Manuscript title

Pharmacological assessment of sepiapterin reductase inhibition on tactile response in the rat

Authors and affiliations

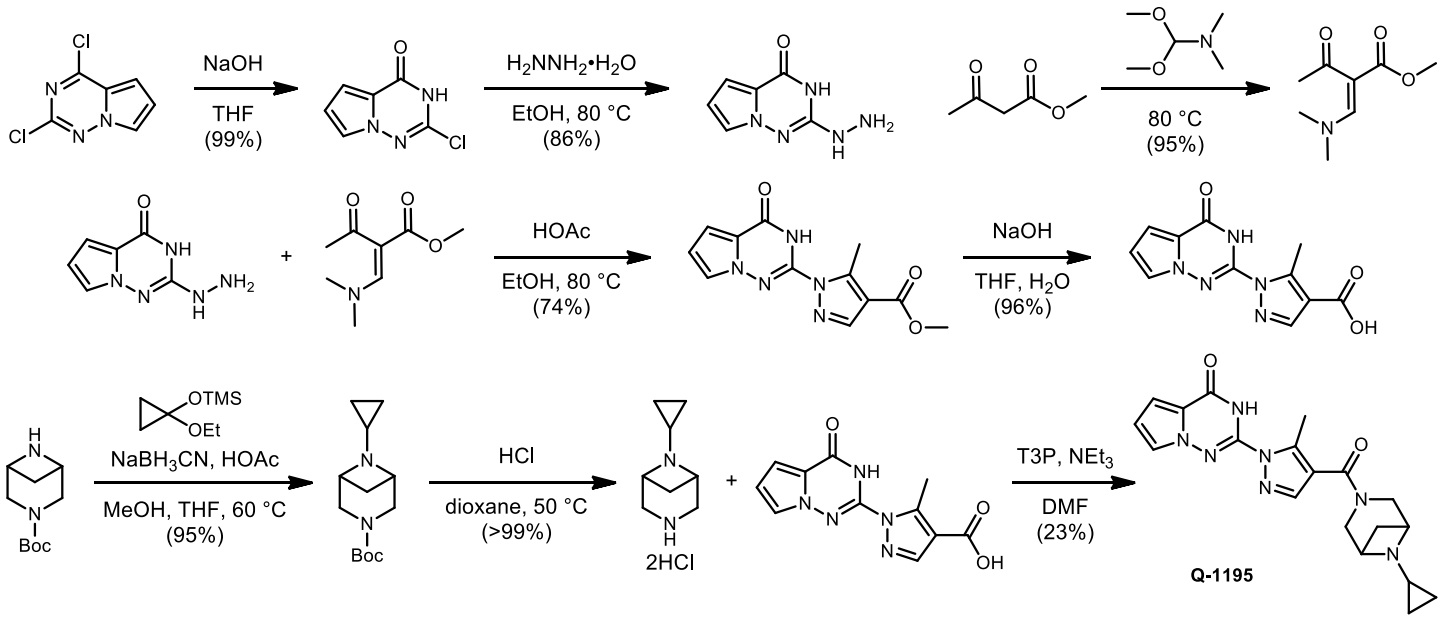
James T. Meyer, Brian A. Sparling, William J. McCarty, Maosheng Zhang, Marcus Soto, Stephen Schneider, Hao Chen, Jonathan Roberts, Helming Tan, Thomas Kornecook, Paul Andrews, Charles G. Knutson.

Amgen Research, One Amgen Center Drive, Thousand Oaks, CA 91320, United States (JTM, MZ, MS, HT, TK)

Amgen Research, 360 Binney Street, Cambridge, MA 02142, United States. (BAS, WJM, SS, HC, JR, PA, CGK)

