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Congo red analogues as potential anti-prion agents

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Abstract

'Transmissible Spongiform Encephalopathies' (TSE) are a group of degenerative, progressive and fatal disorders of CNS which affect both humans and animals, characterised by a long incubation time. The pathogenetic mechanism in TSE is the conversion of normal prion protein (PrP^{sen}) to an altered protease resistant isoform (PrP^{res}) that accumulates in amyloid deposits into the brain; therefore, PrP^{res} is the primary target for therapeutic strategies. The discovery that the sulphonated azo dye Congo red (CR) is able to inhibit the replications of TSE agents and the accumulation of PrP^{res} in animals and in scrapie infected mouse neuroblastoma cells induced us to designe molecules structurally related to CR (**1a**–**f**, **2f**,**g**). The compounds were tested in vitro to evaluate their interaction with 263K PrP^{res}. Six of the tested compounds were found to interact with PrP^{res} molecules and to over-stabilise the PrP^{res} aggregates, as CR does. However, none of them induced the reversion of PrP^{res} to PrP^{sen}.
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Keywords: Congo red; Transmissible spongiform encephalopathies (TSE); Anti-prion-molecules

1. Introduction

Transmissible Spongiform Encephalopathies (TSE) are fatal neurodegenerative disorders in animals and humans. They are characterized by a long incubation period and a slow progression. TSE are represented principally by Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker Syndrome (GSS) and Fatal Familial Insomnia (FFI) in humans and by scrapie and Bovine Spongiform Encephalopathy in animals (BSE).

The BSE epizootic recently spread in UK and the first cases of a new human spongiform encephalopathy, called 'new juvenile variant CJD' (vCJD) [1,2], alarmed the entire scientific community for the risk of bovine prion transmission to human. This hypothesis was also supported by the discovery that vCJD is caused by the same prion found in BSE [3,4].

The pathogenic mechanism of the disease is the conversion of normal prion protein (PrP^{sen}) to a protease resistant isoform (PrP^{res}) that accumulates in amyloid deposits into the brain [5–7]; therefore, PrP^{res} is the primary target for therapeutic strategies. Since neither control tools nor prophilaxis are currently available for viral or bacterial infections, the necessity for therapeutic agents is highly desiderable.

The recent discovery that the azoic dye Congo red (CR) [8] is able to inhibit the deposition of PrP in amyloid plaques [9,10] induced us to project and evaluate new molecules structurally related to CR (see Table 1). We assumed that CR was metabolised by endogenus azareductase to release **1a** that could be the real active agent, in analogy with other azoic prodrugs (e.g. Sulphasalazine [11]). So we considered as reference compound 3,4-diamino-1-naphthalen-1-sulphonic acid (**1b**) as the sodium salt (**1a**) formally derived from CR by hydrogenolysis. However, **1a** was unexpectedly found unstable not only in aqueous solution but also as such thus making the in vitro test unreliable. Therefore, the corresponding acid **1b** was regarded as the parent compound. In addition **1c**, **1d** in which the sulphonic

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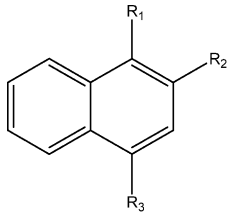
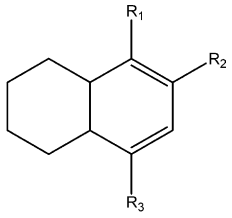
Table 1



1a-f



2f,g

Compounds	R_1	R_2	R_3	Over-stabilising PrP aggregates (WB)
RC				++++
1b	NH_2	NH_2	SO_3H	+++
1c	NH_2	NH_2	COOH	++
1d	NH_2	NH_2	SO_2NH_2	++
1e	NH_2	H	SO_3Na	-
1f	NH_2	NH_2	H	++
2f	NH_2	NH_2	H	++
2g	NH_2	H	SO_3H	-

group of **1b** was, respectively replaced by a carboxyl or sulphonamide group, and **1f** which missed the acidic function were prepared. Finally to verify the importance of the naphthalenic moiety, the tetralin analogues of **1f** (**2f**) and **1g** (**2g**) were synthesized. Unfortunately, any attempt to obtain the partially reduced analogue of **1b** failed.

