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Synthesis of the tritiated isotopomers of enzastaurin and its *N-des*-pyridylmethyl metabolite for use in ADME studies

William J. Wheeler,* and Dean K. Clodfelter

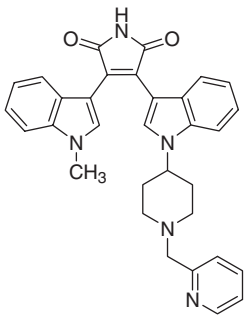
Enzastaurin (3-(1-methyl-1*H*-indol-3-yl)-4-[1-[1-(2-pyridinylmethyl)-4-piperidinyl]-1*H*-indol-3-yl]-1*H*-pyrrole-2,5-dione, 1), an agent with potential utility in the treatment of solid tumors, is currently in phase II clinical trials. Enzastaurin undergoes metabolism *in vitro* and *in vivo* to several products of oxidative metabolism, the major one of which is 3-(1-methyl-1*H*-indol-3-yl)-4-(1-piperidin-4-yl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (2). In a model study, the attempted synthesis 1-[^{2}H] by reaction of 1 with deuterium gas in the presence of Ir[(COD)(Cy_3P)pyr]PF_6 (Crabtree's catalyst) was unsuccessful. Alternatively, it was decided to prepare tritiated 2 as both a final product and the starting material for the tritiation of 1. We have reported herein a route that was developed for use in the preparation of tritium-labeled 2-[^{3}H] and its successful conversion to 1-[^{3}H].

Keywords: PKC; AKT; VEGF; tritiated; enzastaurin

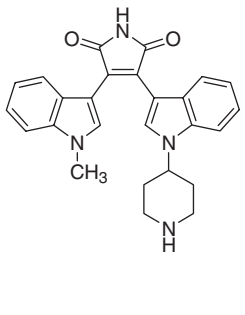
Introduction

Protein kinase C (PKC) is a family of serine-/threonine-specific kinases, which are involved in signal transduction pathways that govern a wide variety of physiological processes.[1] Vascular endothelial growth factor (VEGF) is the most common and direct acting angiogenic factor in cancer patients, and the up-regulation of VEGF receptors has been observed in tumor-associated endothelial cells; there are data to support the premise that PKC activation is directly responsible for the VEGF signaling that leads to neovascularization.[2] Enzastaurin, (3-(1-methyl-1*H*-indol-3-yl)-4-[1-[1-(2-pyridinyl-methyl)-4-piperidinyl]-1*H*-indol-3-yl]-1*H*-pyrrole-2,5-dione, **1**), a selective inhibitor of PKCβ, when dosed to mice-bearing human small cell lung (SW2) and renal cell (Caki01) tumors, resulted in significant reductions in plasma VEGF levels, which was observed 5–7 days after the onset of treatment and persisted for 2–3 weeks after the termination of therapy.[3] This reduction of VEGF was accompanied by a decrease in intratumoral vessel density. A dual center, phase I clinical study was initiated to study the safety and pharmacokinetics of enzastaurin in patients with solid tumors; these studies revealed that the half life of enzastaurin (**1**) ranged from 9 to 25 h with no significant accumulation.[4] The *N-des*-pyridylmethyl metabolite **2** had a longer half life and accumulated upon multiple dosing. Subsequent *in vitro* studies have revealed that **1** robustly induced apoptosis of a variety of human tumor cells by suppressed signaling through the PI3K/AKT pathway.[5]

Oral dosing of **1** to xenograft-bearing mice (HCT116 colon carcinoma and U87MG glioblastoma) suppressed tumor growth. In Phase II clinical studies, enzastaurin showed some promise in the treatment of recurrent glioblastoma (25% of 92 patients).[6] The synthesis of unlabeled enzastaurin[7] as well as carbon-14-labeled enzastaurin (**1-[^{14}C]**) for use in ADME studies in laboratory animals and humans has been reported.[8] In order to gather more data to support further evaluation of **1**, the tritiated isotopomers (**1-[^{3}H]** and **2-[^{3}H]**) were required. In this paper, we will discuss the successful efforts to prepare these tritiated compounds.



1



2

Discussion

In spite of the pyridine-directed tritiation reported by Shu *et al*. (Figure 1)[9], treatment of **1** (as a model for tritiation) with deuterium gas in the presence of 0.5 or 1.0 eq. (two experiments) of Ir[(COD)(Cy_3P)pyr]PF_6 in methylene chloride

Lilly Research Laboratories, A Division of Eli Lilly and Company, Indianapolis, IN 46285, USA

**Correspondence to: William J. Wheeler, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA.*
E-mail: isotopicsolutions@comcast.net

(Crabtree's catalyst) yielded only unreacted starting material (no deuterium incorporation).

