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Synthesis and Characterization of the Selective, Reversible PKC_{β} Inhibitor (9*S*)-9-[(Dimethylamino)methyl]-6,7,10,11-tetrahydro-9*H*,18*H*-5,21:12,17-dimethenodibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione, Ruboxistaurin (LY333531)

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ABSTRACT

The demonstrated role of PKC_{β} in the mediating amphetamine-stimulated dopamine efflux, which regulates amphetamine-induced dopamine transporter trafficking and activity, has promoted the research use of the selective, reversible PKC_{β} inhibitor (9*S*)-9-[(dimethylamino)methyl]-6,7,10,11-tetrahydro-9*H*,18*H*-5,21:12,17-dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione, ruboxistaurin. Despite the interest in development of ruboxistaurin, as the mesylate monohydrate (Arxxant), for the treatment of diabetic retinopathy, macular oedema, and nephoropathy, several crucial details in physicochemical characterization were erroneous or missing. This report describes the synthesis and full characterization of ruboxistaurin free base (as a monohydrate), including *X*-ray crystallography to confirm the absolute configuration, and of the mesylate salt, isolated as a hydrate containing 1.5 moles of water per mole.

KEYWORDS: Addiction, Parkinson's disease, attention deficit disorder, schizophrenia, psychostimulants, Arxxant, ruboxistaurin

1. INTRODUCTION

The dopamine transporter (DAT), which has been implicated in neurodegenerative and psychiatric disorders such as Parkinson's disease,[1, 2] attention deficit/hyperactivity disorder,[2] and schizophrenia,[3] has been identified as a primary target of psychostimulants such as amphetamine, and is responsible for amphetamine addiction.[4] DAT trafficking and activity are regulated by a complicated protein network involving multiple protein kinases and DAT-interacting proteins[5] including protein kinase C (PKC). In particular, it has been shown that amphetamine-stimulated

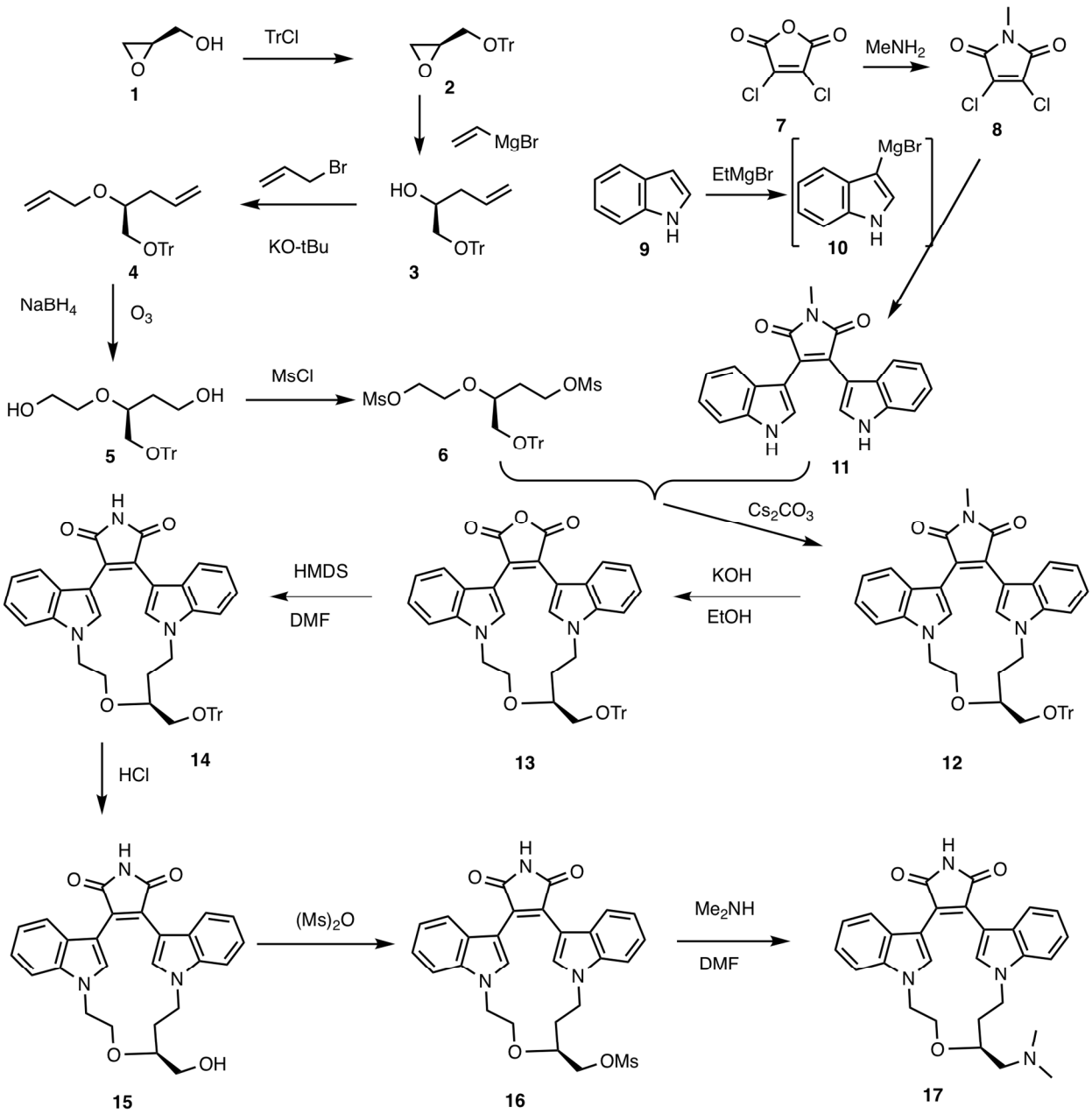
dopamine efflux is mediated by PKC_{β},[6] which regulates amphetamine-induced DAT trafficking and activity.[4] Due to the important role of PKC_{β}, the preparation of (9*S*)-9-[(dimethylamino)methyl]-6,7,10,11-tetrahydro- 9*H*,18*H*-5,21:12,17-dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione, ruboxistaurin (LY333531, **17** in Scheme 1), a selective, reversible inhibitor of PKC_{β}, was carried out. Since the properties of the product isolated by our Laboratory were somewhat different than those reported, the structure was confirmed by *X*-ray crystallography. Additionally, the mesylate salt (Arxxant) was prepared and characterized. Herein we report our results.