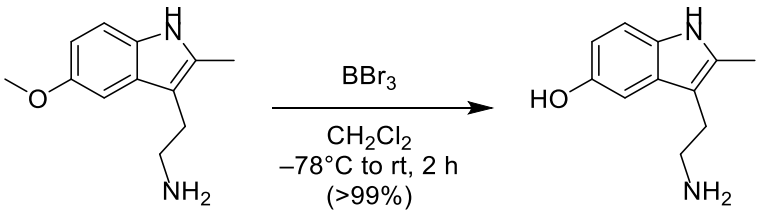
Supplemental Methods

Synthesis Scheme



Supplemental

General Synthetic Procedures, Materials, and Instrumentation. Commercial reagents and solvents were used as received. NMR spectra were recorded with a Bruker AV III 300, 400, and 500 MHz spectrometers, are reported in parts per million (δ), and are calibrated using residual non-deuterated solvent as an internal reference: d_6-DMSO, δ 2.50 (d_5-DMSO). Data for ^{1}H NMR spectra are reported as follows: chemical shift (multiplicity, coupling constants, integration). Multiplicities are reported as follows: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad, or combinations thereof. Preparative separations were performed on a Thar SFC Prep 80 or a Thar SFC Prep 350 instrument.

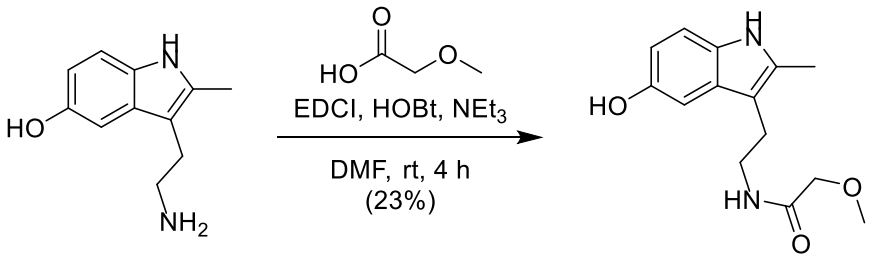


3-(2-Aminoethyl)-2-methyl-1*H*-indol-5-ol:

To a CH_2Cl_2 (375 ml) solution of 5-methoxy-2-methyltryptamine (15 g, 73 mmol) cooled to −78 °C was dropwise added boron tribromide (1 M in CH_2Cl_2, 225 ml, 2380 mmol). The mixture was allowed to reach room temperature and stirred at that temperature for 2 h. The mixture was quenched by adding methanol (130 ml). The pH of the mixture was adjusted to pH 7 with moistened sodium carbonate. The mixture was then filtered, and the filtrate was concentrated under reduced pressure. The residue obtained taken in 10% methanol in chloroform and filtered through a pad of celite. The filtrate collected was concentrated under reduced pressure to afford 3-(2-aminoethyl)-2-methyl-1H-indol-5-ol (14 g, 73.6 mmol, quantitative yield) as a brown gum.

^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.35 (s, 1H), 8.51 (broad s, 1H), 6.99 (dd, J = 8.6, 3.2 Hz, 1H), 6.75 – 6.65 (m, 1H), 6.47 (dt, J = 8.4, 2.8 Hz, 1H), 2.65 (m, 4H), and 2.27 (m, 3H).

MS-ESI (m / z): 191.2 $[M+H]^+$.

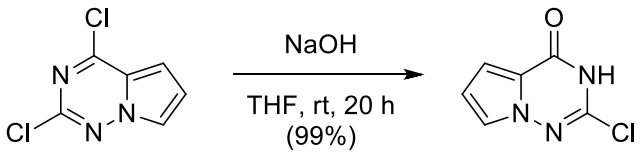


_N_-(2-(5-Hydroxy-2-methyl-1_H_-indol-3-yl)ethyl)-2-methoxyacetamide (SPRi3):

A DMF (150 ml) mixture of 3-(2-aminoethyl)-2-methyl-1*H*-indol-5-ol (14 g, 73.6 mmol), 2-methoxyacetic acid (7.95 g, 88 mmol), 1-hydroxybenzotriazole (16.9 g, 110. mmol), *N*1-((ethylimino)methylene)-*N*3,*N*3-dimethylpropane-1,3-diamine hydrochloride (21.16 g, 110. mmol,) and triethylamine (33.0 ml, 237 mmolequiv) was stirred at room temperature for 4 h. The reaction mixture was then concentrated *in vacuo*. The residue was diluted with water and extracted thrice with EtOAc. The organic extracts were combined, washed with brine, dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated *in vacuo*. The resulting brown oil was purified by reverse phase chromatography eluting with a gradient of 0% to 20% acetonitrile in water to afford *N*-(2-(5-hydroxy-2-methyl-1*H*-indol-3-yl)ethyl)-2-methoxyacetamide (4.5 g, 17.16 mmol, 23% yield) as an off-white solid.