Several alternative methods were evaluated for the use in the tritiation of **1**. Most certainly, **2-[^{3}H]** could be synthesized using chemistry described by Faul *et al*.; however, this would require the alkylation of a suitable indole precursor with CT_3I, followed by several subsequent radioactive steps.[7] Since both **1-[^{3}H]** and **2-[^{3}H]** were required, the most efficient path forward would be to synthesize **1-[^{3}H]** from **2-[^{3}H]**. Pursuant to this option, reaction of **2** with the HBr salt of 2-bromo-methylpyridine in the presence of Hunig's base yielded **1** in 42% yield (Scheme 1). With the means of synthesizing **1** from **2** in hand, the remaining task became the synthesis of **2-[^{3}H]**.

The most straightforward scenario for the synthesis of **2-[^{3}H]** would entail the alkylation of **3** (or an analog of **3**) with CT_3I. Not surprisingly (maleimide pK_a (DMSO) = 10.0 vs indole = 21.0), alkylation of **3** yielded a mixture of regioisomers **4** and **5** (Scheme 2). Using careful silica gel chromatography, the mixture of **4** and **5** was separated. Had the alkylation of **3** yielded even a small amount of material methylated only on the indole nitrogen, it would have provided a means for the synthesis of **2-[^{3}H]**, albeit not the most ideal solution. Based on the results shown in Scheme 2, methylation of **5** with CT_3I might be useful if **4-[^{3}H]** could be easily converted to **2-[^{3}H]**. In the synthesis of ruboxistaurin, another PKC inhibitor, previously under clinical trial for the treatment of diabetic retinopathy, the *N*-methyl maleimide (**6**) was converted to the unsubstituted maleimide (**8**) via the corresponding anhydride (**7**) as shown in Scheme 3.[10]

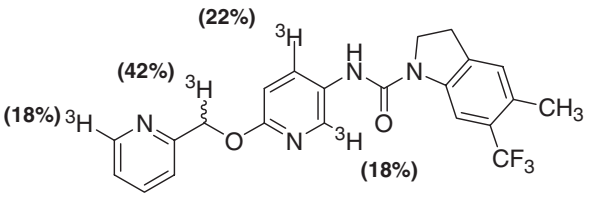
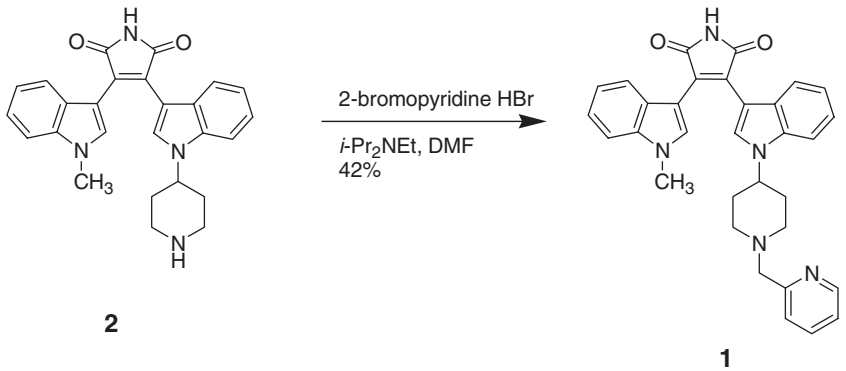


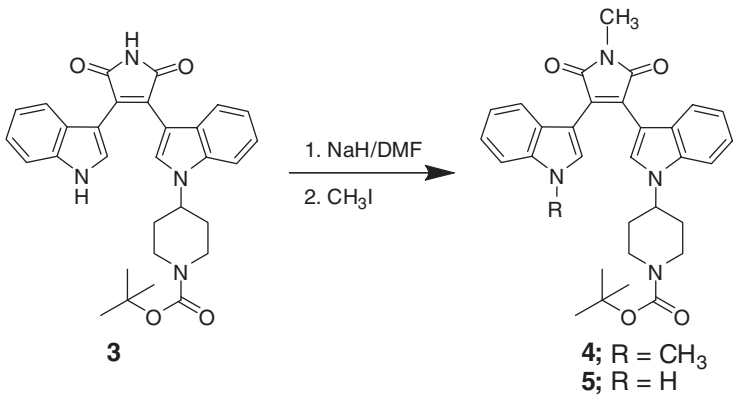
Figure 1. Tritium incorporation resulting from overnight treatment of 1 mg of the substrate (and 1 mg of two other similar compounds) with 3.13 eq. of Crabtree's catalyst/4.6 Ci of tritium in methylene chloride.

Upon further consideration, it seemed probable that starting from the anhydride obtained from **3** (or **5**) would avoid an unnecessary step, thus affording **2-[^{3}H]** in only three steps from readily available starting material. Hydrolysis of the maleimides to ring-opened maleic acid was sluggish (in spite of reports that competitive hydrolysis to the anhydride was observed in the synthesis of **1** when a basic pH was not minimized in the work-up[7]) and never went to completion as was previously shown in the hydrolysis of **6**. Overnight reaction of **3** and **5** with excess potassium hydroxide (10% aqueous) in refluxing dioxane failed to go to completion; the reaction mixture was extracted with EtOAc (to remove unreacted imide) and the aqueous layer was acidified to yield **10** in 36 and 47%, respectively (Scheme 4). Treatment of **9** (*t*- BOC analog of **2**) in the same manner gave **11** in 36% yield.

