Synthesis and bioactivity of some 2-oxo-5-aryl-3-hydrazone and 2-oxo-5-aryl-4-hydrazone pyrrolidine derivatives

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DOI: http://dx.doi.org/10.3998/ark.5550190.0012.932

Abstract

2-Aryl-4,5-dioxopyrrolidine-3-carboxylate and 5-aryl-2,4-dioxopyrrolidine-3-carboxylate derivatives were successfully synthesized *via* carbonyl-based multiple component reaction and Dieckmann cyclization, respectively. Successive functional group transformations which include decarboxylation and hydrazonation afforded 2-oxo-5-aryl-3-hydrazone and 2-oxo-5-aryl-4-hydrazone derivatives. Compound **3d** exhibited activity against human histiocytic lymphoma (U937) and neuroblastoma (SH-SY5Y) cell lines while compound **6** showed neuroprotective ability from oxidative stress medium induced with H_2O_2.

Keywords: Pyrrolidine, hydrazone synthesis, cytotoxicity, neurotoxicity, neuroprotection

Introduction

In our continuous effort towards the chemical investigation of bioactive pyrrolidine type compounds, we have successfully synthesized various novel pyrrolidine derivatives with unique structural moiety.[1-5] Many natural and synthetic compounds with pyrrolidine type moieties have received much attention because of their remarkable biological properties.[6-13] Recognizing the importance of such compounds in drug discovery has prompted us to investigate potential anticancer and neuroprotection activities of the synthesized novel compounds.

The pyrrolidone ring system has been the subject of research for more than three decades. In the early 1970s, piracetam was the first pyrrolidone to come to the attention of clinicians, and since then there has been much pharmaceutical interest in a broad range of indications and in new compounds. Modes of action of the pyrrolidones have revealed various pharmacological effects, as reviewed by Shorvon.[6] There are evidence that the pyrrolidones exert a number of

biological effects and that there is unlikely to be no single predominant mode of action that is common to all pyrrolidone compounds.[6]

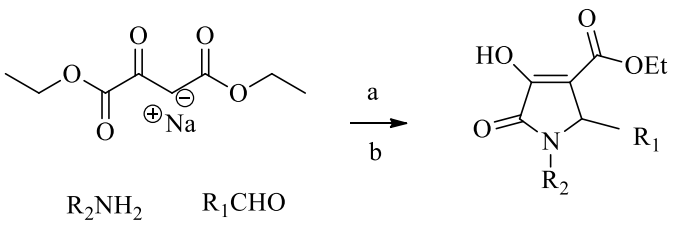
Structural modification on the pyrrolidine ring to produce more bioactive compounds has attracted many research groups. Tsou and co-workers have reported on synthesizing natural product-based small molecule libraries containing 2-aryl pyrrolidine skeleton.[14] Interestingly, the structural modification *via* hydrazonation reactions of the 2,3- or 2,4-dioxo-arylpyrrolidinones has not been reported before. Hydrazone functionalities are important intermediates for the synthesis of some bioactive compounds such as β-lactams[15] and reported to display a variety of interesting biological activities.[7-8] Consequently, novel 2-oxo-5-aryl-3-hydrazone and 2-oxo-5-aryl-4-hydrazone derivatives have been synthesized in our laboratory and their bioactivities are reported in this paper.

Results and Discussion

Chemistry

Previously we have reported[5] that the synthetic intermediate 2,3-dioxo-5-(hetero) arylpyrrolidines, **1a-d,** can be formed by refluxing equimolar diethyl oxalacetate, aromatic aldehyde and amine *via* carbonyl based on Dehaen's multi component reaction protocol.[16] In addition, we have also prepared some new compounds of 2,3-dioxo-5-(hetero) arylpyrrolidines **1e-1j** utilizing different aldehydes and amines (in the case of **1e,** ammonia is used) through the one pot reaction (Table 1). Although the yield of this reaction was not excellent, the expected products however, could easily be isolated without the need of any column chromatography.[5]

It is interesting to note that as racemization is expected to occur at C-5 of the pyrrolidinone ring during the cyclisation process, we found that only one sharp singlet peak of C_5-H (δ 4.95 - 5.00) was observed in the proton spectra. This suggested that the cyclisation reaction might take place in a stereo controlled manner of which the group at C-5 dictates the stereochemistry of the five membered ring system. For the record, similar observation of the stereochemistry at C-5 of the cyclized products was also reported by Dehaen *et al*.[16] In order to validate this, compound **1c** was subjected to decarboxyethylation with concomitant carbonyl reduction to give 3- hydroxyl 5-aryl pyrrolidine ring. Treatment of 1c with Boc anhydride gave 2-oxo-3-OBoc 5-aryl pyrrolidine ring, of which the structure was proved by X-ray analysis (Scheme 2, Figure 1).

