to synthesize the enantiomers of **12** and **13** derived from L-phenylalanine. Thus the dipeptide **15**, from the methyl ester of L-phenylalanine (**14**), and a protected glycine was synthesized in good yield using EDCI as the coupling agent.[13] The diketopiperazine was formed in 73% yield by in situ thermal deprotection[14,15] and cyclization, by heating the protected dipeptide **15** in a sealed tube in a microwave reactor at 160°C. The cyclic dipeptide **16** was then activated with acetic anhydride in refluxing dichloromethane. The choice of the base, triethylamine instead of pyridine, was critical to achieving high yields. The *N*,*N*′-diacetylpiperazinedione (3*S*)-**8** was subjected to the same synthetic sequence described for compound **7** to give the derivatives (3*S*)-**12** and (3*S*)-**13**. It is worth noting that structure (3*S*)-**12** has been previously reported in the patent literature, as a potential herbicide,[16] by an aldol from the *N*,*N*′-dimethylpiperazinedione but as such, no details were reported. The intermediate (5*S*)-**11** was a crystalline solid and X-ray crystallography confirmed the formation of the *Z* alkene. However, the crystal that was selected for analysis was racemic (Fig. 3), indicating some racemization had occurred. It has been reported that epimerization occurs during aldol condensations of these activated piperazinediones, up to 30% in some cases,[17] although these typically use much stronger bases such as LDA or potassium *t*-butoxide. However, analysis of the literature values of the optical rotation of the two preceding intermediates suggests that epimerization may in fact occur during diacetylation. While (3*S*)-**8** has been reported numerous times, the optical rotation has only been reported once, with a value of +7.8°.[18] In our hands, we observe a value of +19.2°. The previously reported method[18] to synthesize (3*S*)-**8** involves heating with sodium acetate in acetic anhydride at 100°C, while we have used milder conditions. It is thus possible that epimerization occurs in the activation and not during the aldol condensation. As the optical rotation is performed in methanol, it is possible that deacetylation could result in formation of **16**, which has a higher optical rotation. However, analysis of this sample by ^{1}H NMR indicated no deacetylation had occurred. The reported optical rotation for **16** also varies, with representative literature values including +97.8°,[19] +60°,[18] +26.6°,[20] and +7.3°;[15] while we observed a value of +48.4°. The cyclization to form

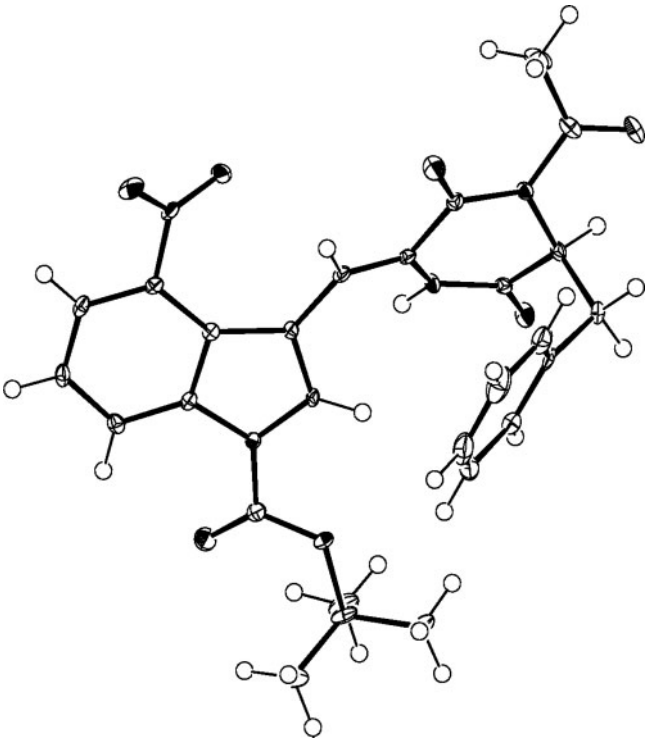
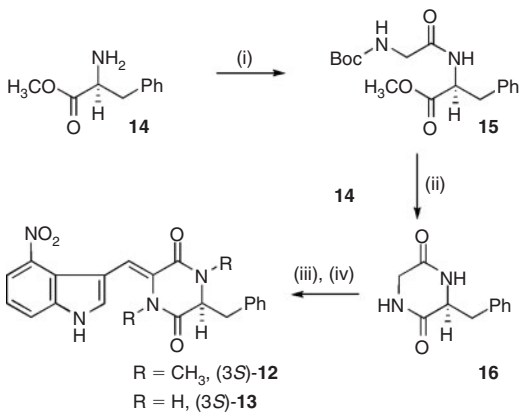


Fig. 3. X-ray crystal structure of racemic **11**; molecules form centrosymmetric dimers through H-bonding involving the amide N-H and the adjacent carbonyl oxygen centre.



Scheme 2. (i) EDCI, NEt_3, Boc-Gly-OH, CH_2Cl_2; (ii) H_2O, EtOH, 160°C, μ-wave; (iii) Ac_2O, NEt_3, DMAP, CH_2Cl_2,; (iv) as per conditions v through x from Scheme 1.

piperazinediones under neutral or acidic conditions does not usually cause racemization, therefore it is likely to occur duirng diacetylation or aldol condensation. Analysis by chiral chromotography at each synthetic stage, is required to trace the source of the racemization in these synthetic sequences. Attempts to resolve the two enantiomers of **12** using NMR chiral shift reagents to determine the extent of epimerization was unsuccessful. While the compound is enriched in one enantiomer, as indicated by the optical rotation ($[\alpha]_D^{20} = -23.1°$ (DMSO)), the extent was not ascertained, as no data was reported for (3*S*)-**12** (Scheme 2).[16]

Phytotoxicity

With the three structural variants synthesized, the phytotoxicity was evaluated in a bioassay using *Arabidopsis thaliania* (Columbia) seedlings as the test subject. *Arabidopsis thaliana* seeds were germinated on agar plates incorporating one of four different concentrations (0.1 μM, 1.0 μM, 10.0 μM, 50.0 μM) of the subject compound. These were each compared with a healthy control. Thaxtomin A (isolated from *S. scabiei* broth) was used for comparison. In each experiment, root length was measured after 7 days of growth. Compound **10**, which lacks the nitro group, was inactive. Encouragingly, the racemic nitro-containing derivatives **12** and **13** inhibited root growth by >80% compared with the control, even at sub-micromolar concentration (Fig. 4). Following this initial screen, a more detailed evaluation was carried out to determine LD_{50} of the compounds, as well as those derived from L-phenylalanine.