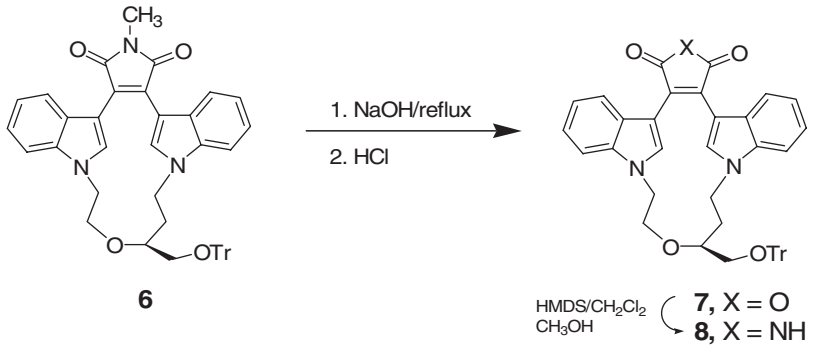
Alkylation of **10** with methyl iodide followed by chromatography on silica gel yielded **11** in 54% yield (Scheme 5). Reaction



Scheme 1



Scheme 2



Scheme 3

Scheme 4

	R	R′	R″		R	R′
3	*t*-BOC	H	H	**10**	*t*-BOC	H
5	*t*-BOC	H	CH_3			
9	*t*-BOC	CH_3	H	**11**	*t*-BOC	CH_3

of **11** with hexamethyldisilazide in DMF/CH_3OH yielded **9** in 95% yield (Scheme 5). Reaction of **9** with 12 N HCl in refluxing CH_3OH yielded **2** in 57% yield. Reaction of the sodium salt (prepared by reaction with NaH/THF) of **10** with [^{3}H]-methyl nosylate yielded **11-[^{3}H]** which was converted, after purification by HPLC, to **9-[^{3}H]** by reaction with HMDS/DMF/CH_3OH.[†] De-protection of **9-[^{3}H]** by reaction with 9:2 TFA/water followed by HPLC purification yielded **2-[^{3}H]** with a specific activity of 80 Ci/mmol (the radiochemical purity (RCP) was 97.4%).

Reaction of **2-[^{3}H]** with 2-bromomethylpyridine in DMF/Hunig's base followed by HPLC purification (Scheme 6) yielded **1-[^{3}H]** with a specific activity of 82 Ci/mmol (the radiochemical purity was 98.2%).

Experimental

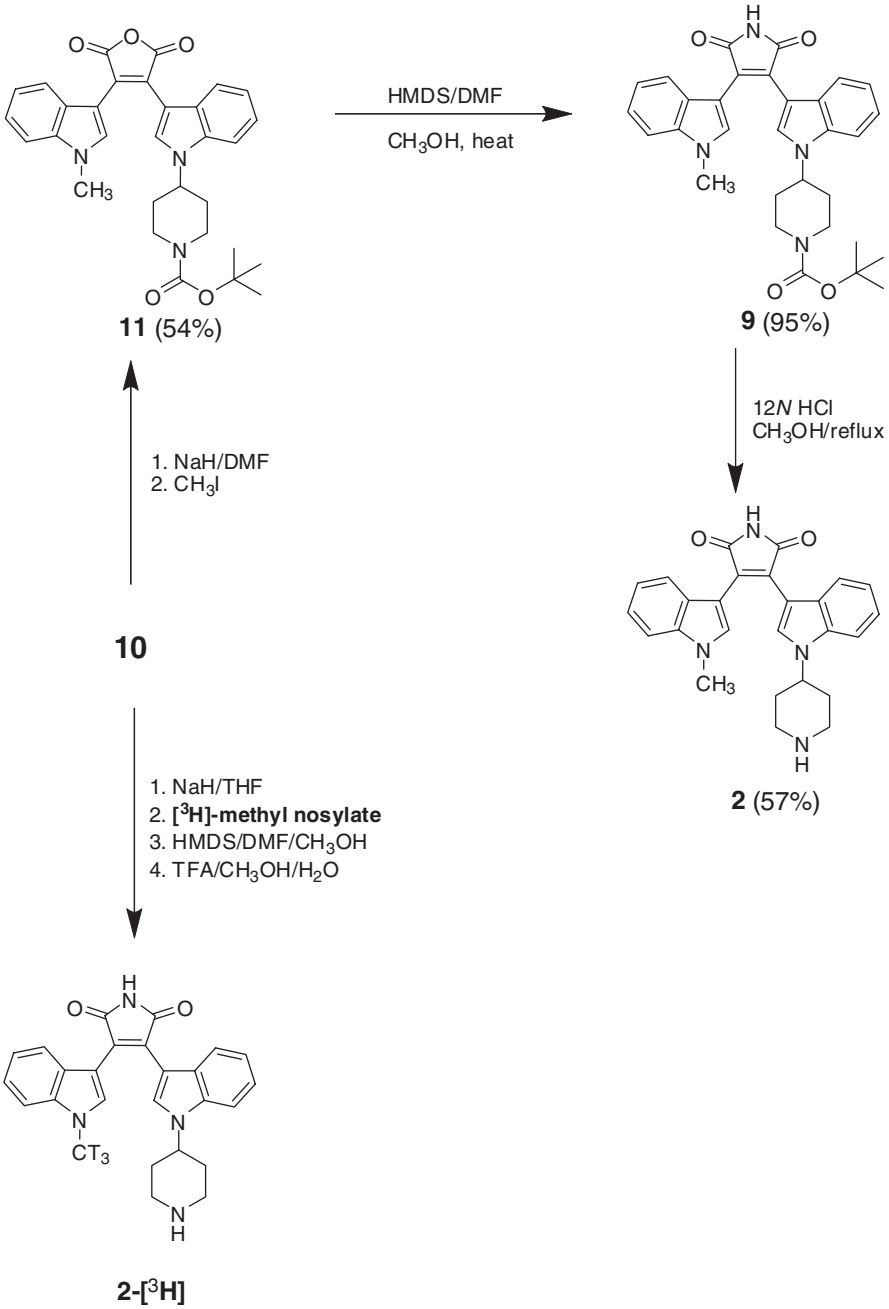
NMR spectra were obtained on a Varian Mercury-400 or Violin-Inova-500 spectrometer at 400 or 500 (^{1}H) and 100 (^{13}C) MHz (numbering system for the NMR's are shown in structure **10**). Peak assignments were made using gradient selected COSY (gCOSY), HSQC and HMQC experiments. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. ES-MS spectra were run on a Waters Micromass ZQ single quadrapole mass spectrometer. Exact masses were determined on a Premier Micromass High Resolution Q-TOF mass spectrometer coupled with Acquity UPLC. Flash chromatography was performed on a Biotage system using silica gel cartridges. Thin layer chromatography was conducted on EMD Chemicals, Inc., silica gel 60 F_{254} plates (5 × 10 cm, 250 μm). The RCP was assessed by radio-HPLC.