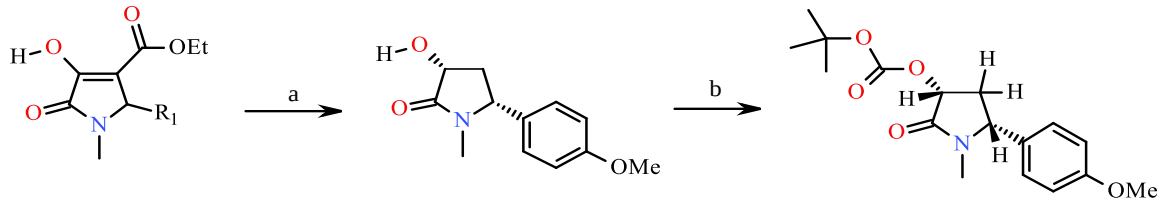


Scheme 1. One pot reaction of 2,3-dioxo-5-(hetero) arylpyrrolidine.

Table 1. Structure and yield of the 2,3-dioxo-5-(hetero) arylpyrrolidines

Compound	R_1	R_2	Yield %
1a	Ph	CH_3	40[a,a2]
1b	4-$CH_3C_6H_4$	CH_3	31[a, a2]
1c	4-$CH_3OC_6H_4$	CH_3	31[a,a1],40[a,a2], 25[b,a2]
1d	4-OHC_6H_4	CH_3	46[a,a2]
1e	4-$CH_3OC_6H_4$	H	54 [a,a2]
1f	4-BrC_6H_4	CH_3	52 [a,a2]
1g	4-allyloxyC_6H_4	CH_3	15 [a,a2]
1h	2-thienyl	CH_3	44 [a,a2]
1i	2-furyl	CH_3	40 [a,a2]
1j	3-$COOHC_6H_4$	CH_3	79 [a,a2]

Method [a] in ethanol [a1] = 33% methylamine in ethanol;
Method [b] in acetic acid [a2] = 40% methylamine in H_2O



1c

Scheme 2. Reagents and conditions: (a) i. acetonitrile/H^+ ii. $NaBH_4$; (b) Boc anhydride, DMAP, THF.