2. Chemistry

The 3,4-diamino-naphthalen-1-sulphonic-acid (**1b**) was synthesised from CR by reduction with stannous chloride in concentrated hydrochloric acid according to a reported method [12]. The corresponding sodium salt **1a** still unreported in literature, was prepared by reduction of CR with hydrazine monohydrate in the presence of catalytic amount of 10% Pd/C in ethanol (see Scheme 1). 3,4-Diamino-naphthalen-1-carboxylic acid (**1c**) was obtained from the known 3-nitro-naphthalen-1-carboxylic acid [13] by reaction with hydroxylamine hydrochloride in the presence of potassium hydroxide (**3**) [14] followed by catalytic (10% Pd/C) reduction (see Scheme 3). Analogously, **1f** (1,2-diaminonaphthalene) was obtained from the commercially available 2-nitro-naphthalene (see Scheme 3). The sulphonamide **1d** was synthesised from CR as the acetyl derivative **4** by reaction with $POCl_3$ and PCl_5 followed by treatment of the thus obtained **5** with 30% NH_4OH to give **6** which was eventually reduced to **1d** as above described for **1a** (see Schemes 1 and 2). The compound **2f** was synthesised starting from the commercially available 5,6,7,8-tetrahydro-1-amino-naphthalene by acetylation to **8** which was nitrated to give a mixture of 2-nitro (**10a**) and 4-nitro (**10b**) derivatives which were separated by flash chromatography. Deacetylation of **10a** with 6 N HCl followed by catalytic reduction gave **2f** isolated as the hydrochloride (see Scheme 4). Chlorosulphonation of **8** followed by deacetylation with 6 N HCl led to **2g** (see Scheme 5). Attempts to synthesise the 5,6,7,8-tetrahydro analogue **2b** of **1b** by nitration of **2g** followed by catalytic reduction, failed (Fig. 1).

3. Biology

All compounds were tested in a simple cell-free system composed of partially purified PrP^{res}, to evaluate their ability in over-stabilising the PrP^{res} and in inducing the reversion of the protease resistant PrP^{res} into the normal protease sensitive PrP^{sen}. CR was used as the reference compound [9].

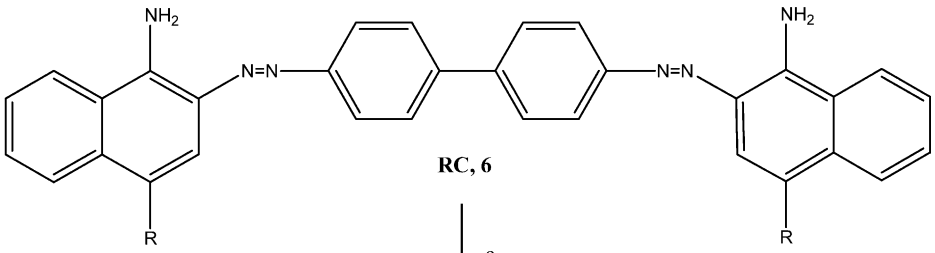
4. Experimental

4.1. Chemistry

Melting points (°C) were determined on a Büchi 510 capillary melting point apparatus and are uncorrected. Analyses indicated by the symbols were within ± 0.4 of the theoretical values. ^{1}H NMR spectra were recorded on a Brucker AC200 spectrometer; chemical shift are reported as δ (ppm) relative to tetramethylsilane (TMS) as internal standard. Dimethyl-d_6 sulphoxide was used as the solvent, unless otherwise noted. The TLC on silica gel plates was used to check product purity. Silica gel 60 (Merck 70–230 Mesh) was used for flash column chromatography. The structure of all compounds were consistent with their analytical and spectroscopic data. The compounds CR and **1e** are commercially available (Sigma–Aldrich) and were used without further purification.