Synthesis of 3-(1-methyl-1*H*-indol-3-yl)-4-[1-[1-(2-pyridinylmethyl)-4-piperidinyl]-1*H*-indol-3-yl]-1*H*-pyrrole-2,5-dione, 1

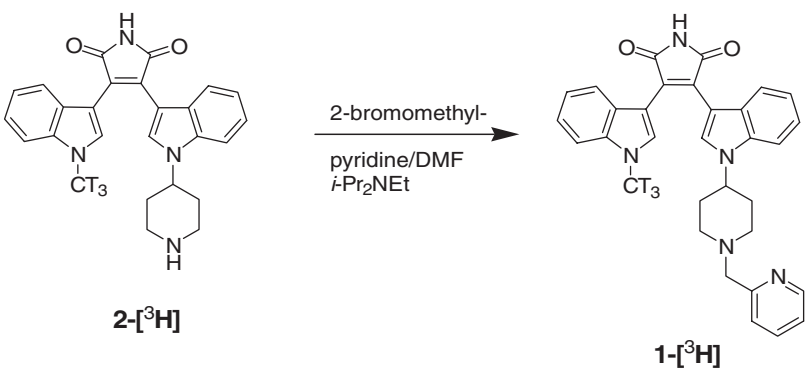
A DMF (2 mL) solution of **2** (10.0 mg, 24 μmol) was treated with 2-bromomethylpyridine hydrobromide (12.0 mg, 48 μmol) and Hunig's base (9.0 mg, 13.0 μL, 72 μmol), and the reaction mixture was stirred at room temperature overnight. Thin layer chromatography (silica gel, CH_2Cl_2/CH_3OH/NH_4OH, 90:10:1) showed loss of **2** and the formation of **1**. The reaction mixture was poured into a mixture of CH_2Cl_2 and saturated sodium bicarbonate. The aqueous layer was extracted with CH_2Cl_2 (2 × 15 mL) and the combined organic layers were washed with saturated brine, dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The red residue was purified by Biotage chromatography on silica gel, eluting with 5 mL fractions of CH_2Cl_2/CH_3OH/NH_4OH (90:10:1). Fractions 5–8 were combined and concentrated to yield **1** (5.4 mg, 45%)

ES-MS: $[M+H]^+$ *m/z* = 516; $[M+Na]^+$ *m/z* = 538. This material was identical in all respects with authentic material.[7] HPLC:

[†]The synthesis and purification of **1** and **2-[^{3}H]** was conducted by Amersham International Plc, Whitchurch, Cardiff, Wales, UK, using methods supplied by the authors.



Scheme 5



Scheme 6

Zorbax SB-C8 (4.6 × 250 mm, 5 μm) at 1 mL/min at ambient temperature with UV detection at 220 nm and gradient elution (Solvent A: 10% CH_3CN/90% H_2O/0.5% H_3PO_4, v/v; Solvent B: 90% CH_3CN/10% H_2O/0.5% H_3PO_4, v/v): 0–5 min: 10% B; 5–35 min: 70% B; 35–45 min: 70% B; 45–46 min: 10% B; 46–60 min: 10% B. The sample was dissolved in 40% CH_3CN/60% H_2O/0.5% H_3PO_4 (v/v) and the injection volume was 20 μL. **1**, R_T = 22.25 min and **2**, R_T = 20.15 min.