2. RESULTS AND DISCUSSION

The synthesis of ruboxistaurin (**17** in Scheme 1), as the hydrochloride salt, was first described in 1996[7] in a publication in which its cellular activity was compared to that of staurosporin. A follow-up publication from the same laboratory provided more detailed synthetic information and described the monohydrate of ruboxistaurin;[8] a patent in 2000[9] described several salts and formulations, including a mesylate salt that was described in greater detail in the chemical literature.[10] Our synthesis of ruboxistaurin (**17**) was carried out following the patent literature[9] (Scheme 1) with most of the intermediates being characterized by ^{1}H NMR analysis. Triphenhylmethyl protection of the hydroxyl functionality of commercially procured (2*R*)-2-oxiranemethanol (**1**) with triphenhylmethyl chloride, followed by reaction of the resulting *(2S*)-2-[(triphenylmethoxy)methyl]oxirane (**2**) with vinylmagnesium bromide gave (2S)1-triphenylmethoxy-4-penten-2-ol (**3**), which was O-alkylated with allyl bromide to give 1,1',1''-[[[(2*S*)-2-(2-propen-1-yloxy)-4-penten-1-yl]oxy]methylidyne]trisbenzene (**4**). Ozonolysis of **4**, followed by reduction with sodium borohydride, afforded (3*S*)-3-(2-hydroxyethoxy)-4-(triphenylmethoxy)-1-butanol (**5**) that was converted to the corresponding bismesylate **6** by

treatment with methylsulfonyl chloride. Cesium carbonate-mediated coupling of **6** with 3,4-di-1*H*-indol-3-yl-1-methyl-1*H*-pyrrole-2,5-dione (**11**) that was prepared by converting 3,4-dichloro-2,5-furandione (**7**) to the corresponding *N*-methyl imide **8** by treatment with methylamine to give **8,** followed by reaction of **8** with the Grignard reagent **10**, prepared by reaction of 1*H*-indole (**9**)

Scheme 1. Synthesis of Ruboxistaurin (17)



with ethylmagnesium bromide, gave (9*S*)-6,7,10,11-tetrahydro-19-methyl-9-[(triphenylmethoxy)methyl]-9*H*,18*H*-5,21:12,17- dimethenodibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione (**12**). Saponification of **12** using ethanolic potassium hydroxide to give **13**, followed by amination with hexamethyldisilazane gave the corresponding imide analog **14** that was O-deprotected by refluxing in a mixture of 6 N hydrochloric acid and ethanol to give (9*S*)-6,7,10,11-tetrahydro-9-(hydroxymethyl)-9*H*, 18*H*-5,21:12,7-dimethenodibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione (**15**). Conversion of the hydroxyl functionality of **15** to the corresponding mesylate **16** by treatment with methylsulfonic anhydride, followed by displacement of the mesyl group by dimethylamine, gave ruboxistaurin (**17**) as a red solid with elemental analysis consistent with the molecular formula $C_{28}H_{28}N_4O_3 \bullet H_2O$ and with ^{1}H NMR spectrum (in deuterated dimethyl sulfoxide) consistent with expectation. Specifically, the total area of the resonances in the aromatic region (δ 7 – 7.5 ppm) corresponded to 10 protons, and resonances in the aliphatic region (δ 1.8 – 4.5 ppm) had a total area corresponding to 17 protons that included a sharp singlet resonance (δ 2.03) with area corresponding to 6 protons, as expected for the *N,N*-dimethylamino group. To our surprise, these ^{1}H NMR data were completely different from the reported ^{1}H NMR spectrum[8] which listed no resonances corresponding to the *N*-methyl groups (expected to be singlet(s) with area corresponding to 6H at δ ~2 ppm) but did report two doublet resonances (δ 7.82 and 7.78), with areas corresponding to 1H each, not seen in our ^{1}H NMR spectrum.

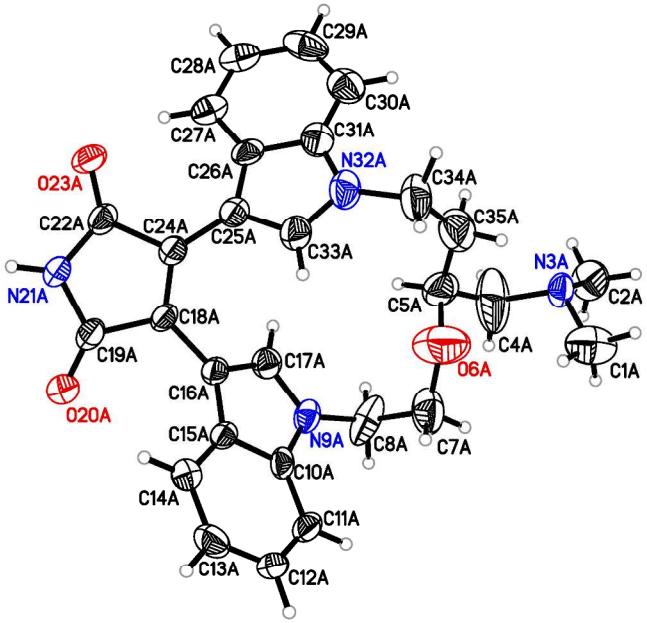
As part of the development of ruboxistaurin for the treatment of diabetic retinopathy, macular oedema, and nephoropathy, the mesylate monohydrate (Arxxant), which is reported to have better aqueous solubility than the corresponding hydrochloride, was identified as the

optimal salt form for clinical studies in a salt selection study.[10] This salt was characterized by polarizing microscopy, hygroscopicity, differential scanning calorimetry (DSC), thermal gravimetric analysis (TGA), and X-ray powder diffraction. [10] Surprisingly, neither the melting point, nor the optical rotation, was reported. Although not explicitly stated, the basis for describing the salt as a monohydrate appears to be the TGA data, which show a weight loss of ca 2.7% between 30–75 °C, consistent with the loss of a mole of water. The DSC data for ruboxistaurin mesylate monohydrate showed a broad endotherm (140–155 °C) that was described as being associated with water loss, followed by a larger endotherm at 260–265 °C, said to represent the melt. [10] The TGA data were interpreted as indicating decomposition of the salt around 270 °C.