Compounds **12**, **13**, (3*S*)-**12**, (3*S*)-**13**, and thaxtomin A were evaluated between 0.01 and 5.0 μM and the LD_{50} determined (Fig. 5). A clear enhancement in activity for the methylated derivative was observed and, surprisingly, the racemic **12** had an LD_{50} of 0.143 μM, compared with 0.039 μM for the natural product thaxtomin A **1**, and 0.699 μM for compound **13**. The enantio-enriched (3*S*)-**12** was 25% (0.110 μM) more active than the racemic derivative, not surprisingly indicating that the enantiomer with the *S* configuration is more active than the *R* configured isomer. These results suggest the active site must have some degree of flexibility, as the conformational change induced by introducing an alkene into the molecule does not substantially alter the toxicity, whereas the stereochemistry at the 3-position is clearly important.

This study has provided histological evidence to suggest these compounds may inhibit plant cell development in a similar manner as thaxtomin A. Thaxtomin A typically induces cell swelling (hypertrophy), and enhances root hair proliferation in treated *A. thaliana* shoot and root tissues (Fig. 6, panel B). Compounds **12** and **13** induced similar morphological changes in *A. thaliana* seedlings (Fig. 6, panels D and C).

Conclusions

We have synthesized two structurally similar analogues of thaxtomin that each exhibit potent phytotoxicity, inhibiting plant growth and development in a similar manner to thaxtomin A. Evidently, there is some conformational freedom in the active site. Alkylation of the nitrogens of the central ring was associated with enhanced activity.

Experimental

General Experimental

See Accessory Publication.

*(3Z)-1-Acetyl-3-benzylidene-piperazine-2,5-dione (**5**)*

To a solution of benzaldehyde (2.80 mL, 27.5 mmol) and 1,4-diacetylpiperazine-2,5-dione **4** (4.951 g, 25.0 mmol), in DMF (50 mL) under an atmosphere of nitrogen, was added potassium carbonate (4.325 g, 31.2 mmol). The reaction was stirred for 18 h before the addition of water. The resulting precipitate was collected by filtration, washed with water, and dried under high vacuum to yield the *title compound* as an off-white solid (5.549 g, 91%), mp 198–200°C (lit.[9] 199–200°C). ν_{max} (solid/cm^{-1}): 681, 768, 999, 1034, 1099, 1203, 1224, 1264, 1359, 1405, 1428, 1494, 1631, 1678, 1698, 3048, 3267. δ_H ($CDCl_3$): 2.66 (s, 3H),

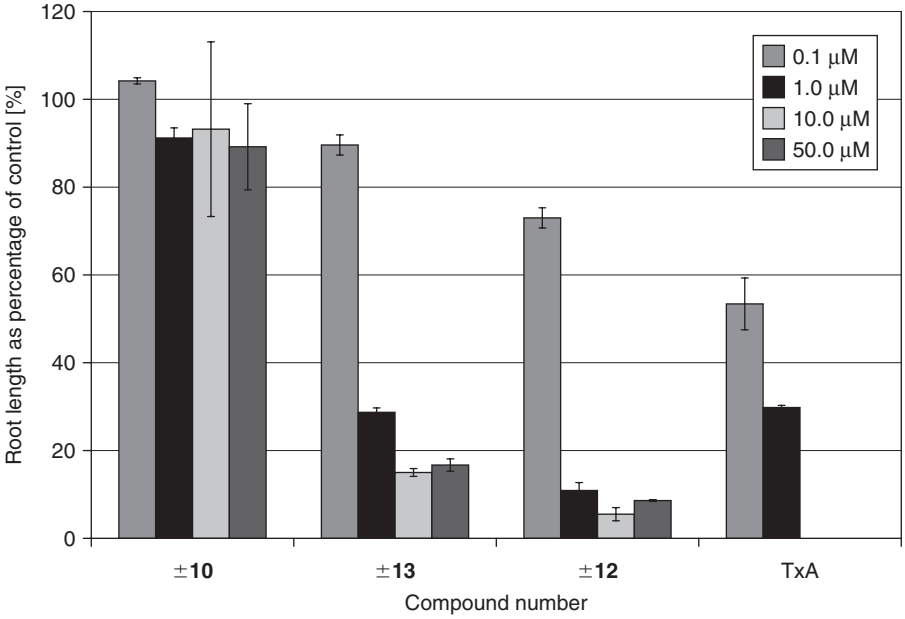


Fig. 4. Phytotoxicity screening results for ±**10**, ±**12**, ±**13**, and thaxtomin A (TxA). Results shown are root length as a percentage of the untreated control. Error bars indicate standard error.