Synthesis of 4-[3-[4-(1*H*-indol-3-yl)-1-methyl-2,5-dioxo-1*H*-pyrrol-3-yl]-indol-1-yl]]-piperidine-1-carboxylic acid, *tert*.-butyl ester, 5

A solution of **3** (1.0 g, 1.95 mmol) was dissolved in anhydrous DMF (30 mL) and stirred under argon. The mixture was treated with a 60% mineral oil dispersion of sodium hydride (0.066 g, 1.65 mmol); the color deepened and stirring was continued for 0.5 h. Methyl iodide (0.426 g, 0.187 mL, 3.0 mmol) was added, the solution cleared to ruby red and stirring was continued for 1 h. TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) showed loss of **3**. The reaction mixture was poured into saturated NH_4Cl and extracted with ethyl acetate. The aqueous layer was extracted with ethyl acetate; the combined organic layers were washed with saturated brine, dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The oily residue was re-dissolved in acetonitrile and washed with hexanes (to remove the mineral oil). The acetonitrile was removed *in vacuo*. ES-MS of the residue showed peaks at 525 ($[M+H]^+$ of **5**) as well as 542 ($[M+NH_4]^+$ of **5**) and 556 ($[M+NH_4]^+$ of **4**). The residue was purified by silica gel chromatography, eluting with CH_2Cl_2; fractions 78–90 were a mixture of **4** and **5** and were re-chromatographed eluting with pentane/Et_2O. Fractions 81–160 were combined, concentrated and crystallized from Et_2O to yield **5** (0.292 g, 29%) as red needles.

ES-MS: ($[M+H]^+$, *m/z* = 525; $[M+NH_4]^+$ *m/z* = 542; ^{1}H NMR ($CDCl_3$/DMSO/d_6): δ 1.51 (s, 9H, OC(C**H**$_3$)$_3$), 1.77 (dd, *J* = 3.9 and 12.7 Hz, 1H, 25 or 29 ax-H), 1.82 (dd, *J* = 3.9 and 12.7 Hz, 1H, 25 or 29 ax-H), 2.05 (d, *J* = 12.7 Hz, 2H, 25 and 29 eq-H), 2.91 (t, *J* = 12.6 Hz, 2H, 26 and 28 ax-H), 3.22 (s, 3H, imide-Me), 4.30 (m, 2H, 26 and 28 eq-H), 4.37 (tt, *J* = 3.9 and 11.8 Hz, 1H 24 ax-H), 6.76 (d, *J* = 7.4 Hz, 1H, 5-H), 6.87 (d, *J* = 7.4 Hz, 1H, 6-H), 6.88 (t, *J* = 7.4, 1H, 18-H), 7.10 (t, *J* = 7.4 Hz, 1H, 4-H), 7.16 (t, *J* = 7.4, 1H, 19-H), 7.22 (d, *J* = 8.3 Hz, 1H, 17-H), 7.36 (d, *J* = 7.9 Hz, 1H, 3-H), 7.37 (d, *J* = 7.9 Hz, 1H, 20-H), 7.61 (s, 1H, 23-H), 7.80(d, *J* = 3.1 Hz, 1H, 9-H) and 8.60 ppm (bs, 1H, N**H**); ^{13}C NMR ($CDCl_3$/DMSO/d_6): δ 24.11 (*N*–C), 28.41 (C(CH_3)$_3$), 32.12 (25 and 29-C), 43.21 (26 and 28-C), 53.78 (24-C), 80.03 (Me_3C), 106.39 (15-C), 107.27 (8-C), 109.28 (3-C), 111.17 (20-C), 120.31 (5-C), 120.45 (18-C), 122.06 (6-C), 122.17 (19-C), 122.57 (17-C), 126.35 (16-C), 127.06 (14-C), 127.83 (10-C), 128.14 (23-C), 128.24 (9-C), 135.70 (21-C), 135.88 (2-C), 154.51 (CO-*t*-Bu) and 172.38 (11 and 13-C).

HRMS: $[M-H]^+$, *m/z* calculated for $C_{31}H_{31}N_4O_4$ 523.2345, found 523.2359.

Fractions 41–60 were concentrated *in vacuo* and crystallized from Et_2O/heptane to yield **4** (4-[3-[1-methyl-4-(1-methyl-1*H*-indol-3-yl)-2,5-dihydro-1*H*-pyrrol-3-yl]indol-1-yl] piperidine-1-carboxylic acid *tert*.-butyl ester) as a red solid (0.045 g, 4%).