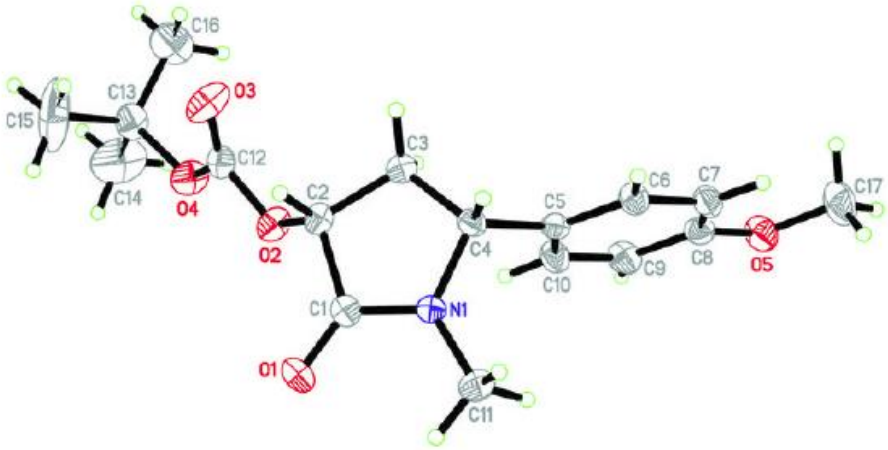
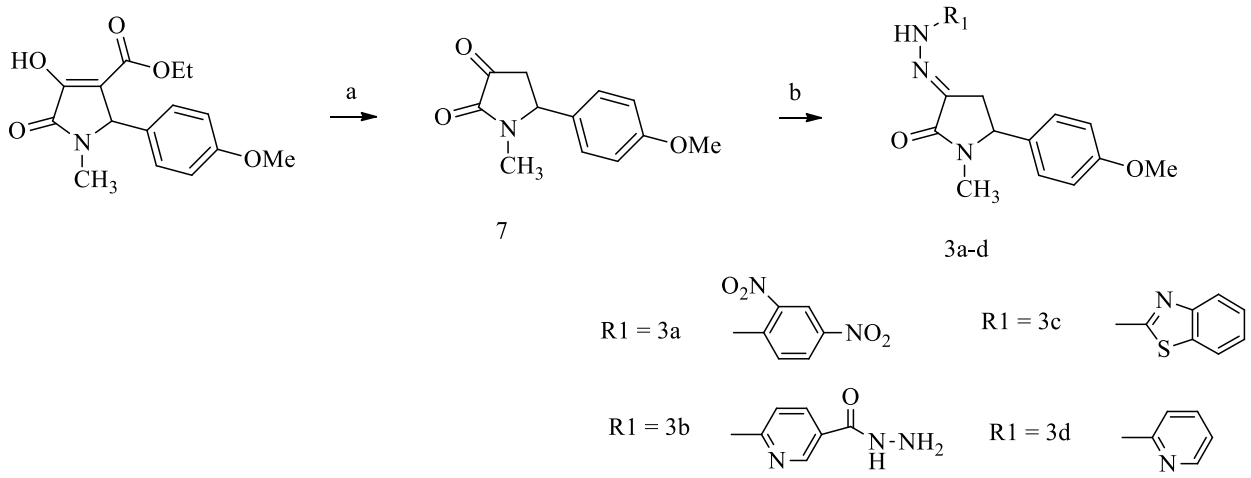


Figure 1. X-ray structure of 2-oxo-3-OBoc 5-aryl pyrrolidine.

The similarity of the carbon skeleton of compound **1c** to that of the natural compound (-)-codonopsinine[14-15] prompted us to further explore the chemistry of this compound. For example, when **1c** was refluxed in 10% HCl, the decarboxylated 2,3-diketo **7** was formed with a yield of 68%. Subsequently, hydrazonation of **7** with various hydrazine salts furnished hydrazone derivatives of 2-oxo-5-aryl-3-hydrazone **3a-d** (Scheme 3). Wang *et al.* reported that pyrrolidine ring bearing hydrazones functionality were shown to display a variety of interesting biological activities.[17]