Our preparation of the mesylate salt of **17**, which followed the literature,[10] gave a rose-colored solid that, after drying under vacuum for 24 hours, had elemental analysis consistent with a hydrate containing 1.5 moles of water per mole of **17** mesylate, not with the salt being a monohydrate, as reported.[10] This salt had a sharp melting point of 244–245 °C, significantly lower than that reported for the monohydrate (based on DSC), consistent with it being a different hydrate than the reported[10] monohydrate. These results imply that the sample isolated in this study may have crystallized as a 1:1 mixture of a monohydrate and a dihydrate. Engel et al. [10] had reported multiple hydrated forms of the hydrochloride salt of **17**, but not of the mesylate salt; it should be noted, however, that the hygroscopicity determination for ruboxistaurin monohydrate demonstrated absorption of 0.6 moles of water at ca 12% relative humidity (RH) and the desorption isotherm showed ca 0.5 moles of water to remain at 5% RH.[10] Taken together with the results of elemental analysis obtained by us it would appear that without drying at 60 °C[10] ruboxistaurin mesylate contains an average of 1.5 mols of water per mole.

Since neither the melting point nor the optical rotation for the free base **17** was reported,[8] it was decided to confirm the structure and stereochemistry of our synthetic product by *X*-ray crystallography. The red crystals of the free base **17**, obtained from methanol, were used for the analysis (Figure 1). Based on the method of Hooft comparing intensities of Bijovet pairs (69% of the pairs were measured)[11] using Bayesian statistics of the data, there is a small chance that the *X*-ray structure could be a racemic twin. However, this possibility was refuted by the observed optical rotation of a methanolic solution of the mesylate salt of **17**. Examination of the asymmetric center in the *X*-ray structure, showed the absolute stereochemistry to be *S,* in agreement with the absolute configuration of the starting material **1**.

Figure 1. X-Ray Structure of Ruboxistaurin (17).
Displacement ellipsoids are at the 50% level. One of the two molecules in the symmetric unit has been omitted for clarity.



At the suggestion of a reviewer, Eli Lilly was contacted and eventually a copy of a ^{1}H NMR spectrum of their ruboxistaurin (in DMSO-d_6) along with a ^{1}H–^{13}C correlation spectrum was provided. The ^{1}H NMR was identical to that reported in this study.

3. CONCLUSIONS

This study provides thorough characterization of the selective, reversible PKC_{β} inhibitor ruboxistaurin (**17**) and its mesylate salt. The structure of ruboxistaurin, which was prepared following the literature procedure,[8] and was found to exhibit a ^{1}H NMR spectrum distinctly different than reported, was characterized by *X*-ray crystallography. The red solid, which based on elemental analysis was a monohydrate, had the expected structure; examination of the asymmetric center showed the absolute stereochemistry to be *S*, as expected. Further characterization was obtained by interpretation of the ^{1}H NMR spectrum; the optical rotation was also determined. The mesylate salt of ruboxistaurin, reported as a monohydrate, was characterized by us as a hydrate containing 1.5 moles of water per mole of **17** mesylate. Both the optical rotation and the melting point of ruboxistaurin mesylate hydrated with 1.5 moles of water/mole have been determined.

4. METHODS

Instrumentation. ^{1}H NMR spectra were acquired on a Bruker Avance 300 MHz spectrometer, or a 500 MHz NMR spectrometer. Low-resolution LC-MS data were obtained using a PerkinElmer API 150 EX mass spectrometer outfitted with an ESI (turbospray) source, coupled to a PerkinElmer 200 Series liquid chromatography system. Melting points were determined using a MEL-TEMP II capilaty melting point apparatus. Optical rotations were measured on an AutoPol III polarimeter from Rudolph Research. Elemental analyses were carried out by Atlantic Microlab Inc., Norcross, GA.

Materials. Chemicals were reagent-grade, obtained from commercial sources, and used without purification.

Synthesis of ((9*S*)-9-[(Dimethylamino)methyl]-6,7,10,11-tetrahydro-9*H*,18*H*-5,21:12,17-dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione, (Ruboxistaurin, 17) Mesylate

*(2*S*)-2-[(Triphenylmethoxy)methyl]oxirane (**2**).* To a solution of triphenylmethyl chloride (50.7 g, 0.182 mol) in CH_2Cl_2 (125 mL), at 0-5 °C, was slowly added Et_3N (18.4 g, 0.182 mol) followed by (2*R*)-2-oxiranemethanol (**1**) (15.0 g, 0.202 mol). After stirring under N_2, at RT for 24 h the mixture was quenched with sat'd NH_4Cl (150 mL) and H_2O (50 mL). The organic phase was separated, dried (Na_2SO_4) and concentrated. To the residual oil was added EtOH and the solvent was evaporated until solids appeared. The collected solids were recrystalized from *i*-PrOH (180 mL) to afford **2** as a white solid (35.6 g, 62%); m.p. 96.5–97.0 °C, (Lit[12] 94-96)$[\alpha]_D$ = -3.77° (c 0.770, MeOH) (Lit[12] -9.7, 1.0 $CHCl_3$).^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.7 (dAB, 2H, OC**H**$_2$CH), 3.20 (d, 1H, OC**H**), 3.3 (dAB, 2H, CH_2OTr), 7.32–7.37 (m, 15H, TrH)

*(2S)-1-Triphenylmethyoxy-4-petnene-2-ol (**3**).* To a solution of vinylmagnesium bromide in THF (110 mL, 1.0 M, 0.110 mol), at -40 °C, was added CuI (0.590 g, 0.0031 mol). After stirring under N_2, for 15 min, a solution of *(2S)*-2-[(triphenylmethoxy)methyl]oxirane (**2**) (19.42 g, 0.0614 mol) in THF (90 mL) was added. The mixture was stirred at - 40 °C for 1 h, then the bath temperature was allowed to rise to -10 °C and the reaction was quenched with sat'd NH_4Cl (100 mL). EtOAc (150 mL) and H_2O (150 mL) was added and the organic phase was separated, washed with NH_4OH/H_2O (1:1), dried (Na_2SO_4), and concentrated *in vacuo* to afford **3** as a light yellow oil (21.1 g, 100%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.28 (quintuplet, 2H, CHC**H$_2$**CH$_2$),

3.18 (ddAB, 2H, CH_2OTr), 3.85 (m, 1H, OC**H**), 5.10 (dd, 2H, $=CH_2$), 5.9 ((quintuplet, 1H, =CH), 7.26-7.49 (m, 15H, TrH).