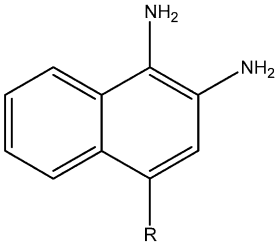
4.1.1. 3,4-Diamino-naphthalene-1-sulphonic acid sodium salt (*1a*)

To a solution of CR (1 g, 1.4 mmol) in EtOH (20 ml) hydrazine hydrate (1 ml, 21 mmol) was added dropwise and the mixture was refluxed for 20 h in the presence of 10% Pd/C (10/1 w/w). After filtration of the catalyst and evaporation in vacuo of the solvent, the residue was triturated in EtOH (2×20 ml) to give a yellow solid which was crystallized from EtOH (76%) $C_{10}H_9N_2NaO_3S$ (260.24) ^{1}H NMR: 8.63 (d, 1H, H–Ar), 7.89 (d, 1H, H–Ar), 7.57 (s, 1H, H-3), 7.05–7.38 (m, 2H, H–Ar), 5.0 (br s, 4H, $2NH_2$ exchangeable with D_2O).



RC, 6

a



RC, 1a R = SO_3Na
6, 1d R = SO_2NH_2

a) NH_2NH_2 H_2O, 10% Pd/C, EtOH, Δ

Scheme 1.

*4.1.2. 3,4-Diamino-naphthalene-1-sulphonic acid (**1b**)*

A solution of stannous chloride (10 g, 52.7 mmol) in concentrated HCl (37.5 ml) was added dropwise to a solution of CR (5 g, 7.18 mmol) in water–EtOH (100 ml 2:1). The mixture was stirred on a warm water bath until decolorization and then further refluxed for 2.5 h. After cooling to 5 °C the yellow solid which separated was filtered and vigorously shaken with MeOH (10 ml). The residue was filtered and crystallized from water to give **1b** (2 g, 59%) $C_{10}H_{10}N_2O_3S$ (238.17) m.p. 274–276 °C ^{1}H NMR: 8.76 (m, 1H, H–Ar), 8.12 (m, 1H, H–Ar), 7.85 (s, 1H, H-3), 7.49 (m, 2H, H–Ar).

*4.1.3. 3-Nitro-4-amino-naphthalene-1-carboxylic acid (**3**)*

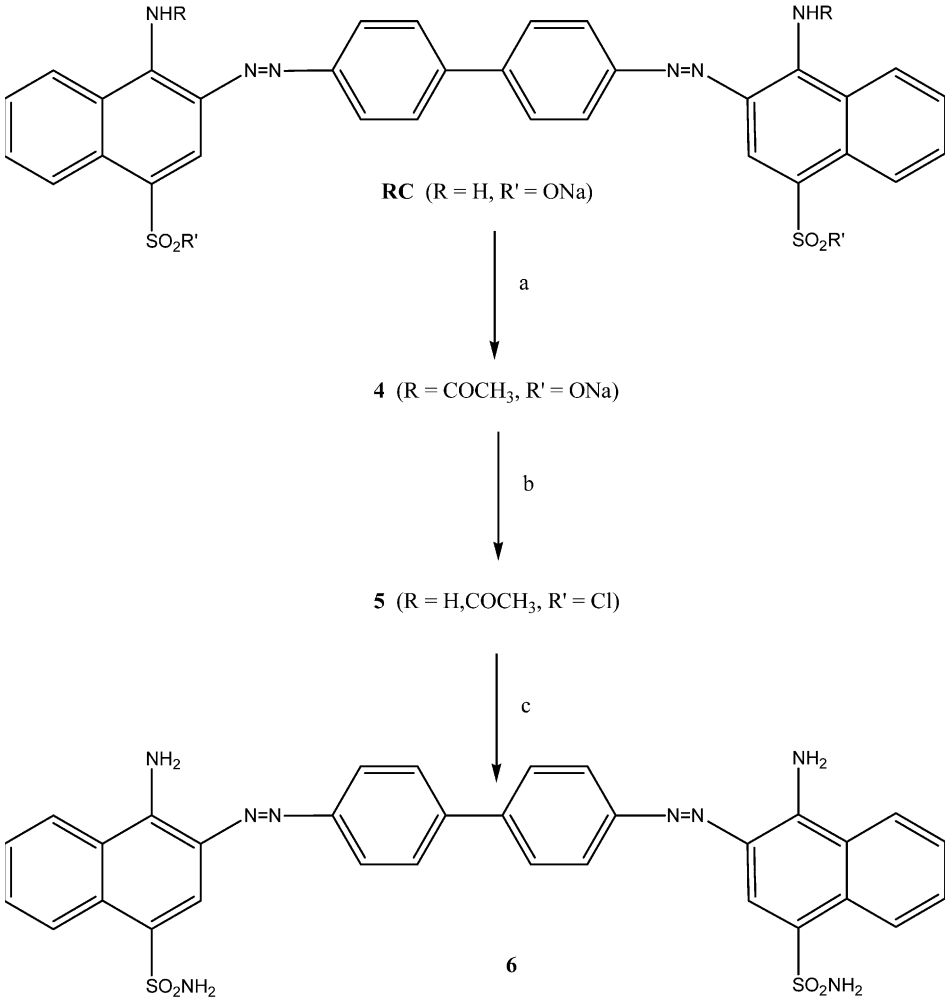
To a solution of 3-nitro-naphthalene-1-carboxylic-acid (0.94 g, 4.3 mmol) and hydroxylamine hydrochloride (1.87 g, 27 mmol) in absolute EtOH (45 ml) a solution of potassium hydroxide (5.61 g, 99.8 mmol) in absolute EtOH (35 ml) was added dropwise. After heating at 50–60 °C for 1 h and cooling at room temperature (r.t.) the reaction mixture was poured into ice and the pH was adjusted to pH 6 with 6 N HCl. The insoluble was filtered and washed with water to give **3** (0.44 g, 44.5%). M.p. 202–204 °C $C_{11}H_8N_2O_4$ (232.19) ^{1}H NMR: 12.8–13.2 (br s, 1H, COOH exchangeable with D_2O), 9.0–9.2 (m, 3H, 1H–Ar+2H NH_2 exchangeable with D_2O), 8.8 (s, 1H, H-2), 8.6–8.7 (m, 1H, H–Ar), 7.6–7.9 (m, 2H, H–Ar).