^{1}H NMR (400 MHz, chloroform-*d*) δ: 7.71 (s, 1H), 7.13 (dd, *J* = 8.7, 2.7 Hz, 1H), 6.96 (s, 1H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.66 (s, 1H), 3.89 (d, *J* = 2.6 Hz, 2H), 3.53 (q, *J* = 7.2, 6.6 Hz, 2H), 3.33 (d, *J* = 2.6 Hz, 3H), 2.88 (dd, *J* = 8.1, 5.5 Hz, 2H), and 2.36 (d, *J* = 2.7 Hz, 3H).

MS-ESI (m / z): 263.2 $[M+H]^+$.

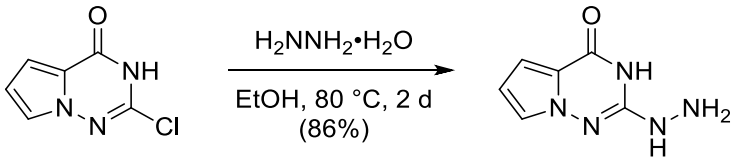


2-Chloropyrrolo[2,1-*f*][1,2,4]triazin-4(3*H*)-one:

A yellow THF (400 ml) solution of 2,4-dichloropyrrolo[1,2-d][1,3,4]triazine (20.2 g, 107 mmol) in a 1-L round-bottom flask was treated with sodium hydroxide (6 M in water, 107 ml, 645 mmol). The mixture was stirred vigorously at rt for 20 h. Volatiles were then removed in vacuo, and the light yellow mixture was acidified with 2 N HCl until pH < 4. The resulting off-white precipitate was collected via filtration and washed with water and then diethyl ether, and 2-chloropyrrolo[2,1-*f*][1,2,4]triazin-4(3*H*)-one (17.97 g, 106 mmol, 99% yield) was used without further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.79 (br s, 1 H), 7.61 (dd, *J*=2.47, 1.69 Hz, 1 H), 6.93 (dd, *J*=4.41, 1.56 Hz, 1 H), 6.55 (dd, *J*=4.41, 2.59 Hz, 1 H).

MS-ESI (m / z): 169.2 $[M+H]^+$.

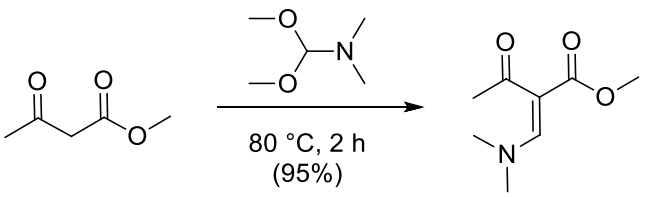


2-Hydrazinylpyrrolo[2,1-*f*][1,2,4]triazin-4(3*H*)-one:

A EtOH (20 ml) slurry of 2-chloropyrrolo[2,1-f][1,2,4]triazin-4(3H)-one (5.0 g, 29.5 mmol) in a 250-ml round-bottom flask was treated with hydrazine monohydrate (4.68 ml, 124 mmol). A findenser was affixed to the flask, and the apparatus was flushed with N_2. The yellow mixture was then heated to 80 °C. The resulting yellow solution was stirred at that temperature for 2 d, whereupon a white precipitate formed. The slurry was cooled to rt, and the precipitate was collected via filtration, washed twice with cold water, and dried under vacuum/N_2 sweep to afford 2-hydrazineylpyrrolo[2,1-*f*][1,2,4]triazin-4(3*H*)-one (4.19 g, 25.4 mmol, 86% yield) as a white amorphous solid.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.27 - 7.32 (m, 1 H), 7.21 (br s, 1 H), 6.72 (dd, *J*=4.28, 1.69 Hz, 1 H), 6.33 (dd, *J*=4.41, 2.59 Hz, 1 H).

MS-ESI (m / z): 166.2 $[M+H]^+$.