*1,1',1''-[[[(2S)-4-Methyl-2-(2-propen-1-yloxy)-4-penten-1-yl]oxy]methylidyne]trisbenzene (**4**)*.To a solution of (2*S*)-1-triphenylmethyoxy-4-petnene-2-ol (**3**) (10.06 g, 0.0292 mol) in THF (40 mL) was added a solution of *t*-BuOK (3.46 g, 0.0308 mol) in THF (40 mL). The reaction mixture was stirred at 45 °C for 45 min, cooled to RT and a solution of allyl bromide (8.83 g, 0.0730 mol) in THF (10 mL) was slowly added. After stirring for an additional 1 h the reaction was quenched with sat'd NH_4Cl and EtOAc was added. The organic phase was separated, dried (Na_2SO_4) and concentrated *in vacuo* to give of an orange oil (10.5 g). The oil was chromatographed on SiO_2, eluting with hexane/EtOAc (9:1), to afford **4** (8.82 g, 79%) as a light yellow oil; $[\alpha]_D$ = -7.63° (c 0.760, MeOH) (Lit[8] -10.07 ° 0.98, MeOH), R_f = 0.63, SiO_2, hexane/EtOAc (9:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.4 (m, 2H, CHC**H$_2$**C**H$_2$**), 3.18 (m, 2H, CH_2OTr), 4.07 (t, 1H, OC**H**), 4.10 (dAB, 2H, OCH_2), 5.04 (m, 4H, $(=CH_2)_2$), 5.8 ((quintuplet, 1H, =CH), 6.0 (quintuplet, 1H, =CH), 7.24–7.53 (m, 15H, TrH).

*(3S)-3-(2-Hydroxyethoxy)-4-(triphenylmethoxy)-1-butanol (**5**)*. To a solution of (*S*)-1-triphenylmethyoxy-[[[2-(2-propenyloxy)-4-pentenyl]oxy]methyne] (**4**) (19.6 g, 0.0509 mol) in MeOH/CH_2Cl_2 (1:1) (120 mL) was added a solution of Sudan red indicator (0.387 g, 0.00102 mol) in CH_2Cl_2/EtOH (3:1) (6 mL). The reaction mixture was cooled to -45 °C and ozone was bubbled through the mixture until a reddish-orange colored solution persisted. This color change usually was seen after 2 h of bubbling ozone into the reaction mixture. Nitrogen was then bubbled into the mixture for 15 min and the solution was then added to an ice-chilled solution of $NaBH_4$ (4.27 g, 0.112 mol) in 0.5 N NaOH (50 mL). The reaction mixture was stirred for 17 h, allowing the bath to come to RT. The mixture was neutralized (pH = 6-7) with 1 N HCl; brine

and EtOAc were then added and the mixture was stirred for 15 min. The organic phase was separated, dried (Na_2SO_4) and concentrated to yield an orange oil (22.0 g) that was purified by column chromatography (SiO_2, EtOAc) to afford **5** as a thick orange oil (12.0 g, 60%).

*(S)-3-(2-Hydroxyethoxy)-4-(triphenylmethoxy)-1-butanol Methanesulfonate (**6**)*. To an ice-chilled solution of (3*S*)-3-(2-hydroxyethoxy)-4-(triphenylmethoxy)-1-butanol (**5**) (14.2 g, 0.0362 mol) in CH_2Cl_2 (200 mL) was added Et_3N (11.3 g, 0.112 mol). After 15 min of stirring, a solution of methanesulfonyl chloride (11.1 g, 0.0969 mol) in CH_2Cl_2 (50 mL) was added and the mixture was stirred at bath temperature for 2 h. The mixture was diluted with CH_2Cl_2 and washed with water and sat'd NH_4Cl. The organic phase was separated, dried (Na_2SO_4) and concentrated to give an orange oil. Addition of mixtures of heptane/EtOAc to the oil and concentration of the solution *in vacuo* (with no heat), resulted in the formation of a solid that was filtered and dried *in vacuo* to afford **6** as a beige solid (16.7 g, 85%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.98 (m, 2H, CHC**H$_2$**CH**$_2$**), 3.03 (s, 3H, S CH_3)), 3.05 (s, 3H, S CH_3)), 3.23 (dd, 2H, CH_2OTr), 3.8 (dAB, 2H, OCH_2), 4.0 (t, 1H, OC**H**), 4.38 (m, 4H, $(CH_2O)_2$), 7.29–7.49 (m, 15H, TrH). **Note:** This product was reacted with **11** within hours of its preparation.

*3,4-Dichloro-1-methylmaleimide (**8**)*. To a solution of 3,4-dichloro2,5-furandione (**7**) (25.0 g, 0.150 mol) and potassium acetate (22.1 g, 0.225 mol) in acetic acid (30 mL) was added methylamine HCl (15.2 g, 0.225 mol) and the mixture was stirred at reflux for 4 h. After cooling to RT the thick mass was slowly poured into ice-chilled 10% $NaHCO_3$ solution. The product was extracted with ether. The combined extract was washed with brine, dried (Na_2SO_4) and concentrated to yield an orange solid (20.2 g) that was purified by column chromatography (SiO_2, hexane/EtOAc (9:1)) to give **8** as a white solid (18.3 g, 68%); mp. 82–83 °C (lit[13] 82-83). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.05 (CH_3).