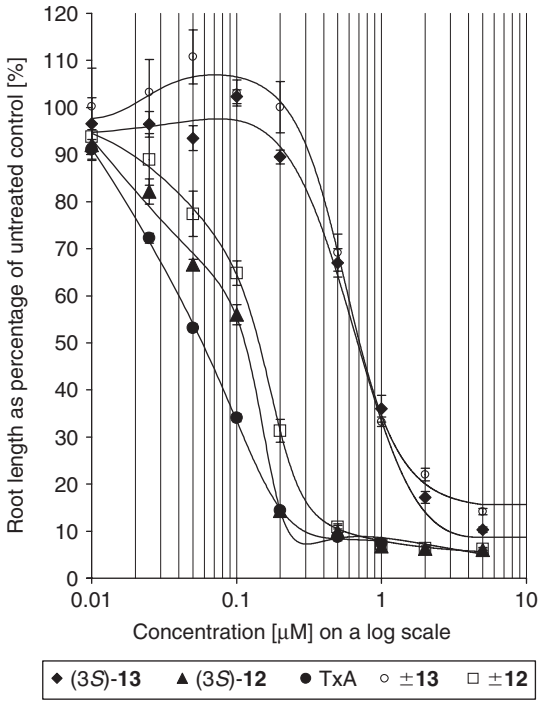


Fig. 5. Comparison of the ability of compounds **12**, **13**, (3*S*)-**12**, (3*S*)-**13** to thaxtomin A to inhibit root growth in *Arabidopsis thaliana* seedlings. Error bars indicate standard error.

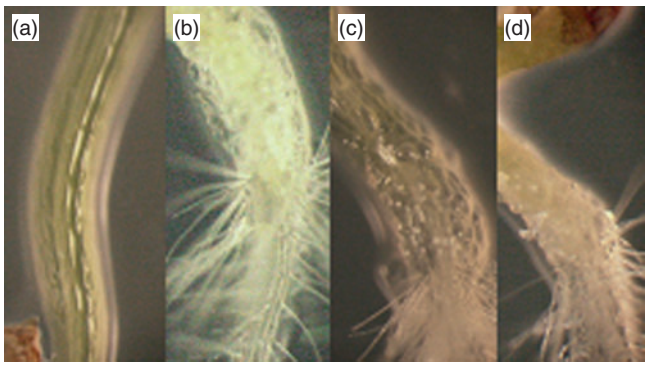


Fig. 6. Seedlings from extended toxicity trials, at 80× magnification. (a) Untreated control; (b) thaxtomin A treatment at 0.05 µM; (c) **13** at 2.0 µM; (d) **12** at 0.2 µM. Photographs were obtained using a Leica Disectting microscope MZ16 fitted with a Nikon Digital Sight DS-Fi1 (Nikon Corporation, Japan).

4.51 (s, 2H), 7.18 (s, 1H), 7.32–7.54 (m, 5H), 7.97 (bs, 1H). δ_C ($CDCl_3$): 27.4, 46.3, 120.2, 125.8, 128.7, 129.6, 129.8, 132.7, 160.2, 162.9, 172.7. *m/z* (EI+): 244 (M^+, 100), 202 (98), 173 (38), 117 (60), 91 (22). HRMS calc. for $C_{13}H_{12}N_2O_3$ [$M^{+\bullet}$] 244.08479, found 244.08447.

*(±)-N-Acetylglycylphenylalanine Methyl Ester (**6**)*

(3*Z*)-3-Benzylidenepiperazine-2,5-dione (200 mg, 0.819 mmol) was dissolved in methanol (20 mL) and stirred with 10% Pd/C (20 mg) under an atmosphere of hydrogen (1 atm, balloon) for 24 h. The solution was filtered through a plug of celite and the solvent removed under reduced pressure, to yield the *title compound* as a colourless glassy solid (0.233 g, 98%), mp 128–130°C. ν_{max} (thin film/cm^{-1}): 669, 1030, 1214, 1279, 1495, 1564, 1729, 1752, 1952, 3063, 3244, 3264. δ_H ($CDCl_3$): 1.98 (s, 3H), 3.04 (dd, *J* 13.9, 6.2 Hz, 1H), 3.14 (dd, *J* 13.9, 6.2 Hz, 1H), 3.70 (s, 3H), 3.76–3.96 (m, 2H), 4.77–4.86 (m, 1H), 6.60 (bs, 1H), 6.90 (d, *J* 7.8 Hz, 1H), 7.10 (add, *J* 7.7, 1.5 Hz, 2H), 7.17–7.30 (m, 3H). δ_C ($CDCl_3$): 22.9, 37.8, 43.1, 52.5, 53.4, 127.2, 128.7, 129.3, 135.8, 168.9, 170.8, 172.0. *m/z* (EI+): 278 (M^+, <1%), 162 (100), 120 (35), 100 (38), 91 (20). HRMS calc. for $C_{14}H_{18}N_2O_4$ [$M^{+\bullet}$] 278.12666, found 278.12659.

*(±)-1-Acetyl-3-benzyl-piperazine-2,5-dione (**7a**) and (±)-1-Acetyl-6-benzyl-piperazine-2,5-dione (**7b**)*

(3*Z*)-3-Benzylidenepiperazine-2,5-dione (4.629 g, 18.9 mmol) was dissolved in THF (400 mL)/acetic acid (40 mL), 10% Pd/C

(400 mg) was added and the mixture stirred at room temperature overnight, under an atmosphere of hydrogen (1 atm, balloon). The residue was filtered through celite with ethyl acetate and the solvent removed under reduced pressure. The residue was purified by elution from a short silica plug with dichloromethane to yield (±)-1-acetyl-6-benzyl-piperazine-2,5-dione as a colourless crystalline solid (2.215 g, 48%). Flushing the plug with ethyl acetate yielded (±)-1-acetyl-3-benzyl-piperazine-2,5-dione as colourless crystalline solid (1.672 g, 36%) (84% combined yield).

(±)-1-Acetyl-3-benzyl-piperazine-2,5-dione (**7a**). mp 151–153°C (lit.[9] 136–138°C). ν_{max} (thin film/cm^{-1}): 1097, 1205, 1258, 1367, 1436, 1684, 1704, 3246. δ_H ($CDCl_3$): 2.59 (s, 3H), 3.12 (dd, *J* 13.9, 7.2 Hz, 1H), 3.26 (dd, *J* 13.9, 4.1 Hz, 1H), 3.47 (d, *J* 18.2 Hz, 1H), 4.21 (d, *J* 18.2 Hz, 1H), 4.37 (ddd, *J* 7.2, 4.1, 2.6 Hz, 1H), 6.53 (bs, 1H), 7.15–7.21 (m, 2H), 7.30–7.36 (m, 3H). δ_C ($CDCl_3$): 27.4, 40.3, 45.6, 58.1, 128.2, 129.4, 129.7, 134.5, 166.4, 168.4, 171.9. *m/z* (EI+): 264 (M^+, 11%), 204 (9), 91 (100). HRMS calc. for $C_{13}H_{14}N_2O_3$ [$M^{+\bullet}$] 246.10044, found 246.10049.