ES-MS: $[M+NH_4]^+$ *m/z* = 556; ^{1}H NMR (DMSO/d_6): δ 1.39 (s, 9H, OC(C**H**$_3$)$_3$), 1.59 (dd, *J* = 3.6 and 11.7 Hz, 1H, 25 or 29 ax-H), 1.65 (dd, *J* = 3.6 and 11.7 Hz, 1H, 25 or 29 ax-H), 1.87 (d, *J* = 12.6 Hz, 2H, 25 and 29eq-H), 2.84 (m, 2H, 26 and 28 ax-H), 3.03 (s, 3H, imide-Me), 3.86 (s, 3H, indole-Me), 3.89–4.16 (m, 2H, 26 and 28 eq-H), 4.61 (tt, *J* = 3.5 and 12.2, 1H, 24 ax-H), 6.52 (d, *J* = 8.1 Hz, 1H, 6-H), 6.62 (t, *J* = 8.1 Hz, 1H, 5-H), 6.80 (t, *J* = 8.1 Hz, 1H, 18-H), 7.04 (t, *J* = 8.1 Hz, 1H, 4-H), 7.06 (t, *J* = 8.3, 1H, 19-H), 7.11 (d, *J* = 7.3 Hz, 1H, 17-H), 7.42 (d, *J* = 8.1 Hz, 1H, 3-H), 7.59 (d, *J* = 8.1 Hz, 1H, 20-H), 7.63 (s, 1H, 23-H) and 7.89(s, 1H, 9-H).

HRMS: $[M+H]^+$; *m/z* calculated for $C_{32}H_{35}N_4O_4$ 539.2658, found 539.2637.

Synthesis of 4-[3-4-(1*H*-indol-3-yl)-2,5-dioxo-2,5-dihydrofuran-3-yl]indol-1-yl]-piperidine-1-carboxylic acid, *tert*.-butyl ester, 10

A dioxane solution (5 mL) of **5** (0.250 g, 0.486 mmol) was treated with 10% potassium hydroxide (10 mL) and stirred at reflux overnight. The initial deep red color of the solution gradually lightened to a pale red. The reaction mixture was poured into water and extracted with EtOAc (5 × 25 mL); the aqueous layer was acidified to pH 2.4 with HCl (5 N) and then extracted with EtOAc (5 × 40 mL). The combined organic extracts were washed with saturated brine, dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to yield **10** (0.114 g, 46%). This material was a single spot on TLC (silica gel, 7:3 Et_2O/heptane), R_f = 0.35. This material was mixed with some less pure material (0.052 g) from a previous run and purified by silica gel chromatography, eluting with 20 mL fractions of 7:3 Et_2O/heptane; fractions 16–25 were concentrated *in vacuo* (0.101 g) and recrystallized from Et_2O to yield **10** as a red crystalline solid (0.078 g).

^{1}H NMR ($CDCl_3$/DMSO/d_6): δ 1.34 (s, 9H, OC(C**H**$_3$)$_3$), 1.56 (dd, *J* = 3.8 and 11.5, 1H, 25 or 29 ax-H), 1.61 (dd, *J* = 3.8 and 11.5, 1H, 25 or 29 ax-H), 1.89 (d, *J* = 11.5 Hz, 2H, 25 and 29eq-H), 2.77 (t, *J* = 12.3 Hz, 2H, 26 and 28 ax-H), 4.12 (m, 2H, 26 and 28 ax), 4.26 (tt, *J* = 3.2 and 8.3 Hz, 1H, 24 ax-H), 6.59 (d, *J* = 6.8 Hz, 1H, 6-H), 6.61 (t, *J* = 7.7 Hz, 1H, 5-H), 6.78 (t, *J* = 7.9 Hz, 1H, 18-H), 6.94 (ddd, *J* = 1.8, 6.1 and 7.9 Hz, 1H, 4-H), 7.04 (t, *J* = 7.5 Hz, 1H, 19-H), 7.13 (d, *J* = 8.1 Hz, 1H, 17-H), 7.27 (d, *J* = 8.1 Hz, 1H, 20-H), 7.28 (d, *J* = 7.1 Hz, 1H, 3-H), 7.44 (s, 1H, 23-H), 7.75 (d, *J* = 3.3 Hz, 1H, 9-H) and 10.78 ppm (bs, 1H, N**H**); ^{13}C NMR ($CDCl_3$/DMSO/d_6): δ 28.33 (C(CH_3)$_3$), 31.94 (25 and 29-C), 43.16 (26 and 28-C), 53.90 (24-C), 79.96 (Me_3C), 105.33 (8-C), 105.41 (15-C), 109.66 (20-C), 112.05 (3-C), 120.21 (6-C), 120.78 (18-C), 121.83 (5-C), 122.40 (4-C), 122.38 (17-C), 122.50 (19-C), 124.15 (7-C), 126.77 (16-C), 127.66 (14-C), 128.84 (10-C), 128.97 (23-C), 134.20 (9-C), 135.62 (21-C), 136.52 (2-C), 154.27 (CO-*t*-Bu) 166.54 (11 or 13-C) and 166.66 ppm (11 or 13-C); ES-MS: $[M+NH_4]^+$, *m/z* = 529.

HRMS: $[M-H]^+$; *m/z* calculated for $C_{30}H_{28}N_3O_5$ 510.2029, found 510.2005.