*3,4-di-1*H-*Indol-3-yl-1-methyl-1*H-*pyrrole-2,5-dione(**11**)*. A solution of indole (**9**) (39.1 g, 0.330 mol) in toluene (200 mL) was added to a solution of EtMgBr (330 mL, 1.0 M, 0.330 mol) in THF, under N_2, at RT. The mixture was heated to 60 °C for 1 h and a solution of 3,4-dichloro-1-methyl-1-*H*-pyrrole-2,5-dione (**8**) (16.2 g, 0.090 mol) in toluene (200 mL) was added. After stirring at 90 °C for 17 h the reaction mixture was cooled to RT and sat'd NH_4Cl (300 mL) was added, followed by water and EtOAc. The organic phase was separated, dried (Na_2SO_4) and concentrated to give a thick red oil. Ether was added and the heterogeneous mixture was stirred for15 min, then filtered to get **11 (**9.6 g). The mother liquor was subjected to column chromatography on SiO_2, eluting with hexane/EtOAc (1:1), to afford additional **11 (**4.5 g) for a total yield of 14.1 g (46%); R_f = 0.48, SiO_2, hexane/EtOAc (1:1), *m/z* for $C_{21}H_{15}N_3O_2$ calcd: 341.12; found: 341. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.03 (s, 3H, CH_3), 6.61 (m, 2H, ArH), 6.78 (d, 2H, ArH), 6.96 (m, 2H, ArH), 7.35, (d, 2H, ArH), 7.73 (d, 2H, (ArH), 11.66 (d, 2H, NH).

*(9S)-6,7,10,11-Tetrahydro-19-methyl-9-[(triphenylmethoxy)methyl]-9H,18H-5,21:12,17-dimethenodibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20(19H)-dione (**12**)*. To a suspension of cesium carbonate (16.9 g, 0.0519 mol) and 3,4-di-1*H*-indol-3-yl-1-methyl-1*H*-pyrrole-2,5-dione (**11**) (8.07 g,0.0236 mol) in DMF (500 mL) at 100 °C was added a solution of (*S*)-3-(2-hydroxyethoxy)-4-(triphenylmethoxy)-1-butanol methanesulfonate (**6**) (16.7 g, 0.0304 mol) in DMF (200 mL). The mixture was stirred at 100 °C for 17 h, cooled to 50 °C and Hyflo (15 g) was added. The resulting mixture was stirred at 50 °C for 15 min then filtered. The filter cake was washed with DMF and the filtrate was concentrated *in vacuo*. To the residue was added CH_3CN and the solution was concentrated. This process was repeated two more times, then a slurry of the solid in CH_3CN was chilled for 2 h. The solids were collected by filtration

and dried to give **12** as a reddish violet solid (12.26 g, 58%) m.p. 205–208 °C, R_f = 0.63, SiO_2, hexane/EtOAc (6.5:3.5).

*(9S)-6,7,10,11-Tetrahydro-9-[(triphenylmethoxy)methyl]-5,21:12,17-dimetheno-9H-dibenzo[e,k]furo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20-dione (**13**)*. A suspension of (9*S*)-6,7,10,11-tetrahydro-19-methyl-9-[(triphenylmethoxy)methyl]-9*H*,18*H*-5,21:12,17-dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione (**12**) (18.37 g, 0.0263 mol) and KOH (14.7 g, 0.263 mol) in EtOH (200 mL) was stirred at gentle reflux for 22 h. The mixture was cooled to RT and treated with CH_2Cl_2 (200 mL) and water (350 mL). The organic phase was separated and treated with 20% citric acid (300 mL). Water was added and the organic phase was separated and dried (Na_2SO_4). Concentration gave a thick oil that was dissolved in EtOH and the volatiles were evaporated This was repeated two more times, then a slurry of the solid residue in EtOH was chilled for 1 h. The solid was collected by filtration, washed with EtOH and dried to give **13** as a purple solid (14.7 g, 82%); mp 223–225 °C, R_f = 0.44, SiO_2, hexane/EtOAc (65:35). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.2 (m, 2H, CHC**H$_2$**CH**$_2$**), 3.14 (d, 2H, CH_2), 3.6 (dAB 2H, CH_2)), 3.8 (m 1H, CH), 4.3 (m, 4H, $(CH_2)_2$), 7.21–7.37 (m, 19H, ArH+TrH), 7.46 (d, 1H, ArH), 7.7.61(d, 1H, ArH), 7.71 (d, 2H, ArH), 7.9 (dd, 2H, ArH).

*(9S)-6,7,10,11-Tetrahydro-9-[(triphenylmethoxy)methyl]-9H,18H-5,21:12,17-dimethenodibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20(19H)-dione (**14**)*. To a solution of (9*S*)-6,7,10,11-tetrahydro-9-[(triphenylmethoxy)methyl]-5,21:12,17-dimetheno-9*H*-dibenzo[*e,k*]furo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20-dione (**13**) (7.90 g, 0.0115 mol) in DMF (75 mL) was added a premixed solution of hexamethyldisilazane (18.6 g, 0.115 mol) in MeOH (2.5 mL). The reaction mixture was heated at 80 °C for 5 h. Analysis by TLC

(SiO_2, hexane/EtOAc (6.5:3.5)) showed no starting material remaining. The reaction mixture was cooled to RT, diluted with CH_2Cl_2 (200 mL) and quenched with 1 N HCl (80 mL). Water was added and the organic phase was separated, dried (Na_2SO_4) and concentrated in vacuo. To the residue was added EtOH and the mixture was re-concentrated. More EtOH was added and the slurry was chilled with ice and allowed to stand for 30 min. The dark mixture was filtered to get **14 (**4.66 g). The mother liquor was subjected to column chromatography (SiO_2, hexane/EtOAc (6.5:3.5)), to give additional **14** (1.82 g). The solids were combined for a total of 6.48 g (82%) of **14** as reddish-violet solid; mp 145–151 °C (softened at 142 °C), $R_f = 0.43$, SiO_2, hexane/EtOAc (6.5:3.5).