(±)-1-Acetyl-6-benzyl-piperazine-2,5-dione (**7b**). ν_{max} (thin film/cm^{-1}): 703, 1097, 1205, 1258, 1367, 1439, 1680, 1699, 3254. δ_H ($CDCl_3$): 2.56 (s, 3H), 3.18 (m, 2H), 3.24 (d, *J* 18.3 Hz, 1H), 4.18 (d, *J* 18.3 Hz, 1H), 4.39 (td, *J* 5.4, 2.6 Hz, 1H), 7.16–7.19 (m, 2H), 7.29–7.38 (m, 3H), 7.39 (bs, 1H). δ_C ($CDCl_3$): 27.4, 40.4, 45.4, 58.2, 128.1, 129.3, 129.9, 134.5, 167.2, 168.5, 171.9. *m/z* (EI+): 246 (M^+, 8%), 204 (10), 91 (100). HRMS calc. for $C_{13}H_{14}N_2O_3$ [$M^{+\bullet}$] 246.10044, found 246.10053.

*(±)-1,4-Diacetyl-3-benzyl-piperazine-2,5-dione (**8**)*

Pyridine (0.66 mL, 8.15 mmol), acetic anhydride (0.71 mL, 7.47 mmol), and DMAP (105 mg, 0.852 mmol) were added sequentially to a solution of **7a** (1.672 g, 6.79 mmol) in dry dichloromethane (30 mL), under an atmosphere of nitrogen. After 18 h the reaction was quenched with saturated potassium hydrogen sulfate solution (30 mL), the layers separated and the aqueous phase extracted with additional dichloromethane (2 × 25 mL). The combined organic layers were washed with saturated potassium hydrogen sulfate (30 mL), 2 M sodium carbonate solution (30 mL), brine (40 mL), and then dried over magnesium sulfate. Filtration through a plug of silica gel, eluted with dichloromethane, yielded the *title compound* as a colourless crystalline solid (1.559 g, 95% – based on recovered starting material), mp 83–85°C (lit.[18] 84–85°C). Unreacted starting material was recovered by elution with ethyl acetate (0.2700 g, 1.09 mmol).

Alternately **8** could be prepared from compound **7b**. Pyridine (0.87 mL, 10.8 mmol), acetic anhydride (0.94 mL, 9.89 mmol), and DMAP (177.6 mg, 1.45 mmol) were added sequentially to (±)-1-acetyl-6-benzylpiperazine-2,5-dione (2.215 g, 8.99 mmol) in dry dichloromethane (40 mL), using the method described above for (±)-1-acetyl-3-benzyl-piperazine-2,5-dione **7a**, yielding the title product as a colourless crystalline solid (2.258 g, Quant. – based on recovered starting material). Unreacted starting material was recovered by elution with ethyl acetate (0.269 g, 1.09 mmol).

ν_{max} (thin film/cm^{-1}): 704, 743, 965, 1040, 1138, 1206, 1218, 1368, 1695, 1712, 1735, 2938, 3008. δ_H ($CDCl_3$): 2.44 (d, *J* 19.0 Hz, 1H), 2.55 (s, 3H), 2.57 (s, 3H), 3.20 (dd, *J* 14.0, 5.5 Hz, 1H), 3.35 (dd, *J* 14.0, 4.4 Hz, 1H), 4.48 (d, *J* 19.0 Hz, 1H), 5.43 (dd, *J* 5.5, 4.4 Hz, 1H), 7.02–7.08 (m, 2H), 7.22–7.32 (m, 3H). δ_C ($CDCl_3$): 27.2, 27.5, 39.0, 46.3, 59.4, 128.5, 129.4, 130.0, 134.6, 166.3, 168.3, 171.3, 171.5. *m/z* (EI+): 288 (M^+, 20%), 246 (33), 203 (11), 131 (35), 91 (100). HRMS calc. for $C_{15}H_{16}N_2O_4$ [$M^{+\bullet}$] 288.11101, found 288.11110.

*(±)-t-Butyl-3-[(Z)-(4-acetyl-5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]indole-1-carboxylate (**9**)*