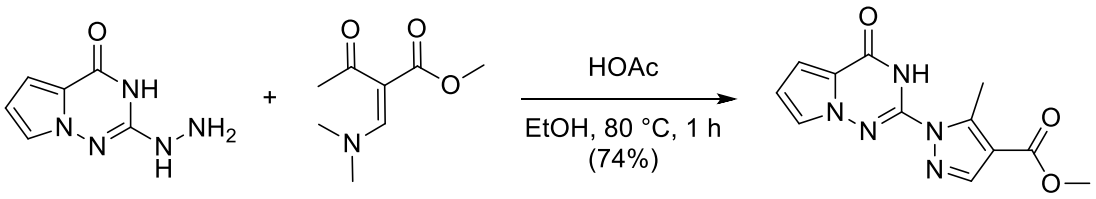


Methyl 2-((dimethylamino)methylene)-3-oxobutanoate:

A yellow mixture of methyl acetoacetate (5.0 ml, 46 mmol) and *N*,*N*-dimethylformamide dimethyl acetal (7.4 ml, 56 mmol) in a 40-ml vial was stirred at 80 °C for 2 h. Volatiles were removed *in vacuo* from the resulting black-red syrup, and the concentrate was placed under vacuum for >24 h, producing methyl 2-((dimethylamino)methylene)-3-oxobutanoate (7.57 g, 44.2 mmol, 95% yield) as a red-black amorphous solid that was used without further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.63 (s, 1 H), 3.63 (s, 3 H), 3.08 - 3.36 (m, 3 H), 2.68 - 2.97 (m, 3 H), 2.13 (s, 3 H).

MS-ESI (m / z): 172.2 $[M+H]^+$.



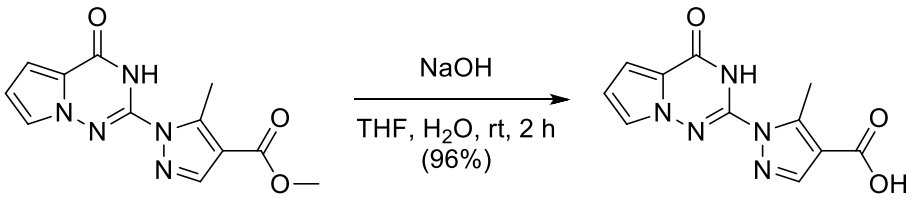
Methyl 5-methyl-1-(4-oxo-3,4-dihydropyrrolo[2,1-*f*][1,2,4]triazin-2-yl)-1*H*-pyrazole-4-carboxylate:

A EtOH (36 ml) slurry of 2-hydrazineylpyrrolo[2,1-*f*][1,2,4]triazin-4(3*H*)-one (4.19 g, 25.4 mmol), methyl 2-((dimethylamino)methylene)-3-oxobutanoate (4.34 g, 25.4 mmol), and acetic acid (5.08 ml, 89 mmol) in a 100-

ml round-bottom flask outfitted with a findenser under N_2 was heated to 80 °C. The orange-yellow slurry was stirred at that temperature for 1 h. The orange slurry was then cooled to rt and filtered to isolate a light orange precipitate, which was washed with two small portions of cold EtOH. Drying under vacuum/N_2 sweep afforded methyl 5-methyl-1-(4-oxo-3,4-dihydropyrrolo[2,1-f][1,2,4]triazin-2-yl)-1H-pyrazole-4-carboxylate (5.119 g, 18.73 mmol, 74% yield) as a light orange amorphous solid that was used without further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.74 - 12.89 (m, 1 H), 8.16 (s, 1 H), 7.69 (s, 1 H), 7.01 (d, *J*=4.15 Hz, 1 H), 6.52 - 6.72 (m, 1 H), 3.81 (s, 3 H), 2.74 (s, 3 H).

MS-ESI (m / z): 274.2 $[M+H]^+$.