*(9S)-6,7,10,11-Tetrahydro-9-(hydroxymethyl)-9H, 18H-5,21:12,7-dimethenodibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20(19H)-dione (**15**)*. A suspension of (9*S*)-6,7,10,11-tetrahydro-9-[(triphenylmethoxy)methyl]-9*H*,18*H*,-5,21:12,17-dimethenodibenzo*[e,k]*pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione (**14**) (6.48 g, 0.00947 mol) in EtOH (70 mL) and 6 N HCl (70 mL) was heated at reflux for 2 h. The mixture was cooled to RT and filtered. The solid cake was washed with water and with a small amount of CH_2Cl_2. The purple solids were dried under high vacuum to yield **15** as a purple solid (3.74 g, 89%); $R_f = 0.50$, SiO_2, EtOAc, mp >260 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.04 (dAB, 2H, CHC**H$_2$**CH**$_2$**), 3.3 (m, 4H, CH_2)$_2$), 3.75 (dAB, 2H, CH_2)), 3.8 (m 1H, CH), 4.0 (dAB, 2H, CH_2), 4.7 (b, 1H, CH), 7.09–7.22 (m, 4H, ArH), 7.35 (m, 4H, ArH), 7.8 1(m, 2H, ArH), 10.82 (s, 2H, NH).

*(9S)-6,7,10,11-Tetrahydro-9-{[(methylsulfonyl)oxy]methyl}-9H,18H-5,21:12,7-dimethenodibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20(19H)-dione (**16**)*. To THF (80 mL) was added (9*S)*-6,7,10,11-tetrahydro-9-(hydroxymethyl)-9*H*, 18*H*-5,21:12,7-

dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione (**15**) (1.08 g, 0.00245 mol), pyridine (0.87 g, 0.0110 mol) and methanesulfonic acid anhydride (1.28 g, 0.00735 mol). After stirring for 90 min at 70 °C the mixture was cooled to RT and brine (75 mL) and 1 N HCl (25 mL) was added. The mixture was extracted with EtOAc and the organic extract was dried (Na_2SO_4) and concentrated *in vacuo* to give a purple solid (1.42 g). The solid was chromatographed (SiO_2, EtOAc/hexane (7:3)) to afford **16** as a purple solid (0.75 g, 59%); R_f = 0.50, SiO_2, EtOAc/hexane (7:3), *m/z* calcd for $C_{27}H_{25}N_3O_6S$: 519.57; found: 519. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.2 (dAB, 2H, CHC**H$_2$**CH$_2$), 3.24 (s, 3H, CH_3), 3.7 (m, 2H, CH_2), 4.0 (m, 1H, CH), 4.26 (m, 4H, $(CH_2)_2$), 4.45 (d, 2H, CH_2), 7.16–7.24 (m, 4H, ArH), 7.53–7.60 (m, 4H, ArH), 7.83–7.90 (m, 2H, ArH), 11.0 (s, 2H, NH).

*(9S)-9-[(Dimethylamino)methyl]-6,7,10,11-tetrahydro-9H,18H-5,21:12,17-dimethenodibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20(19H)-dione, Ruboxistaurin (**17**)*. To a solution of Me_2NH in DMF (30 mL, 5.7 M, 0.171 mol) was added (9*S*)-6,7,10,11-tetrahydro-9-{[(methylsulfonyl)oxy]methyl}-9*H*,18*H*-5,21:12,7-dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-18,20(19*H*)-dione (**16**). (2.67 g, 0.00514 mol) and the mixture was placed in a sealed reaction vessel. The mixture was stirred at 60 °C for 22 h, cooled to RT and concentrated *in vacuo*. To the residue was added MeOH (2x), the mixture was concentrated and the resulting solids were triturated with 10% $NaHCO_3$, filtered, washed with water and dried under high vacuum overnight to yield a red solid (2.1 g, 88%). Solids from previous runs were combined (4.60 g) and dissolved in MeOH (1500 mL). The solution was concentrated to 800 mL and allowed stand overnight at RT. The precipitated solid was collected by filtration (2.94 g of a red solid) and the ML was concentrated to 300 mL. After standing overnight a second crop (0.257 g) of red solid was collected. The ML

was concentrated and the residue was chromatographed (SiO_2, MeOH/CH_2Cl_2 (1:1)) to afford additional red solid (0.46 g). Analysis of the three solids by TLC and ^{1}H NMR indicated they were all the same. The solids were combined and dried under vacuum for two days to afford **17** as a red solid (3.64 g); mp = 235–239 °C. R_f = 0.41, SiO_2, $CHCl_3$/MeOH/ NH_4OH/CH_2Cl_2; (40:10:0.1:50) *m/z* calcd for $C_{28}H_{28}N_4O_3$: 468: found: 468. ^{1}H NMR (DMSO-d_6) δ 1.80–2.30 (m, 4H, $(CH_2)_2$), 2.03 (s, 6H, $N(CH_3)_2$), 3.42 (bm, 1H, OCH), 3.57 (m, 1H, NCH), 3.83 (bm, 1H, NCH), 4.05–4.38 (m, 4H, $(CH_2)_2$), 7.06–7.24 (m, 4H, ArH), 7.41–7.46 (m, 4H, ArH), 7.80 (m, 2H, ArH). Anal. calcd for $C_{28}H_{28}N_4O_3 \bullet H_2O$: C, 69.12; H, 6.21; N, 11.51; found: C, 69.3; H, 5.79; N, 11.37.

*Ruboxistaurin (**17**) Mesylate.* To a slurry of ruboxistaurin (**17**) (3.42 g, 0.00703 mol) in acetone (90 mL) was added a solution of methanesulfonic acid (0.68 g, 0.00703 mol) in H_2O (10 mL) and the heterogeneous mixture was stirred at RT for 15 min. The solids were collected by filtration and were rinsed with acetone (25 mL). The rosy red solids were dried under high-vacuum for 24 h to yield ruboxistaurin mesylate (3.00 g, 72%); mp 244–245 °C, Rf = 0.68, SiO_2, CH_2Cl_2/$CHCl_3$MeOH/NH_4OH (50/40/10/0.1), *m/z* calcd for $C_{28}H_{28}N_4O$: 468; found: 469 (M+H); $[\alpha]_{633}$ = -19.0 (c 0.345, MeOH). Anal. for $C_{29}H_{32}N_4SO_6 \bullet 1.5H_2O$ calcd: C, 58.87; H, 5.96; N, 9.47; found: C, 58.60, H, 5.86; N, 9.31. Solubility: water, >4 mg/mL; very soluble in DMSO and MeOH.