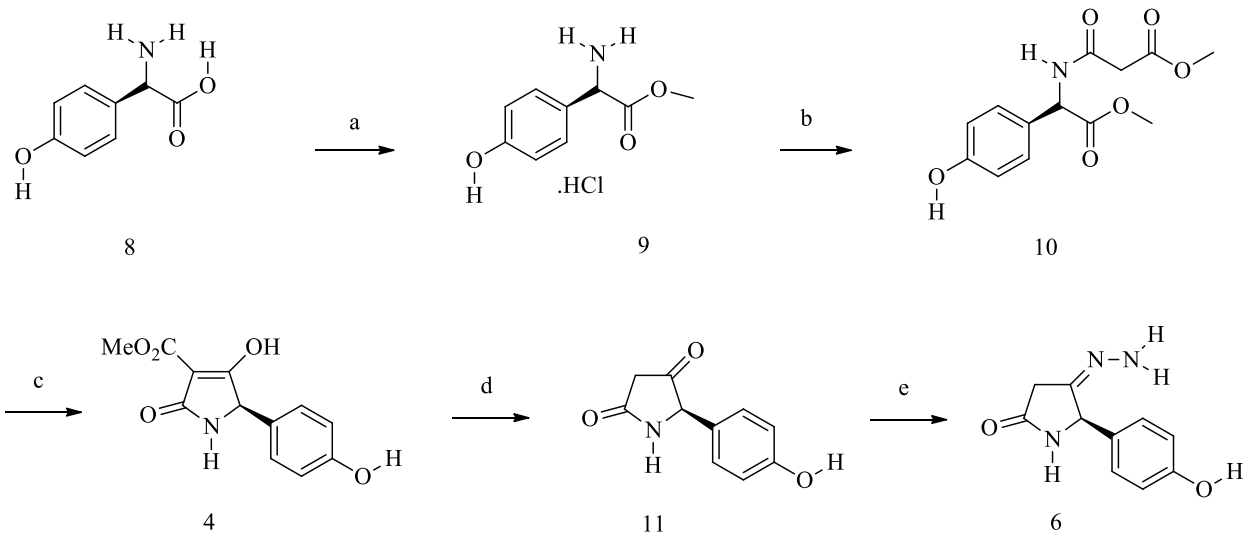


Scheme 3. Reagents and conditions: (a) 10% HCl solution reflux 7h; (b) hydrazine salts, EtOH, reflux 3 h.

In another development, 2,4-dioxo-3-alkoxy-5-aryl-pyrrolidines were synthesized initially by reacting *D*-4-hydroxyphenylglycine **8** with thionyl chloride to give a known compound glycine methyl ester hydrochloride salt **9** in a quantitative yield.[18] Treatment of **9** with methyl malonate potassium salt in the presence of dicyclohexylcarbodiimide (DCC) furnished compound **10**.[19] Dieckmann cyclisation reaction of **10** using excess sodium methoxide in methanol[20] gave the cyclised product 2,4-dioxo-3-carboxy-5-hydroxyphenyl pyrrolidine **4** in good yield. Since both the N and OH groups of compound **10** were not protected the cyclisation reaction must have occurred via the formation of a trianion intermediate.

Jouin and Wang independently reported that epimerization at C-5 had occurred though chiral amino acid was employed during the Dieckmann cyclisation reaction.[21-22] It was suggested that the diastereomeric ratio of the products was directly related to the sodium methoxide concentration and reaction time used.[21-22] However, in our case no racemisation was shown to occur since only one singlet at δ 5.00 (C_5-H) was observed in the proton spectra. The ^{1}H-NMR spectra also suggests that compound **4** exists in its enol form although the hydroxyl signal at C-4 was not detected presumably due to fast interchange of the keto-enol tautomer.[23]

The decarboxylation of **4** in refluxing acetonitrile gave compound **11** with high yield although excess solvent and longer reaction time are required.[24] Fortunately, these problems were overcome by adding some mineral acid to the reaction mixture thus accelerating the hydrolysis and decarboxylation to give the desired 2,4-dioxo 5 aryl pyrrolidinone compound **11**. Finally, treatment of **11** with hydrazine hydrate gave novel 2-oxo-5-aryl-4-hydrazone pyrrolidine **6**, (Scheme 4). It is worth mentioning that no column chromatography separation was required in both synthetic strategies towards 2-oxo-5-aryl-3-hydrazone and 2-oxo-5-aryl-4-hydrazone pyrrolidine derivatives.



Scheme 4. Reagents and conditions: a: $SOCl_2$, MeOH, r.t 10 h, quantitative. b: methyl malonate potassium salt, DCC, 0 °C 2 h; 65%. c: NaOMe (5 equiv), MeOH r.t 1 h; 71%. d: MeCN, H^+ (cat), reflux 5 h, quantitative. e: hydrazin .HCl ıte, EtOH, reflux 1 h, quantitative.

Some of the derivatives of compounds **1**, **3**, **4** and **6** were then subjected to biological evaluation for their cytotoxicity, neurotoxicity and neuroprotection activities.

Bioactivity evaluation of pyrrolidine derivatives

Compounds **1a-1d**, **3a**, **3d**, **4** and **6** were tested and evaluated for their cytotoxic ability in human cancer cell lines including neuroblastoma (SH-SY5Y), lymphoma (U937), and lung cancer (A549), *in vitro*.

All compounds were inactive except **3d** which showed activity against U937 and SH-SY5Y cell lines with IC_{50} values of 137 and 227 μM, respectively. The present findings showed that concentration ranges from 1nM to 1mM of compounds **4** and **6** did not show neurotoxicity effects to differentiated SH-SY5Y cells. Compound **6** significantly protected cells from H_2O_2 insults at 100nM to 1mM. The above results did not show any potential for cancer treatment, but

give us insights for therapeutic application in neurodegenerative, that may have potentials in clinical utility.