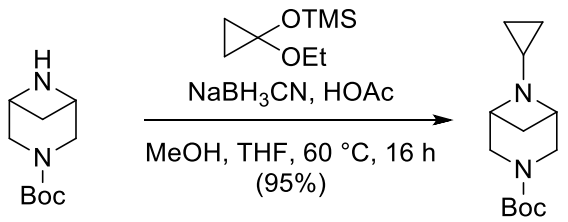


5-Methyl-1-(4-oxo-3,4-dihydropyrrolo[2,1-*f*][1,2,4]triazin-2-yl)-1*H*-pyrazole-4-carboxylic acid:

A THF (30 ml) slurry of methyl 5-methyl-1-(4-oxo-3,4-dihydropyrrolo[2,1-*f*][1,2,4]triazin-2-yl)-1*H*-pyrazole-4-carboxylate (5.119 g, 18.73 mmol) in a 250-ml round-bottom flask was treated with sodium hydroxide (3 M in water, 1.0 ml, 25 mmol) followed by water (15 ml). The dark red slurry was stirred at rt for 2 h. 1 N HCl was then added until pH < 6, causing the formation of a precipitate. This precipitate was collected via filtration, washed twice with TBME, and then dried via vacuum/N_2 sweep to afford 5-methyl-1-(4-oxo-3,4-dihydropyrrolo[2,1-*f*][1,2,4]triazin-2-yl)-1*H*-pyrazole-4-carboxylic acid (4.677 g, 18.04 mmol, 96% yield) as a tan amorphous solid that was used without further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.67 - 12.96 (m, 1 H), 12.26 - 12.60 (m, 1 H), 8.10 (s, 1 H), 7.69 (dd, *J*=2.60, 1.56 Hz, 1 H), 7.01 (dd, *J*=4.41, 1.56 Hz, 1 H), 6.63 (dd, *J*=4.28, 2.72 Hz, 1 H), 2.73 (s, 3 H).

MS-ESI (m / z): 260.2 $[M+H]^+$.

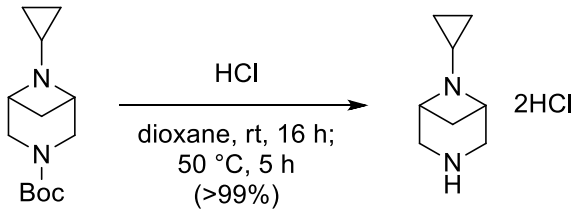


***tert*-Butyl 6-cyclopropyl-3,6-diazabicyclo[3.1.1]heptane-3-carboxylate:**

A MeOH (15 ml)/THF (15 ml) solution of 3-Boc-3,6-diaza-bicyclo[3.1.1]heptane (2.5 g, 12.6 mmol) and (1-ethoxycyclopropoxy)trimethylsilane (5.1 ml, 25 mmol) in a 200-ml recovery flask was treated with sodium cyanoborohydride (1.189 g, 18.91 mmol) and acetic acid (3.6 ml, 63 mmol). The colorless solution was flushed with N_2 and was then heated to 60 °C for 16 h. The reaction was then cooled to rt and treated with water (2 ml). After stirring for 5 min, the mixture was treated with NaOH (1 N in water, 5 ml). After stirring for at least another 15 min, volatiles were removed *in vacuo*. The aqueous residue was extracted with CH_2Cl_2, and this CH_2Cl_2 extract was washed with 1 N NaOH. Aqueous layers were combined and extracted twice with CH_2Cl_2. The organic extracts were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to provide *tert*-butyl 6-cyclopropyl-3,6-diazabicyclo[3.1.1]heptane-3-carboxylate (2.86 g, 12.0 mmol, 95 % yield) as a slight yellow oil that was used without further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.64 (br d, *J*=12.46 Hz, 1 H), 3.57 (br d, *J*=12.46 Hz, 1 H), 3.43 - 3.49 (m, 1 H), 3.38 - 3.43 (m, 1 H), 3.30 - 3.35 (m, 2 H), 3.27 (dd, *J*=12.46, 1.30 Hz, 1 H), 2.18 - 2.28 (m, 1 H), 1.80 (tt, *J*=6.55, 3.31 Hz, 1 H), 1.43 (s, 9 H), 0.34 - 0.41 (m, 2 H), 0.20 (quintet, *J*=3.31 Hz, 2 H).

MS-ESI (m / z): 239.2 $[M+H]^+$.