X-ray Crystal Data on (9*S*)-9-[(Dimethylamino)methyl]-6,7,10,11-tetrahydro-9*H*,18*H*-5,21:12,17-dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-18,20(19*H*)-dione, Ruboxistaurin (17)

Single-crystal *X*-ray diffraction data on (9*S*)-9-[(dimethylamino)methyl]-6,7,10,11-tetrahydro-9*H*,18*H*-5,21:12,17-dimethenodibenzo[*e,k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecine-

18,20(19*H*)-dione, Ruboxistaurin (17), were collected using Cu Kα radiation and a Bruker Photon 100 area detector. The crystal was prepared for data collection by coating with high viscosity microscope oil. The oil-coated crystal was mounted on a micro-mesh mount (MiteGen, Inc.), transferred to the diffractometer and a data set was collected at 150 °K. The 0.315 × 0.074 × 0.067 mm^3crystal was monoclinic in space group $P2_1$, with unit cell dimensions a = 9.2036(2) Å, b = 22.0461(5) Å, c = 11.8589(3) Å, $\alpha = 90°$, $\beta = 90.0670(10)°$, and $\gamma = 90°$. Data were 89.2% complete to 67.67° θ (~0.80 Å) with an average redundancy of 2.43. The final anisotropic full matrix least-squares refinement on F^2 with 636 variables converged at R_1 = 8.89%, for the observed data and wR_2 = 21.34% for all data. The structure was solved by direct methods and refined by full-matrix least squares on F^2 values using the programs found in the SHELXL suite (Bruker, SHELXL v2014.7, 2014, Bruker AXS Inc., Madison, WI). Corrections were applied for Lorentz, polarization, and absorption effects. Parameters refined included atomic coordinates and anisotropic thermal parameters for all non-hydrogen atoms. The H atoms were included using a riding model. Complete information on data collection and refinement is available in the supplemental material.

Assignment of absolute configuration was confirmed using Bayesian statistics of the Bijvoet pairs. Results of this analysis were: P2(true) = 1.000, P3(true) = 0.952, P3(rac-twin) = 0.058, and P3(false) = 0.9×10^{-8}. These results confirm that the absolute configuration has been properly assigned (*i.e.* both P2(true) and P3(true) are ~ 1) and no (or very minimal) racemic twinning is present (P3(rac-twin) = 0.058). It should be noted that neither the Flack parameter nor Hooft(y) parameter was definitive even though the Bayesian statistics are clear.

Atomic coordinates have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 1583495). Copies of the data can be obtained, free of charge, on

application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax: +44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk.

SUPPORTING INORMATION

Crystal data and structure refinement for **17**; Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($Å^2 \times 10^3$) for **17**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor; Bond lengths [Å] and angles [°] for **17**; Isotropic displacement parameters ($Å^2 \times 10^3$) for **17**; Hydrogen coordinates ($\times 10^4$) for **17**; Torsion angles [°] for **17**.

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Author Contributions

The research described in this manuscript was directed by A.H.L., S.W.M., P.A.R, H.H.S., and F.I.C.; the synthesis of **17** was performed by L.B.; *X*-ray crystallography was by J.D. and G.H.I.; the manuscript was written by A.H.L. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

DAT, dopamine transpoter; PKC, protein kinase C; ^{1}H NMR, proton nuclear magnetic resonance; DSC, differential scanning calorimetry; TGA, thermal gravimetric analysis; Et_3N, triethyamine; CH_2Cl_2, methylene chloride; RT, room temperature; NH_4Cl, ammonium chloride; H_2O, water; Na_2SO_4, sodium sulfate; EtOH, ethanol; i-PrOH, *iso*propanol; m.p., melting point; CuI, cuprous iodide; THF, tetrahydrofuran; EtOAc, ethyl acetate; NH_3, ammonia; s, singlet; d, doublet; dd, doublet of doublets; m, multiplet; *t*-BuOK, potassium *t*-butoxide; $NaBH_4$, sodium borohydride; NaOH, sodium hydroxide; HCl, hydrochloric acid; $CDCl_3$, deuterochloroform; EtMgBr, ethylmagnesium bromide; DMF, dimethylformamide; CH_3CN, acetonitrile; KOH, potassium hydroxide; DMSO-d_6, perdeuterated dimethylsulfoxide; SiO_2, silica gel; Me_2NH, dimethylamine; ML, mother liquor.

REFERENCES

[1] Brooks, D. J. (2016) Molecular imaging of dopamine transporters, *Ageing Res Rev 30*, 114-121.

[2] Vaughan, R. A., and Foster, J. D. (2013) Mechanisms of dopamine transporter regulation in normal and disease states, *Trends Pharmacol Sci 34*, 489-496.

[3] Brunelin, J., Fecteau, S., and Suaud-Chagny, M. F. (2013) Abnormal striatal dopamine transmission in schizophrenia, *Current medicinal chemistry 20*, 397-404.

[4] Chen, R., Furman, C. A., Zhang, M., Kim, M. N., Gereau, R. W. t., Leitges, M., and Gnegy, M. E. (2009) Protein kinase Cbeta is a critical regulator of dopamine transporter

trafficking and regulates the behavioral response to amphetamine in mice, *The Journal of pharmacology and experimental therapeutics 328*, 912-920.

[5] Torres, G. E. (2006) The dopamine transporter proteome, *J Neurochem 97 Suppl 1*, 3-10.

[6] Johnson, L. A., Guptaroy, B., Lund, D., Shamban, S., and Gnegy, M. E. (2005) Regulation of amphetamine-stimulated dopamine efflux by protein kinase C beta, *J Biol Chem 280*, 10914-10919.