Experimental Section

General. Melting points were determined on an automatic FP62 melting point apparatus from Mettler Toledo and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian NMR Spectrometer instrument operating at 300 MHz at room temperature, in $CDCl_3$ or DMSO solutions. Chemical shifts are given in δ units (ppm) relative to TMS as internal standard. Elemental analyses were performed on Flash Elemental Analyzer 110 series. IR spectra (4000-400 $cm^{-1)}$ were recorded on Varian Excalibur 3100 FT-IR spectrometer, using ATR. Mass spectra were recorded on Agilent 1200 LCMS-QTOF instrument. The progress of the reactions was routinely monitored by thin layer chromatography (TLC) on silica gel GF254 and the products were visualized with an ultraviolet lamp (254 and 365 nm). All reagents and starting materials were purchased from Sigma-Aldrich Co. and Merck Chemical Co.

General procedure. Synthesis of 2,3-dioxo-5-(hetero)arylpyrrolidines (1)
To a stirred solution of diethyl oxalate (47.6 mmol) in ethanol (100 ml), amine (47.6 mmol) and aromatic aldehyde (47.6 mmol) were added. The reaction mixture was heated under reflux for 1 h. After cooling, ice-water was added to the mixture and then acidified with HCl. The precipitate formed was filtered, washed with water and diethyl ether to remove traces of aldehyde to furnish the product.

Ethyl 4-hydroxy-2-(4-methoxyphenyl)-5-oxo-2,5-dihydro-1*H*-pyrrole-3-carboxylate (1e). M.p. 147-148 °C. IR *v* cm^{-1} : (3316 N-H), (1688 C=O), (1611 C=O); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.12-1.16 (t, 3H, *J* = 7.05 Hz, CH_3), 3.77 (s, 3H, OCH_3), 4.10-4.17 (q, 2H, *J* = 6.9 Hz, OCH_2), 5.19 (s, 1H, ArC*H*NH), 6.81- 6.84 (d, 2H, *J* = 8.4 Hz, ArCH), 7.13- 7.16 (d, 2H, *J* = 8.1 Hz, ArCH), 7.38 (s, 1H, NH), 9.11 (s br, 1H, OH); ^{13}C ($CDCl_3$, 75 MHz): 13.9 (CH_3), 55.2 (OCH_3), 56.6 (Ar*C*HNH), 61.1 (OCH_2), 113.9 (ArCH), 114.8 (*C*CO), 127.7 (quat. Ar), 128.3 (ArCH), 157.1 (C=O), 159.7 (quat. Ar), 165.3 (C=O), 165.9 (COH). MS-QTOF: *m/z* = calcd. for $C_{14}H_{15}NO_5$: 278.1028 $[MH]^+$; found 278.1024 $[MH]^+$.
Ethyl 2-(4-bromophenyl)-4-hydroxy-1-methyl-5-oxo-2,5-dihydro-1*H*-pyrrole-3-carboxylate (1f). M.p. 158-159 °C. IR *v* cm^{-1} : (1710 C=O), (1691 C=O) ; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.06-1.11 (t, 3H, *J* = 7.05 Hz, CH_3), 2.75 (s, 3H, NCH_3), 4.04-4.11 (q, 2H, *J* = 6.9 Hz, OCH_2), 4.91 (s, 1H, ArC*H*NCH_3), 6.98-7.00 (d, 2H, *J* = 8.1 Hz, ArCH), 7.41-7.44 (d, 2H, *J* = 8.1 Hz, ArCH), 9.14 (s br, 1H, OH); ^{13}C ($CDCl_3$, 75 MHz): 13.9 (CH_3), 27.7 (NCH_3), 61.1 (Ar*C*HNCH_3), 61.9 (OCH_2), 112.4 (*C*CO), 122.7 (quat. Ar), 129.2 (ArCH), 132.0 (ArCH), 133.8 (quat. Ar), 157.6 (C=O), 163.7 (C=O), 164.8 (COH); Anal. Calcd for $C_{14}H_{14}BrNO_4$ (340.17) : C, 49.43; H, 4.15; N, 4.12. Found : C, 48.45; H, 4.44; N, 3.84 %.