Synthesis of 4-[3-4-(1*H*-indol-3-yl)-2,5-dioxo-2,5-dihydrofuran-3-yl]indol-1-yl]-piperidine-1-carboxylic acid, *tert*.-butyl ester, 10

A dioxane solution (10 mL) of **3** (0.250 g, 0.490 mmol) was treated with 10% potassium hydroxide (10 mL) and stirred at reflux overnight. The work-up of the reaction mixture as described above yielded **10** (0.091 g, 36%) as a red crystalline solid. TLC (silica gel, 7:3 Et_2O/heptane) showed a single spot at R_f = 0.35, which co-eluted with the material prepared from **5** as described above.

NMR ($CDCl_3$): superimposable with the spectrum obtained from **10** prepared from **5**; ES-MS: $[M+Na]^+$, *m/z* = 534.

Synthesis of 4-[3-[4-(1-methyl-1*H*-indol-3-yl)-2,5-dioxo-2,5-dihydrofuran-3-yl]indol-1-yl]piperidine-1-carboxylic acid, *tert*.-butyl ester, 11

A dioxane solution (10 mL) of **9** (0.250 g, 0.476 mmol) was treated with was treated with 10% potassium hydroxide (10 mL) and stirred at reflux overnight. The work-up of the reaction mixture as described above, yielded **11** (60 mg, 24%) as a red crystalline solid. TLC (silica gel, 7:3 Et_2O/heptane) showed a single spot at $R_f = 0.49$.

ES-MS showed a $[M+NH_4]^+$ at $m/z = 543$; ^1H NMR ($CDCl_3$): δ 1.48 (s, 9H, OC($\mathbf{H}_3$)$_3$), 1.72 (dd, $J = 4.1$, 12.4 Hz, 1H, 25 or 29 ax-H), 1.78 (dd, $J = 4.1$, 12.4 Hz, 1H, 25 or 29 ax-H), 2.03 (d, $J = 12.4$ Hz, 2H, 25 and 29 eq-H), 2.89 (t, $J = 13.2$ Hz, 2H, 26 and 28 ax-H), 3.88 (s, 3H, indole-Me), 4.29 (d, $J = 11.3$, 2H, 26 and 28 eq-H), 4.38 (tt, $J = 3.9$ and 11.8 Hz, 1H 24 ax-H), 6.73 (dd, $J = 1.6$ and 7.9 Hz, 1H, 6-H), 6.76 (td, $J = 0.9$ and 7.9 Hz, 1H, 5-H), 6.93 (ddd, $J = 0.9$, 2.1 and 7.9 Hz, 1H, 18-H), 7.15 (ddd, $J = 1.7$, 6.6 and 8.2, 1H, 4-H), 7.19 (ddd, $J = 1.0$, 7.2 and 8.2, 1H, 19-H), 7.28 (d, $J = 8.2$ Hz, 1H, 17-H), 7.32 (d, $J = 8.5$ Hz, 1H, 20-H), 7.38 (d, $J = 8.2$ Hz, 1H, 3-H), 7.60 (s, 1H, 23-H) and 7.83 (s, 1H, 9-H); ^{13}C NMR ($CDCl_3$): δ 28.41 (C(CH_3)$_3$), 32.47 (indole-CH_3), 32.49 (25 and 29-C), 43.16 (26 and 28-C), 53.96 (24-C), 80.14 (Me_3C), 104.96 (8-C), 105.73 (15-C), 109.67 (C-20), 109.87 (3-C), 120.61 (5-C), 120.96 (18-C), 122.51 (6-C), 122.70 (4-C, 17-C, 19-C), 125.01 (7-C), 126.12 (16-C), 127.44 (14-C), 128.10 (10-C), 128.97 (23-C), 134.20 (9-C), 135.70 (21-C), 137.1 (2-C), 154.6 (CO-*t*-Bu), 166.62 (11 or 13-C) and 166.92 (11 or 13-C).

HRMS: $[M+H]^+$; *m/z* calculated for $C_{31}H_{32}N_3O_5$ 526.2342, found 526.2347.

Synthesis of 4-[3-[4-(1-methyl-1*H*-indol-3-yl)-2,5-dioxo-2,5-dihydrofuran-3-yl]indol-1-yl]piperidine-1-carboxylic acid, *tert*.-butyl ester, 11

A mixture of **10** (38 mg, 74 μmol) and sodium hydride (60% mineral oil dispersion, 1.1 eq., 3.2 mg, 81 μmol) was dissolved in anhydrous THF (10 mL (under argon) and stirred for 0.5 h. To the resulting solution was added a THF (2 mL) solution of methyl iodide (1.1 eq., 12 mg, 5 μL, 81 μmol). The resulting solution was stirred overnight at room temperature. The mixture was acidified with HCl (1 N) and diluted with EtOAc/water. The organic layer was washed with saturated brine, dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The residue was triturated with heptane to remove the mineral oil; the heptane was extracted with CH_3CN. The CH_3CN extract was added the original residue and concentrated *in vacuo*. TLC (silica gel, 7:3 Et_2O/heptane) of the residual product showed a major spot running slightly ahead of the starting material as well as two very minor impurities. The red semi-solid was purified by Biotage on silica gel, eluting with 5 mL fractions of Et_2O/heptane (7:3). Fractions 12–28 were concentrated to yield **11** (21 mg, 54%).