*4.1.4. 3,4-Diamino-naphthalene-1-carboxylic-acid (**1c**)*

A mixture of **3** (0.84 g, 3.62 mmol) and 10% Pd–C (0.17 g) in absolute EtOH (100 ml) was hydrogenated at r.t. The catalyst was filtered off and the solution was acidified with HCl in Et_2O and evaporated to give **1c** (0.82 g, 95%). $C_{11}H_{10}N_2O_2 \cdot HCl$ (238.67) m.p. > 280 °C. ^{1}H NMR: 10.2–11.0 (br s, 1H, COOH exchangeable with D_2O), 9.1–9.2 (m, 1H, H–Ar), 8.35–8.4 (m, 1H, H–Ar), 8.3 (s, 1H, H-2), 7.4–7.8 (m, 2H, H–Ar).

*4.1.5. 3,3′-{[1,1′-Biphenyl]-4,4′-diylbis(azo)}bis(4-acetyl-amino)-naphthalene-1-sulphonic acid sodium salt (**4**)*

To a mixture of CR (2 g, 2.9 mmol) in AcOH (20 ml) acetic anhydride (0.57 ml, 5.8 mmol) was added dropwise. The solution was refluxed for 1 h. After cooling, the separated solid was filtered and washed with acetone (2 × 5 ml) to yield **4** (2.26 g in almost quantitative yield). M.p. > 300 °C $C_{36}H_{26}N_6Na_2O_6S_2$ (780.73) ^{1}H NMR: 8.8–8.9 (m, 2H, H–Ar), 8.5–8.6 (m, 2H, H–Ar), 8.3 (s, 2H, H-2), 8.1–8.2 (m, 4H, H–benzidine), 8.0–8.1 (m,



a) $(CH_3CO)_2O$, CH_3COOH, Δ ; b) $POCl_3$, PCl_5, Δ ; c) 30% NH_4OH EtOH, Δ.

Scheme 2.

4H, H–benzidine), 7.5–7.6 (m, 4H, H–Ar), 1.95 (s, 6H, $COCH_3$).