Ethyl 2-(4-(allyloxy)phenyl)-4-hydroxy-1-methyl-5-oxo-2,5-dihydro-1*H*-pyrrole-3-carboxylate (1g). M.p 128-129 ºC. IR *v* cm^{-1} : (1693 C=O), (1673 C=O); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.09-1.14 (t, 3H, *J* = 7.2 Hz, CH_3), 2.78 (s, 3H, NCH_3), 4.08-4.15 (q, 2H, *J* = 7.35 Hz, OCH_2), 4.50-4.52 (d, 2H, *J* = 5.1 Hz, OCH_2), 4.94 (s, 1H, ArC*H*NCH_3), 5.26-5.29 (d, 1H, *J* = 9.3 Hz, CH), 5.36-5.42 (d, 1H, *J* = 15.6 Hz, CH), 5.96-6.09 (m, 1H, CH), 6.85-6.88 (d, 2H, *J* = 8.4 Hz, ArCH), 7.04-7.07 (d, 2H, *J* = 8.4 Hz, ArCH), 9.15 (s br, 1H, OH); ^{13}C ($CDCl_3$, 75 MHz): 13.9 (CH_3), 27.5 (NCH_3), 60.9 (Ar*C*HNCH_3), 62.0 (OCH_2), 68.8 (OCH_2), 112.8 (*C*CO), 114.9 (ArCH), 117.8 (CH), 126.5 (quat. Ar), 128.7 (ArCH), 132.9 (CH), 157.5 (C=O), 158.8 (quat. Ar), 163.7 (C=O), 165.1 (COH); Anal. Calcd for $C_{16}H_{17}NO_5$ (303.31) : C, 63.36; H, 5.65; N, 4.62. Found : C, 63.44; H, 6.66; N, 4.23 %.

Ethyl 4-hydroxy-1-methyl-5-oxo-2-(thiophen-2-yl)-2,5-dihydro-1*H*-pyrrole-3-carboxylate (1h). M.p 147-148 ºC. IR *v* cm^{-1} : (1708 C=O), (1665 C=O); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.12-1.17 (t, 3H, *J* = 7.05 Hz, CH_3), 2.87 (s, 3H, NCH_3), 4.15-4.22 (m, 2H, OCH_2), 5.32 (s, 1H, ArC*H*NCH_3), 6.96-6.98 (t, 1H, *J* = 3.75 Hz, ArCH), 7.08 (s, 1H, ArCH), 7.25-7.29 (t, 1H, *J* = 5.55 Hz, ArCH), 9.20 (s br, 1H, OH); ^{13}C ($CDCl_3$, 75 MHz): 13.9 (CH_3), 27.5 (NCH_3), 57.8 (Ar*C*HNCH_3), 61.0 (OCH_2), 112.2 (*C*CO), 126.2 (ArCH), 126.8 (ArCH), 127.8 (ArCH), 138.2 (quat. Ar), 157.7 (C=O), 163.1 (C=O), 164.9 (COH); Calcd for $C_{12}H_{13}NO_4S$ (267.30) : C, 53.92; H, 4.90; N, 5.24. Found : C, 53.18; H, 5.01; N, 5.29 %.

Ethyl 2-(furan-2-yl)-4-hydroxy-1-methyl-5-oxo-2,5-dihydro-1*H*-pyrrole-3-carboxylate (1i). M.p 131-132 ºC. IR *v* cm^{-1} : (1692 C=O), (1661 C=O); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.14-1.19 (t, 3H, *J* = 6.9 Hz, CH_3), 2.86 (s, 3H, NCH_3), 4.10-4.27 (m, 2H, OCH_2), 5.14 (s, 1H, ArC*H*NCH_3), 6.36 (s, 2H, ArCH), 7.35 (s, 1H, ArCH), 9.26 (s br, 1H, OH); ^{13}C ($CDCl_3$, 75 MHz): 14.0 (CH_3), 27.7 (NCH_3), 55.8 (Ar*C*HNCH_3), 61.0 (OCH_2), 109.3 (*C*CO), 109.9 (ArCH), 110.5 (ArCH), 143.1 (ArCH), 147.3 (quat. Ar), 158.2 (C=O), 163.3 (C=O), 164.9 (COH); Calcd for $C_{12}H_{13}NO_5$ (251.24) : C, 57.37; H, 5.22; N, 5.58. Found : C, 57.08; H, 5.25; N, 5.72 %.