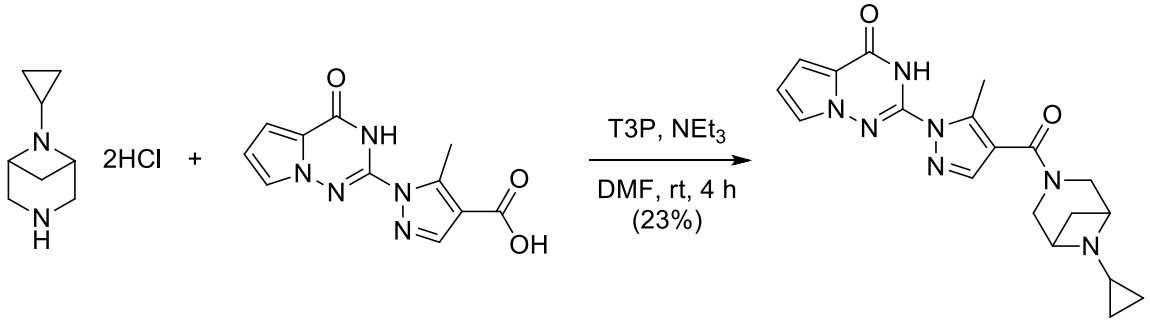


6-Cyclopropyl-3,6-diazabicyclo[3.1.1]heptane dihydrochloride:

tert-Butyl 6-cyclopropyl-3,6-diazabicyclo[3.1.1]heptane-3-carboxylate (2.86 g, 12.0 mmol) was taken up in dioxane (40 ml) in a 250-ml round-bottom flask and treated with hydrogen chloride (4.0 M in dioxane, 14.2 ml, 56.7 mmol). After stirring 16 h at rt and then at 50 °C for 5 h, solvent was decanted off, and the residue was dried *in vacuo* to afford 6-cyclopropyl-3,6-diazabicyclo[3.1.1]heptane dihydrochloride (2.96 g, >99% yield) as a white solid, which was used without further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.60 - 12.17 (m, 1 H), 10.46 - 10.91 (m, 1 H), 9.94 - 10.32 (m, 1 H), 4.39 (br s, 2 H), 4.12 - 4.29 (m, 2 H), 3.82 - 3.95 (m, 1 H), 3.62 - 3.78 (m, 2 H), 3.35 - 3.51 (m, 1 H), 2.33 - 2.49 (m, 1 H), 0.96 - 1.22 (m, 2 H), 0.84 (br d, *J*=6.49 Hz, 1 H).

MS-ESI (m / z): 139.2 $[M+H]^+$.



2-(4-(6-Cyclopropyl-3,6-diazabicyclo[3.1.1]heptane-3-carbonyl)-5-methyl-1*H*-pyrazol-1-yl)pyrrolo[2,1-*f*][1,2,4]triazin-4(3*H*)-one (Q-1195):

A DMF (35 ml) solution of 5-methyl-1-(4-oxo-3,4-dihydropyrrolo[2,1-*f*][1,2,4]triazin-2-yl)-1*H*-pyrazole-4-carboxylic acid (2.121 g, 8.18 mmol), 6-cyclopropyl-3,6-diazabicyclo[3.1.1]heptane dihydrochloride (1.90 g, 9.00 mmol), and triethylamine (5.75 ml, 41 mmol) in a 250-ml round-bottom flask in a rt water bath was treated with 1-propanephosphonic acid cyclic anhydride (50 wt% in DMF, 7.81 g, 12.3 mmol). The red orange slurry was stirred at rt for 4 h and then concentrated *in vacuo*. Purification via SFC (DEAP 30mm x 150mm column, 5-40% MeOH, 100 ml/min) afforded 2-(4-(6-cyclopropyl-3,6-diazabicyclo[3.1.1]heptane-3-carbonyl)-5-methyl-1*H*-pyrazol-1-yl)pyrrolo[2,1-*f*][1,2,4]triazin-4(3*H*)-one (0.72 g, 1.90 mmol, 23% yield) as an off-white amorphous solid.

^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.05 - 12.65 (m, 1 H), 8.14 (s, 1 H), 7.65 (s, 1 H), 6.98 (d, *J*=3.63 Hz, 1 H), 6.51 - 6.72 (m, 1 H), 3.95 (br d, *J*=11.16 Hz, 1 H), 3.80 (br d, *J*=13.49 Hz, 1 H), 3.66 (br d, *J*=14.27 Hz, 1 H), 3.54 - 3.61 (m, 2 H), 3.48 (br d, *J*=1.82 Hz, 1 H), 2.57 (s, 3 H), 2.30 (br d, *J*=7.27 Hz, 1 H), 1.97 (br d, *J*=2.08 Hz, 1 H), 1.44 (d, *J*=8.82 Hz, 1 H), 0.42 (br d, *J*=6.75 Hz, 2 H), 0.26 (br d, *J*=1.56 Hz, 2 H).