A solution of (±)-1,4-diacetyl-3-benzyl-piperazine-2,5-dione (**7**) (0.803 g, 2.78 mmol), *N*-Boc-indole-3-carboxaldehyde (0.751 g, 3.06 mmol), and cesium carbonate (1.273 g, 3.91 mmol), in dry DMF (25 mL) under an atmosphere of nitrogen, was stirred for 18 h protected from light. The reaction was quenched with water (30 mL), and extracted with ethyl acetate (3 × 75 mL). The combined organic layers were washed with water (2 × 75 mL) and brine (2 × 70 mL), dried on magnesium sulfate, filtered and the solvent removed under reduced pressure, yielding the *title compound* as a solid which was used without further purification, mp 181–183°C dec. (1.084 g, 94%). ν_{max} (solid/cm^{-1}): 700, 750, 1091, 1150, 1221, 1309, 1336, 1368, 1453, 1631, 1677, 1712, 1735, 2985, 3155, 3256. δ_H (d_6-DMSO): 1.67 (s, 9H), 2.49 (s, 3H), 3.07 (at, *J* 5.2 Hz, 2H), 5.08 (at, *J* 5.0 Hz, 1H), 6.69 (s, 1H), 6.96–7.14 (m, 5H), 7.31 (at, *J* 7.3 Hz, 1H), 7.38 (at, *J* 7.6 Hz, 1H), 7.58 (d, *J* 7.9 Hz, 1H), 7.99 (s, 1H), 8.03 (d, *J* 8.4 Hz, 1H), 10.35 (bs, 1H). δ_C (d_6-DMSO): 27.5, 28.3, 38.9, 58.1, 85.3, 110.0, 113.2, 115.5, 119.7, 123.9, 125.7, 126.6, 127.3, 127.7, 128.9, 130.5, 134.6, 135.4, 139.1, 149.5, 161.8, 166.3, 172.4. *m/z* (EI+): 473 (M^+, <1%), 373 (30), 331 (13), 240 (100), 212 (15), 155 (45). HRMS (LSIMS with mNBA) calc. for $C_{27}H_{28}N_3O_5$ [$MH^{+\bullet}$] 474.19507, found 474.20327. Crystals suitable for X-ray crystallography were grown from ethyl acetate/hexanes. $C_{27}H_{27}N_3O_5$, $M = 473.52$, $T = 193$ K, monoclinic, space group $P2_1/c$, $a = 10.442(5)$, $b = 19.799(3)$, $c = 12.147(3)$ Å, $\beta = 108.83(3)$, $V = 2376.8(13)$ Å^3, $Z = 4$, $D_c = 1.323$ g cm^{-3}, specimen: yellow block, 0.45 × 0.45 × 0.32 mm, 4603 measured reflections, $R_{int} = 0.0597$, $r = 0.0881$ for 2442 observed data ($(I) > 2\sigma(I)$), $wR = 0.2494$, and GOOF = 1.065 for all data (4172).

*(±)-(6Z)-3-Benzyl-6-(1H-indol-3-ylmethylene)piperazine-2,5-dione (**10**)*

Hydrazine hydrate (330 μL, 6.75 mmol) was added dropwise to a solution (±)-*t*-butyl-3-[(*Z*)-(4-acetyl-5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]indole-1-carboxylate (282 mg, 0.675 mmol) in DMF (5 mL), protected from light and stirred for 18 h. The reaction was quenched with water (15 mL), and the precipitated solid collected by filtration, washed with *t*-butyl methyl ether and dried under high vacuum, yielding the *title compound* as a yellow solid (141 mg, 63%), mp 278–280°C dec. ν_{max} (solid/cm^{-1}): 756, 1097, 1139, 1235, 1253, 1383, 1422, 1442, 1620, 1661, 3060, 3208. δ_H (d_6-DMSO): 2.95 (dd, *J* 13.7, 5.2 Hz, 1H), 3.13 (dd, *J* 13.7, 4.5 Hz, 1H), 4.35 (m, 1H), 6.70 (s, 1H), 7.00–7.29 (m, 6H), 7.38 (d, *J* 7.9 Hz, 1H), 7.46 (d, *J* 7.9 Hz, 1H), 7.71 (d, *J* 2.5 Hz, 1H), 7.93 (s, 1H), 8.27 (s, 1H), 9.30 (s, 1H), 11.54 (s, 1H). δ_C (d_6-DMSO): 36.4, 56.8, 107.7, 108.4, 112.4, 118.8, 120.4, 122.6, 122.8, 126.8, 127.3, 127.4, 128.7, 130.6, 136.2, 136.3, 161.1, 166.5. *m/z* (EI+): 331 (M^+, 100), 240 (95), 212 (18), 155 (58), 130 (13), 91 (9). HRMS calc. for $C_{18}H_{19}N_3O_4$ [$M^{+\bullet}$] 331.11716, found 331.13176.

*(±)-t-Butyl 3-[(Z)-(4-Acetyl-5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (**11**)*

To solution of (±)-1,4-diacetyl-3-benzylpiperazine-2,5-dione (829 mg, 2.87 mmol) and 4-nitroindole-3-carboxaldehyde

(874 mg, 3.01 mmol) in dry DMF (25 mL), under an atmosphere of nitrogen, was added cesium carbonate (1.18 g, 3.65 mmol). After protection from light and stirring for 18 h, the reaction was quenched with water (20 mL). The precipitated solid was collected by filtration and washed with *t*-butyl methyl ether, yielding the *title compound* as a yellow solid (919 mg, 61%), mp 191–192°C dec. ν_{max} (thin film/cm^{-1}): 748, 1094, 1150, 1227, 1355, 1370, 1441, 1524, 1676, 1705, 1758, 2980, 3201. δ_H ($CDCl_3$): 1.76 (m, 9H), 2.66 (s, 3H), 3.27 (ad, *J* 4.0 Hz, 1H), 5.35 (at, *J* 4.2 Hz, 1H), 6.76 (s, 1H), 7.05–7.19 (m, 2H), 7.20–7.31 (m, 3H), 7.46 (t, *J* 8.2 Hz, 1H), 7.52 (s, 1H), 7.56 (bs, 1H), 7.98 (d, *J* 8.0 Hz, 1H), 8.57 (d, *J* 7.9 Hz, 1H). δ_C ($CDCl_3$): 27.4, 28.3, 39.3, 58.1, 86.6, 113.4, 120.6, 121.3, 121.9, 125.4, 126.0, 127.9, 128.0, 128.8, 128.9, 130.7, 134.5, 138.0, 142.5, 148.7, 160.0, 166.1, 173.2. *m/z* (EI+): 518 (M^+, <1%), 460 (12), 418 (40), 285 (100), 158 (28), 91 (29).