3-(3-(Ethoxycarbonyl)-4-hydroxy-1-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-2-yl)benzoic acid (1j). M.p. 210-211 ºC. IR *v* cm^{-1} : (1681 C=O), (1655 C=O); ^{1}H NMR (DMSO, 300 MHz): δ 0.97-1.02 11 (t, 3H, *J* = 7.05 Hz, CH_3), 2.62 (s, 3H, NCH_3), 3.85-3.97 (m, 2H, OCH_2), 5.24 (s, 1H, ArC*H*NCH_3), 7.39-7.49 (m, 2H, ArCH), 7.71 (s, 1H, ArCH), 7.85-7.87 (d, 1H, *J* = 7.2 Hz, ArCH), 11.71 (br s, 1H, COOH); ^{13}C (DMSO, 75 MHz): 14.3 (CH_3), 27.7 (NCH_3), 59.9 (Ar*C*HNCH_3), 61.9 (OCH_2), 111.5 (*C*CO), 128.8 (ArCH), 129.4 (ArCH), 129.6 (ArCH), 131.4 (quat. Ar), 132.5 (ArCH), 137.5 (quat. Ar), 154.5 (C=O), 162.3 (C=O), 165.0 (COOH), 167.4 (COH); Calcd for $C_{15}H_{15}NO_6$ (305.28) : C, 59.01; H, 4.95; N, 4.59. Found : C, 58.51; H, 4.99; N, 4.72 %.

Synthesis of methyl amino(4-hydroxyphenyl) acetate (9). 4-Hydroxy-*D*-phenylglycine **8** (10.0 g, 60.0 mmol) was suspended in methanol (200 ml) and thionyl chloride (8 ml) was added dropwise. After the resulting mixture was stirred at room temperature for 10 h, the solvent was removed by rotary evaporation and the residue was washed twice with ether (50 ml) to yield **9** as a white solid (12.8 g, 98%), m.p. 187-188 °C. (lit[18]. m.p. 187-190 °C)

Methyl 4-hydroxy-5-(4-hydroxyphenyl)-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carboxylate (4). A solution of **10** (2.8 g, 9.94 mmol) in dry methanol (12 ml) was added to a solution of sodium methoxide (2.78 g, 49.7 mmol) in dry methanol (100 ml) and left stirring at room temperature for 1 h. The solvent was evaporated under reduced pressure and the crude product was redissolved in toluene. The organic extract was then diluted with water and two phases were separated. The aqueous layers were collected and acidified with concentrated HCl. The product **4** slowly precipitated out as brownish powder (1.75 g, 71%). m.p. 123-124 °C. IR *v* cm^{-1}: 1664 (C=O), 1613 (C=O); ^{1}H NMR (DMSO, 300 MHz): δ 3.65 (s, 3H, OCH_3), 4.91 (s, 1H, ArC*H*NH), 6.71-6.74 (d, 2H, *J* = 9 Hz, ArCH), 7.01-7.03 (d, 2H, *J* = 9 Hz, ArCH), 8.22 (s, 1H, NH), 9.21 (S, 1H, OH); ^{13}C (DMSO, 75 MHz): 55.5 (OCH_3), 67.5 (CH), 114.7 (ArCH), 126.1 (quat. C), 127.4 (quat. Ar), 128.3 (ArCH), 165.4 (quat. Ar), 167.7 (C=O), 187.9 (C=O), 217.5 (COH); Calcd for $C_{12}H_{11}NO_5$ (249.22) : C, 57.83; H, 4.45; N, 5.62. Found : C, 58.01; H, 4.51; N, 5.60%.