MS-ESI (m / z): 380.2 $[M+H]^+$.

Supplemental Table 1. MS/MS Parameter settings for BH4, BIO, SEP, RHAM and TOL.

Compound	Q1 Mass	Q3 Mass	Dwell (msec)	DP	CE	CXP
Biopterin	235.9	191.7	100	-65	-18	-9
Sepiapterin	235.9	163.7	100	-70	-24	-11
Rhamnopterin	266	191.9	25	-55	-20	-11
Tolbutamide	269	169.8	25	-65	-22	-11

Supplemental Table 2. MS/MS parameter settings for Q-1195 and VER

Compound	Q1 Mass	Q3 Mass	Dwell (msec)	DP	CE	CXP
Q-1195	380.3	241.9	100	81	31	14
Verapamil	455.4	165	50	125	52	12

Supplemental Table 3. LC-MS/MS inter-assay precision and accuracy

Biopterin in Plasma [ng/ml]

Nominal [ng/ml]	Mean Measured *	SDev	Precision %	Accuracy %
2.5	2.51	0.1	6	100
5.0	5.27	1.1	21	105
10	10.7	1.0	9	107
25	26.1	2.7	10	104
50	51.8	1.7	3	104
100	105	13	13	105
250	256	7.4	3	102

* mean of n=6

Sepiapterin in Plasma [ng/ml]

Nominal [ng/ml]	Mean Measured *	SDev	Precision %	Accuracy %
1.0	0.99	0.2	18	99
2.5	2.37	0.5	21	95
5.0	4.60	0.6	14	92
10	9.50	1.2	13	95
25	23.6	0.7	3	95
50	51.6	4.3	8	103
100	103	1.5	1	103
250	259	18	7	104

* mean of n=6

BH4 in Plasma [ng/ml]

Nominal [ng/ml]	Mean Measured *	SDev	Precision %	Accuracy %
10	10.8	2.4	22	108
15	15.1	1.9	12	100
25	23.2	3.9	17	93
50	51.5	9.8	19	103
100	104	9.4	9	104
250	248	22	9	99
500	477	48	10	95
1000	989	57	6	99

* mean of n=4

Biopterin in DRG [ng/g]

Nominal [ng/ml]	Mean Measured **	SDev	Precision %	Accuracy %
10	11.0	2.4	22	110
25	26.0	6.8	26	104
50	48.3	6.1	13	97
100	92.1	17	19	92
250	250	15	6	100
500	463	102	22	93
1000	983	152	15	98

** mean of n=9

Sepiapterin in DRG [ng/g]

Nominal [ng/ml]	Mean Measured **	SDev	Precision %	Accuracy %
15	16.5	2.3	14	110
25	22.1	3.5	16	88
50	43.6	7.6	17	87
100	94.2	7.6	8	94
150	150	10	7	100
250	257	27	11	103

** mean of n=5

BH4 in DRG [ng/g]

Nominal [ng/ml]	Mean Measured *	SDev	Precision %	Accuracy %
10	10.6	1.0	9	106
15	14.9	2.8	19	99
25	27.8	4.9	17	111
50	48.9	5.7	12	98
100	105	15	14	105
250	254	24	9	102
500	482	58	12	96
1000	976	122	13	98

** mean of n=8

Precision = (Sdev/Mean) *100

Accuracy = (measured/nominal) *100

Supplemental Table 4. Pterin biomarker standard curve performance

Biomarker	Plasma [ng/ml]					DRG [ng/g]				
	Range	RSq	n	Precision %	Accuracy %	Range	RSq	n	Precision %	Accuracy %
Biopterin	2.5 - 250	0.9903	6	3 - 21	100 - 107	10 - 1,000	0.9742	9	6 - 26	92 - 110
Sepiapterin	1 - 250	0.9818	6	2 - 21	92 - 104	15 - 250	0.9846	5	7 - 17	88 - 110
BH4	10 - 1,000	0.9743	4	6 - 23	93 - 108	10 - 1,000	0.9768	8	9 - 19	98 - 111