*4.1.6. 3,3′-{[1,1′-Biphenyl]-4,4′-dilbis(azo)-}bis(4-amino)-naphthalene-1-sulphonamide (**6**)*

A mixture of **4** (2.0 g, 2.6 mmol), phosphorus pentachloride (2.0 g, 9.6 mmol) and phosphorus oxychloride (3 ml, 16 mmol) was refluxed for 1 h. After cooling, the residue was treated with CH_2Cl_2 (2 × 20 ml) and evaporated to dryness. The black solid isolated (2 g) constituted by crude **5** was directly dissolved in EtOH (12 ml) and to the solution 30% ammonium hydroxide (1.5 ml) was slowly added on cooling until pH 10. The resulting red solution was refluxed for 1.5 h. After cooling, the insoluble red solid was filtered and triturated with potassium bicarbonate (10 ml) and then with acetone (10 ml) to give **6** (1.16 g, 62%) m.p. > 300 °C $C_{32}H_{26}N_8O_4S_2$ (650.72). ^{1}H NMR: 8.84 (d, 2H, H–Ar); 8.52 (br s, 4H, SO_2NH_2); 8.49 (d, 2H, H–Ar); 8.36 (s, 2H, H-2); 8.18 (d, 4H, H–benzidine); 8.02 (d, 4H, H–benzidine); 7.8 (br s, 4H, NH_2 exchangeable with D_2O); 7.59–7.8 (m, 4H, H–Ar).

*4.1.7. 3,4-Diamino-naphthalene-1-sulphonamide (**1d**)*

Compound **1d** was prepared by hydrogenolysis starting from **6** as above reported for **1a** (35%) m.p. 172–173 °C $C_{10}H_{11}N_3O_2S$ (237.28) ^{1}H NMR: 8.43 (d, 1H, H–Ar): 8.06 (d, 1H, H–Ar); 7.74 (s, 1H, H-2); 7.35–7.40 (m, 2H, H-6, H-7); 7.14 (s, 2H exchangeable with D_2O SO_2NH_2); 5.61 (s, 2H exchangeable with D_2O NH_2); 5.00 (s, 2H exchangeable with D_2O NH_2).

*4.1.8. 1,2-Diamino-naphthalene hydrochloride (**1f**)*

Compound **1f** was prepared from the commercial available 2-nitro-napthalene by reaction with NH_2OH

followed by catalytic (10% Pd/C) reduction of the thus obtained **7**, as above reported for **1c**.

For **7** (45.8%) m.p. 144 °C $C_{10}H_8N_2O_2$ (188.18) ^{1}H NMR: 8.13 (d, 1H, H–Ar); 7.95 (d, 1H, H–Ar); 7.79 (d, 1H, H–Ar); 7.62–7.80 (m, 2H, H-6, H-7); 7.38 (s, 2H exchangeable with D_2O NH_2); 7.10 (d, 1H, H–Ar).

For **1f** (72%) m.p. 267 °C $C_{10}H_{12}Cl_2N_2$ (231.11) ^{1}H NMR: 8.56 (s, 6H exchangeable with D_2O NH_2HCl); 8.15 (d, 1H, H–Ar); 7.94 (d, 1H, H–Ar); 7.48–7.60 (m, 2H, H-6, H-7); 7.36 (dd, 2H, H-5, H-8).

*4.1.9. 1-Acetylamino 5,6,7,8-tetrahydro-naphthalene (**8**)*

Compound **8** was obtained by acetylation of the corresponding amine as above reported for **4** (87%) m.p. 151–152 °C $C_{12}H_{15}NO$ (189.26). ^{1}H NMR: 7.52 (d, 2H, H–Ar); 7.00 (m, 2H, H–Ar, H-exchangeable with D_2O $NHCOCH_3$); 2.76–2.85 (m, 2H, H-5, H-8); 2.57–2.65 (m, 2H, H-5, H-8); 2.18 (s, 3H, CH_3); 1.78–1.96 (m, 4H, 2H-6, 2H-7).