[7] Jirousek, R. M., Gillig, J. R., Gonzalez, C. M., Heath, W. F., McDonald, I. J. H. N., D.A., Rito, C. A., Singh, U., Stramm, L. E., Melikian-Badalian, A., Barevsky, M., Balas, L. M., Hall, S. E., Winneroski, L. L., and Faul, M. M. (1996) (S)-13-(dimethylamino)methyl-10,11,13,15-tetrahydro-4,9,16,21-dimetheno-1H,13H-dibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexdecene-1,3(2H)-dione (LY333531) and related analogues: Isozyme selective inhibitors of protein knase Cb, *J. Med. Chem. 39*, 2664-2671.

[8] Faul, M. M., Winneroski, L. L., Krumrich, C. A., Sullivan, K. A., Gillig, J. R., Neel, D. A., Rito, C. J., and Jirousek, M. R. (1998) Macrocyclic Bisindolylmaleimides: Synthesis by Inter- and Intramolecular Alkylation, *J. Org. Chem. 63*, 1961-1973.

[9] Engel, G., Farid, N., Faul, M., Jirousek, M., Richardson, L., and Winneroski, L. (2000) Protein Kinase C Inhibitor, (Office, U. P., Ed.), Eli Lilly and Company, USA.

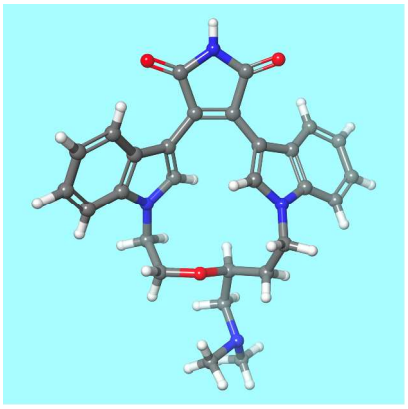
[10] Engel, G. L., Farid, N. A., Faul, M. M., Richardson, L. A., and Winneroski, L. L. (2000) Salt form selection and characterization of LY333531 mesylate monohydrate, *Int. J. Pharm. 198*, 239-247.

[11] Hooft, R. W., Straver, L. H., and Spek, A. L. (2008) Determination of absolute structure using Bayesian statistics on Bijvoet differences, *J Appl Crystallogr 41*, 96-103.

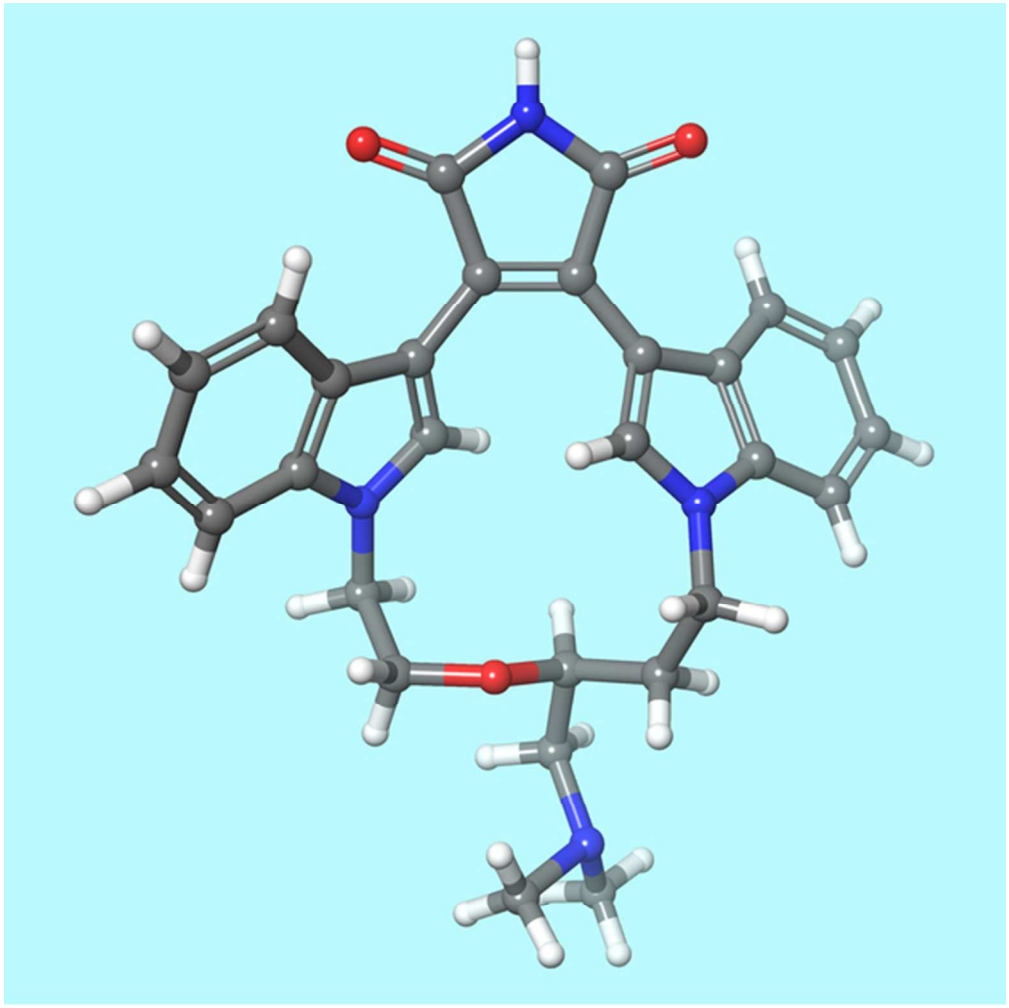
[12] Schweizer, E., Gaich, T., Brecker L, and Mulzer, J. (2007) Synthetic Studies Towards the Total Synthesis of Proveidencin, *Synthesis*, 3807-3814.

[13] Slater, M. J., Cockerill, S., Baxter, R., Bonser, R. W., Gohil, K., Gowrie, C., Robinson, J. E., Littler, E., Parry, N., Randall, R., and Snowden, W. (1999) Indolocarbazoles: potent, selective inhibitors of human cytomegalovirus replication, *Bioorg Med Chem 7*, 1067-1074.

Table of Contents graphic



Ruboxistaurin



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