NMR ($CDCl_3$): superimposable with the spectrum obtained from **11** prepared from **9**; ES-MS: $[M+Na]^+$, $m/z = 548$.

Synthesis of 4-[3-[4-(1-methyl-[$^3H_3$]-1*H*-indol-3-yl)-2,5-dihydrofuran-3-yl]indol-1-yl]piperidine-1-carboxylic acid *tert*.-butyl ester (11-[^{3}H])

Sodium hydride (1 mg) was added to a THF solution of **10** (5 mg, 9.77 μmol); the resulting solution was stirred for 5 min at room temperature and then added to methyl-[$^3H_3$] nosylate (800 mCi, 10 μmol) and stirred overnight. The crude reaction mixture was purified directly on an Ultrasphere ODS column (10 × 250 mm) eluting with a CH_3OH/water/triethylamine gradient system to yield **11-[^{3}H]**.

Synthesis of 4-[3-[4-(1-methyl-1*H*-indol-3-yl)-2,5-dihydro-1*H*-pyrrolo-3-yl]indol-1-yl]piperidine-1-carboxylic acid *tert*.-butyl ester (9)

A DMF (2 mL) of **11** (20 mg, 38 μmol) was blanketed with argon and treated with hexamethyldisilazane (100 μL, 0.480 mmol), followed by the addition of CH_3OH (10 μL). The resulting mixture was stirred at 50–55°C overnight. An aliquot of the reaction mixture was treated with EtOAc/H_2O, TLC (7:3 Et_2O/heptane), and showed a spot to spot conversion to the desired product. The remainder of the reaction mixture was allowed to cool to room temperature and was concentrated *in vacuo*. The residue was dissolved in EtOAc and was washed with water. The EtOAc solution was washed with brine, dried (anhydrous $MgSO_4$) and concentrated to yield **9** (19 mg, 95%); ES-MS: $[M+H]^+$, $m/z = 525$, $[M+NH_4]^+$, $m/z = 542$.

Synthesis of 3-(1-methyl-1*H*-indol-3-yl)-4-(1-piperidin-4-yl-1*H*-indol-3-yl)-pyrrole-2,5-dione (2)

A CH_3OH solution (2 mL) of **9** (19 mg, 36 μmol) was treated with HCl (12 N, 4 μL, 38 μmol) and stirred at reflux for 1 h. Stirring at room temperature was continued overnight. TLC (silica gel, 7:3 Et_2O/heptane) showed some unreacted **9** remained. The mixture was heated to reflux and stirred an additional 2 h; TLC showed complete loss of **9**. TLC (silica gel, CH_2Cl_2/CH_3OH/NH_4OH, 90:10:1) showed material which co-eluted with an authentic sample of **2**.[7] The reaction mixture was diluted with EtOAc and was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to yield **2** (10 mg, 57%). This material was identical in all respects to authentic **2**, prepared as described by Faul *et al*.[7]

Synthesis of 3-(1-methyl-[$^3H_3$]-1*H*-indol-3-yl)-4-(1-piperidin-4-yl-1*H*-indol-3-yl)-pyrrole-2,5-dione (2-[$^3H_3$])

A DMF solution (1 mL) of **11-[^{3}H]** was treated with hexamethyldisilazane (100 μL, 0.480 mmol) and CH_3OH (10 μL) and heated at 50°C for 1 h and then stirred at room temperature overnight. The crude reaction mixture was concentrated *in vacuo* to yield **9-[^{3}H]**. The residue was re-dissolved in 9:1 TFA/H_2O (2 mL) and stirred at room temperature for 1 h and then evaporated to dryness. The crude product was purified on an Ultrasphere ODS column (10 × 250 mm) eluting with a water/acetonitrile/TFA gradient system to yield **2-[^{3}H]** (65 mCi, 8.1% overall for the three steps). The specific activity (as determined by mass spectrometry) was 80 Ci/mmol; the RCP was 97.4%.

Synthesis of 3-(1-methyl-[$^3H_3$]-1*H*-indol-3-yl)-4-(1-piperidin-4-yl-1*H*-indol-3-yl)-pyrrole-2,5-dione (1-[^{3}H])

A DMF solution (0.5 mL) of **2-[^{3}H]** (55 mCi, 0.69 μmol) was treated with 2-bromomethylpyridine (0.47 g, 27 μmol) and di-*iso*-propyl-ethylamine (10 μL, 57 μmol) and stirred at room temperature for 3 h. The reaction mixture was concentrated *in vacuo* and purified by HPLC chromatography on a YMC Pack C8 column (4.6 × 250 mm), eluting with a water/acetonitrile/TFA gradient system to yield **1-[^{3}H]** (23 mCi, 42%). The specific activity (as determined by mass spectrometry) was 82 Ci/mmol; the RCP was 98.2%.

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