(±)-(6Z)-3-Benzyl-1,4-dimethyl-6-[(4-nitro-1H-indol-3-yl)methylene]piperazine-2,5-dione (**12**)

Hydrazine hydrate (62 μL, 1.27 mmol) was added dropwise to a solution of *t*-butyl (±)-3-[(*Z*)-(4-acetyl-5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (657 mg, 1.27 mmol) in DMF (5 mL), protected from light and stirred for 18 h. The reaction was quenched with water (15 mL), and the precipitated solid collected by filtration, washed with *t*-butyl methyl ether and dried under high vacuum to yield (±)-*t*-butyl 3-[(*Z*)-(5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate as yellow powder (577 mg, 95%), mp 187–189°C dec. Insolubility in common NMR solvents prevented full characterization, the compound was used without further purification. ν_{max} (solid/cm^{-1}): 697, 743, 790, 1091, 1154, 1278, 1434, 1526, 1634, 1676, 1743, 3069, 3187. *m/z* (EI+): 476 (M^+, <1%), 376 (100), 330 (54), 285 (78), 158 (48). HRMS calc. for $C_{25}H_{24}N_4O_6$ [$M^{+\bullet}$] 476.16958, found 476.16886.

Iodomethane (1.54 mL, 10.9 mmol) was added to a suspension of potassium carbonate (1.716 g, 12.4 mmol) and (±)-*t*-butyl 3-[(*Z*)-(5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (519 mg, 1.09 mmol) in dry DMF (15 mL), under an atmosphere of nitrogen. After stirring for 72 h the reaction was quenched with water (15 mL) and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with water (3 × 10 mL), and brine (2 × 25 mL), dried over magnesium sulfate, filtered and the solvent removed under reduced pressure. The residue was purified by column chromatography (60% ethyl acetate/hexanes), yielding (±)-*t*-butyl 3-[(*Z*)-(5-benzyl-1,4-dimethyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate as a crystalline yellow solid (435 mg, 79%), mp 130–132°C. ν_{max} (thin film/cm^{-1}): 733, 919, 1101, 1258, 1285, 1315, 1352, 1433, 1522, 1635, 1682, 1743, 2934. δ_H ($CDCl_3$): 1.71 (s, 9H), 2.76 (s, 3H), 3.05 (s, 3H), 3.20 (d, *J* 4.7 Hz, 2H), 4.32 (t, *J* 4.7 Hz, 1H), 6.76 (d, *J* 1.5 Hz, 1H), 7.04–7.30 (m, 6H), 7.37 (at, *J* 8.2 Hz, 1H), 7.86 (d, *J* 8.1 Hz, 1H), 8.45 (d, *J* 8.4 Hz, 1H). δ_C ($CDCl_3$): 28.3, 33.4, 34.8, 37.8, 64.6, 86.1, 112.5, 113.1, 120.1, 120.9, 124.5, 127.7, 129.0, 129.2, 130.1, 130.3, 135.2, 137.1, 142.9, 148.7, 160.9, 166.6. *m/z* (EI+): 504 (M^+, <1%), 404 (40), 313 (100), 158 (30). HRMS calc. for $C_{27}H_{28}N_4O_6$ [$M^{+\bullet}$] 504.20088, found 504.19859.

Hydrazine hydrate (50 μL, 1.03 mmol) was added to a solution of (±)-*t*-butyl 3-[(*Z*)-(5-benzyl-1,4-dimethyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (104 mg, 0.206 mmol) in DMF (5 mL), and stirred for 24 h protected from light. After quenching with water, the resultant precipitate was collected by filtration, washed with *t*-butyl methyl ether, and dried under high vacuum, yielding the *title compound* as an orange solid (38 mg, 46%), mp 210–212°C dec. ν_{max} (solid/cm^{-1}): 691, 730, 795, 975, 1119, 1252, 1286, 1323, 1377, 1424, 1516, 1615, 1675, 2923, 3027, 3183. δ_H (d_6-DMSO): 2.64 (s, 3H), 2.90 (s, 3H), 3.05 (dd, *J* 13.4, 5.1 Hz, 1H), 3.18 (dd, *J* 13.4, 5.2 Hz, 1H), 4.43 (at, *J* 5.3 Hz, 1H), 6.87 (s, 1H), 7.06 (s, 1H), 7.11–7.32 (m, 6H), 7.82–7.85 (m, 2H), 12.22 (bs, 1H). δ_C (d_6-DMSO): 33.2, 34.7, 37.8, 64.1, 107.9, 114.3, 118.3, 118.5, 119.5, 121.7, 127.6, 128.8, 129.1, 130.4, 132.0, 136.4, 139.1, 142.3, 161.6, 166.9. *m/z* (EI+): 404 (M^+, 42%), 313 (100), 285 (20), 158 (33), 130 (22). HRMS calc. for $C_{22}H_{20}N_4O_4$ [$M^{+\bullet}$] 404.14846, found 404.14861.

(±)-(6Z)-3-Benzyl-6-[(4-nitro-1H-indol-3-yl)methylene]piperazine-2,5-dione (**13**)

Hydrazine hydrate (200 μL, 4.16 mmol) was added dropwise to a solution of **10** (215.9 mg, 0.416 mmol) in DMF (3 mL) and stirred for 18 h protected from light. The reaction was quenched with water (15 mL), the precipitated solid collected by filtration, washed with *t*-butyl methyl ether and dried under high vacuum, yielding the *title compound* as a red solid (131 mg, 84%), mp 285–287°C dec. ν_{max} (solid/cm^{-1}): 759, 806, 981, 1136, 1209, 1317, 1359, 1435, 1510, 1618, 1659, 3033, 3281. δ_H (d_6-DMSO): 2.98 (dd, *J* 13.8, 4.8 Hz, 1H) 3.14 (dd, *J* 13.5, 3.6 Hz, 1H), 4.30 (m, 1H), 6.61 (s, 1H), 7.03–7.36 (m, 6H), 7.53 (s, 1H), 7.79 (m, 2H), 8.29 (s, 1H), 9.53 (s, 1H), 12.19 (s, 1H). δ_C (d_6-DMSO): 40.1, 56.8, 107.0, 109.4, 118.0, 118.6, 119.0, 121.4, 124.7, 127.3, 128.7, 130.7, 131.6, 136.3, 139.2, 142.6, 160.7, 166.4. *m/z* (EI+): 376 (M^+, 100%), 330 (51), 285 (78), 158 (61), 91 (91). HRMS calc. for $C_{20}H_{16}N_4O_4$ [$M^{+\bullet}$] 376.11716, found 376.11747.