5-(4-hydroxyphenyl) pyrrolidine-2,4-dione (11). Compound **4** (0.3 g, 1.2 mmol) was dispersed in acetonitrile and heated under reflux for 5 h. The ester dissolved gradually to give a clear light yellow solution. The solvent was evaporated to give **11** as a brown solid (0.22 g, 99%). m.p. 193-194°C. IR *v* cm^{-1}: 1734 (C=O), 1653 (C=O); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.02-3.03 (d, 2H, *J* = 3 Hz, CH_2), 4.95 (s, 1H, ArC*H*NH), 6.84-6.91 (d, 2H, *J* = 9 Hz, ArCH), 7.19-7.22 (d, 2H, *J* = 9 Hz, ArCH), 7.54 (s, 1H, NH); ^{13}C ($CDCl_3$, 75 MHz): 40.9 (CH_2), 56.4 (CH), 116.1 (ArCH), 127.9 (ArCH), 128.8 (quat. Ar), 156.6 (quat. Ar), 169.7 (C=O), 171.4 (C=O); Calcd for $C_{10}H_9NO_3$ (191.18) : C, 62.82; H, 4.74; N, 7.33. Found : C, 61.97; H, 4.93; N, 7.92 %.

(4*Z*)-4-hydrazinylidene-5-(4-hydroxyphenyl)pyrrolidin-2-one (6). Hydrazine hydrate (0.157 ml, 3.13 mmol) was added to a stirred solution of **11** (0.5 g, 2.6 mmol) in ethanol. The mixture was reflux for 1h and the precipitate formed was filtered and washed with DCM to yield the hydrazone **6** as yellowish solid (0.56 g, 99%). IR *v* cm^{-1}: 1691 (C=O); ^{1}H NMR (DMSO, 300 MHz): δ 2.87 (s, 2H, CH_2), 4.96 (s, 1H, ArC*H*NH), 5.86 (s br, 2H, NH_2), 6.66-6.69 (d, 2H, *J* = 9 Hz, ArCH), 6.98-7.01 (d, 2H, *J* = 9 Hz, ArCH), 8.38 (s, 1H, NH), 9.35 (s br, 1H, OH); ^{13}C (DMSO, 75 MHz): 36.2 (CH_2), 66.2 (CH), 120.0 (ArCH), 132.9 (quat. Ar), 137.1 (ArCH), 152.1 (C=N), 161.8 (quat. Ar), 177.7 (C=O); Calcd for $C_{10}H_{11}N_3O_2$ (205.21) : C, 58.53; H, 5.40; N, 20.48. Found : C, 58.19; H, 5.65; N, 20.29 %.

Biological assays

Compounds **1a**, **1b**, **1c**, **1d**, **3a**, and **3d** were synthesized and reported previously.[5] All derivatives **1a**, **1b**, **1c**, **1d**, **3a**, **3d**, **4**, and **6** were dissolved in dimethyl sulfoxide at100 mM and then diluted with complete medium prior each experiment.

Assay for cytotoxic activity. Cells (1×10^5 cells/ml) were seeded in 96-well plates in specific media with 1% non-essential amino acids (100 ×), 1% L-glutamine (200 mM), 1% gentamicin

(10 mg/mL), and supplemented with 10% fetal bovine serum (FBS). All cell lines were maintained in an incubator at 37ºC in a 5% CO_2 atmosphere with 95% humidity. Cell viability was determined after 24 h at 37ºC by CellTiter 96® AQ_{ueous} Assay uses the novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS assay) using Glomax multi detection system and read at 490 nm.[25]

Neurotoxicity and neuroprotection analysis. Retinoic acid will induce the differentiation of the neuroblastoma cells to behave like neuron-phenotypic cells. Compounds **4** and **6** were tested for their neurotoxicity effects. The differentiated cells were exposed to the concentration ranging from 1nM to 1mM. The neurotoxicity effects were determined by MTS assay. In case of neuroprotection, those compounds were added to each well at final concentrations ranging from 1 nM to 1 mM and incubated for 2 h. Then, treated cultures were exposed to 98 µM hydrogen peroxide (H_2O_2, 30%), which caused 50% reduction in cell viability (cell's IC_{50} value). The cultures were further incubated for 24 h, and then cell viability test was performed by MTS assay.

Acknowledgements

We thank Pharmacogenomics Centre UiTM (PROMISE) for the High Resolution MS analysis and the Ministry of Higher Education for the financial support (FRGS fund {600-RMI/ST/FRGS 5/3/Fst (11/2008).

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