Methyl (2S)-2-[[2-(t-Butoxycarbonylamino)acetyl]amino]-3-phenyl-propanoate (N-Boc-Gly-Phe-OMe) (**15**)

Triethylamine (5.70 mL, 40.9 mmol), then EDAC (7.32 g, 37.6 mmol) were added to a solution of L-phenylalanine methyl ester (**14**) (5.62 g, 31.4 mmol) and *N*-Boc-glycine (6.0887 g, 34.5 mmol) in dry dichloromethane (100 mL), under an atmosphere of nitrogen. The reaction was stirred for 18 h, diluted with dichloromethane (50 mL), and the organic phase washed with 0.5 M potassium hydrogen sulfate solution (2 × 75 mL) and saturated sodium carbonate solution (1 × 75 mL). The organic layer was dried with magnesium sulfate and filtered through a plug of silica gel (eluting with 60% ethyl acetate/hexanes), and the solvent removed under reduced pressure, to give the *title compound* as a viscous oil (10.15 g, 96%). δ_H ($CDCl_3$): 1.40 (s, 9H), 2.95–3.15 (m, 2H), 3.64 (s, 3H), 3.67–3.77 (m, 2H), 4.84 (aq, *J* 6.8 Hz, 1H), 5.41 (at, *J* 5.4 Hz, 1H), 6.85 (bd, *J* 7.4 Hz, 1H), 7.02–7.09 (m, 2H), 7.14–7.26 (m, 3H). δ_C ($CDCl_3$): 28.4, 38.1, 44.2, 52.5, 53.3, 80.2, 127.3, 128.7, 129.4, 135.9, 156.2, 169.6, 172.0. *m/z* (EI+): 336 (M^+, <1%), 280 (8), 203 (12), 162 (100), 120 (38). HRMS calc. for $C_{17}H_{24}N_2O_5$ [$M^{+\bullet}$] 336.16852, found 336.16799.

(3S)-3-Benzylpiperazine-2,5-dione (**16**)

Six batches (2 mL each) of *N*-Boc-Gly-Phe-OMe (4.67 g, 13.9 mmol), dissolved in ethanol (18.0 mL), were diluted with water (2 mL) and heated to 160°C for 1 h in a sealed tube using a microwave reactor (CEM Discover microwave reactor). The six

batches were combined and cooled on ice, the resulting precipitate collected by filtration and dried under high vacuum, yielding the *title compound* as a colourless solid (2.07 g, 73%), mp 256–267°C (lit.[19] 270°C). $[\alpha]_D^{21} = +48.4$ (*c* 1.0 in DMSO) lit.[19] +60 (*c* 0.15, DMSO). ν_{max} (solid/cm^{-1}): 701, 851, 1335, 1462, 1670, 2887, 3050, 3196. δ_H (d_6-DMSO): 2.74 (d, *J* 17.4 Hz, 1H), 2.86 (dd, *J* 13.5, 4.9 Hz, 1H), 3.08 (dd, *J* 13.5, 4.5 Hz, 1H), 3.34 (dd, *J* 17.4, 2.9 Hz, 1H), 4.05 (m, 1H), 7.10–7.21 (m, 2H), 7.22–7.32 (m, 3H), 7.88 (bs, 1H), 8.14 (s, 1H). δ_C (d_6-DMSO): 49.0, 60.9, 132.1, 133.5, 135.4, 141.4, 171.0, 172.5. *m/z* (EI+): 204 (M^+, 18%), 91 (100). HRMS calc. for $C_{11}H_{12}N_2O_2$ [$M^{+\bullet}$] 204.08988, found 204.08959.

*(3S)-1,4-Diacetyl-3-benzyl-piperazine-2,5-dione ((+)-**8**)*

Triethylamine (2.92 mL, 20.9 mmol), acetic anhydride (1.83 mL, 19.3 mmol), and DMAP (63.0 mg, 0.276 mmol) were added sequentially to a suspension of (3*S*)-3-benzylpiperazine-2,5-dione (0.6571 g, 3.22 mmol) in dry dichloromethane (30 mL) and refluxed for 6 h. The reaction mixture was diluted with dichloromethane (20 mL), and washed with saturated potassium hydrogen sulfate solution (2 × 30 mL) and 2 M sodium carbonate solution (1 × 30 mL). The organic layer was dried over magnesium sulfate, filtered through a plug of silica gel (eluting with dichloromethane), and the solvent removed under reduced pressure, yielding the *title compound* as a crystalline colourless solid (0.660 g, 71%). The compound was identical by spectroscopy to the racemic material (**7**), $[\alpha]_D^{21} = +19.2$ (*c* 1.4, MeOH) lit.[19] +7.8 (*c* 0.52, MeOH).

t-*Butyl 3-[(3S)-(Z)-(4-Acetyl-5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate ((3S)-**11**)*

(3*S*)-1,4-Diacetyl-3-benzyl-piperazine-2,5-dione (629 mg, 2.18 mmol) was reacted with *N*-Boc-4-nitroindole-3-carboxaldehyde (0.6568 g, 2.29 mmol) and cesium carbonate (899 mg, 2.76 mmol) in DMF (15 mL), using the method described for the racemic compound above, yielding the *title compound* as an off-yellow solid (738 mg, 65%). The compound was identical by spectroscopy to the racemic material (**11**). Crystals suitable for X-ray crystallography were grown from $CDCl_3$. $C_{27}H_{26}N_4O_7$, $M = 518.52$, $T = 100$ K, monoclinic, space group $P2_1/n$, $a = 13.3800(11)$, $b = 8.4840(8)$, $c = 22.9030(14)$ Å, $\beta = 105.4840(10)$, $V = 2505.5(3)$ Å^3, $Z = 4$, $D_c = 1.375$ g cm^{-3}, specimen: yellow block, 0.15 × 0.15 × 0.05 mm, 25381 measured reflections, $R_{int} = 0.0481$, $r = 0.0384$ for 3785 observed data ($(I) > 2\sigma(I)$), $wR = 0.0997$, and GOOF = 1.051 for all data (3567).

*(3S)-(6Z)-3-Benzyl-1,4-dimethyl-6-[(4-nitro-1H-indol-3-yl)methylene]piperazine-2,5-dione ((3S)-**12**)*

t-Butyl 3-[(3*S*)-(*Z*)-(4-acetyl-5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (539 mg, 1.04 mmol) was reacted with hydrazine hydrate (51.0 μL, 1.04 mmol) in DMF (5 mL), using the method described for the racemic compound above, yielding *t*-butyl 3-[(5*S*)-(*Z*)-(5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate as a yellow solid (443 mg, 89%).

Iodomethane (0.440 mL, 6.93 mmol) was added to a suspension of potassium carbonate (1.014 g, 7.33 mmol) and *t*-butyl 3-[(5*S*)-(*Z*)-(5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (332 mg, 0.693 mmol) in dry DMF (15 mL), under an atmosphere of nitrogen. After stirring until TLC showed only one product (5 days), the reaction was quenched with water (50 mL) and extracted with ethyl acetate (3 × 25 mL). The combined organic layers were washed with water (3 × 25 mL) and brine (2 × 50 mL), dried over magnesium sulfate, filtered, and the solvent removed under reduced pressure. The residue was purified by column chromatography (60% ethyl acetate/hexanes) yielding *t*-butyl 3-[(5*S*)-(*Z*)-(5-benzyl-1,4-dimethyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate as crystalline yellow solid (245 mg, 70%). The compound was identical by spectroscopy to the racemic material (**11**), $[\alpha]_D^{21} = -2.24$ (dichloromethane).

t-Butyl 3-[(3*S*)-(*Z*)-(5-benzyl-1,4-dimethyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (245 mg, 0.487 mmol) was reacted with hydrazine hydrate (120 μL, 2.43 mmol) in DMF (5 mL), using the method described for the racemic compound above, yielding the *title compound* as an orange solid (160 mg, 81%).The compound was identical by spectroscopy to the racemic material (**12**), $[\alpha]_D^{21} = -23.1$ (*c* 2.2 in DMSO).

*(3S)-(6Z)-3-Benzyl-6-[(4-nitro-1H-indol-3-yl)methylene]piperazine-2,5-dione ((3S)-**13**)*

t-Butyl 3-[(3*S*)-(*Z*)-(4-acetyl-5-benzyl-3,6-dioxo-piperazin-2-ylidene)methyl]-4-nitroindole-1-carboxylate (195 mg, 0.376 mmol) was reacted with hydrazine hydrate (185 μL, 3.76 mmol) in DMF (3 mL), using the method described for the racemic compound above, yielding (3*S*)-(6*Z*)-3-benzyl-6-[(4-nitro-1*H*-indol-3-yl)methylene]piperazine-2,5-dione as a deep red solid (128 mg, 91%). The compound was identical by spectroscopy to the racemic material (**13**).

Compound Toxicity Screening

Compounds (**10**, **12**, and **13**) were assayed for phytotoxicity using *Arabidopsis thaliana* seedlings at four concentrations (0.1 μM, 1.0 μM, 10.0 μM, 50.0 μM). Each batch had five test compounds which were trialled at four concentrations (0.1 μM, 1.0 μM, 10.0 μM, 50.0 μM), against untreated controls containing the same volume of DMSO. For comparison, plates containing thaxtomin A at four concentrations (0.1 μM, 0.2 μM, 1.0 μM, 2.0 μM) were also used. For each treatment two replicated plates were prepared and 20 vernalised *Arabidopsis thaliana* seedlings per plate were pipetted directly onto the surface of the cooled growth medium. Seeds were placed 15 mm from the top of dish, and were individually spaced evenly across the plate. Plates were wrapped, grouped by replicate, and transferred to a growth chamber, orientated to an upright position of 85°, with the seeds at the top of the plate. The seeds were then allowed to grow along the surface of the agar for 7 days at an ambient temperature of 22 ± 1°C, with a 16 h day length (60 μmol m^{-2} s^{-1}). After 7 days, the root length of 15 random seedlings (per plate) was measured ($n = 30$) and compared with the controls.

Compound Toxicity Range Experiments

Compounds (**12** and **13**) were tested to obtain IC_{50} values in three batches using *Arabidopsis thaliana* seedlings. The two compounds were trialled at nine concentrations (0.01 μM, 0.025 μM, 0.050 μM, 0.10 μM, 0.2 μM, 0.5 μM, 1.0 μM, 2.0 μM, 5.0 μM), in comparison to untreated controls containing an equivalent volume of DMSO. For comparison, plates containing thaxtomin A at seven concentrations (0.01 μM, 0.025 μM,

0.050 μM, 0.10 μM, 0.2 μM, 0.5 μM, 1.0 μM) were also run. For each treatment four replicated plates were prepared. Vernalized *Arabidopsis thaliana* seedlings (20 per plate) were pipetted directly onto the surface of the cooled growth medium. Seeds were placed 15 mm from the top of dish, and were evenly spaced across the plate. Plates were wrapped, randomized, and transferred to the growth chamber, orientated to an upright position of 85°, with the seeds at the top of the plate. The seeds were then allowed to grow along the surface of the agar for 7 days (unless otherwise stated) at an ambient temperature of 22 ± 1°C, with a 16 h day length (60 μmol m^{-2} s^{-1}). After 7 days, the root length of 15 random seedlings (per plate) was measured ($n = 60$ in total) and compared with the controls.

Accessory Publication

General experimental information and NMR spectra for new compounds are available on the Journal's website.

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