*4.1.10. 2-Nitro-1-acetylamino-5,6,7,8-tetrahydro-naphthalene (**10a**)*

To a solution of **8** (10 g, 53 mmol) in AcOH (75 ml) cooled at 5 °C, fuming HNO_3 (9 ml) was added dropwise. After heating at 40 °C for 3 h the reaction mixture was cooled at r.t., poured into ice and extracted with EtOAc (3 × 20 ml). The organic layers were dried over Na_2SO_4, filtered and evaporated in vacuo to give a mixture of **10a** and **10b** which were separated by silica gel flash chromatography eluting with 1:1 petroleum ether–EtOAc. **10a** (2 g, 16%) m.p. 162–164 °C $C_{12}H_{14}N_2O_3$ (234.26). ^{1}H NMR: 9.38 (s, 1H exchangeable with D_2O $NHCOCH_3$); 7.80 (dd, 2H, H-3, H-4); 2.90–2.95 (m, 2H, H-5, H-8); 2.7–2.85 (m, 2H, H-5, H-8); 2.18 (s, 3H CH_3); 1.78–1.96 (m, 4H, 2H-6, 2H-7).

*4.1.11. 2-Nitro-1-amino-5,6,7,8-tetrahydro-naphthalene (**11**)*

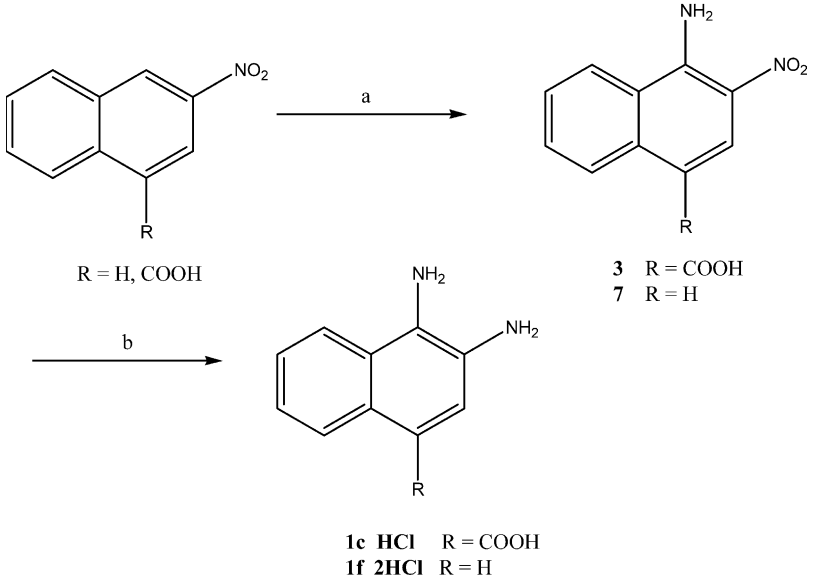
A solution of **10a** (0.5 g, 2.1 mmol) in 6 N HCl (25 ml) was refluxed for 2 h. After cooling, the pH of the mixture was adjusted to 10 with 5 N NaOH then extracted with CH_2Cl_2 (3 × 7 ml). The organic layers were dried over Na_2SO_4, filtered and evaporated in vacuo to give **11** (0.40 g, in almost quantitative yield). M.p. 195–197 °C $C_{10}H_{12}N_2O_2$ (192.21) ^{1}H NMR ($CDCl_3$): 7.84 (d, 1H, H–Ar); 6.70 (d, 1H, H–Ar); 4.18 (br s, 2H exchangeable with D_2O NH_2); 2.9–3.0 (m, 2H, H-5, H-8); 2.43–2.55 (m, 2H, H-5, H-8); 1.78–1.96 (m, 4H, 2H-6, 2H-7).

*4.1.12. 1,2-Diamino-5,6,7,8-tetrahydro-naphthalene (**2f**)*

Compound **2f** was prepared by catalytic hydrogenation starting from **11** as above reported for **1c** (86%) m.p. 234–235 °C $C_{10}H_{16}N_2Cl_2$ (235.04). ^{1}H NMR ($CDCl_3$): 9.0–10.0 (br s, 6H, $2NH_2HCl$); 7.26 (d, H, H-4); 6.52 (d, 1H, H-3); 2.70–3.01 (m, 4H, 2H-5, 2H-8); 1.62–1.93 (m, 4H, 2H-6, 2H-7).

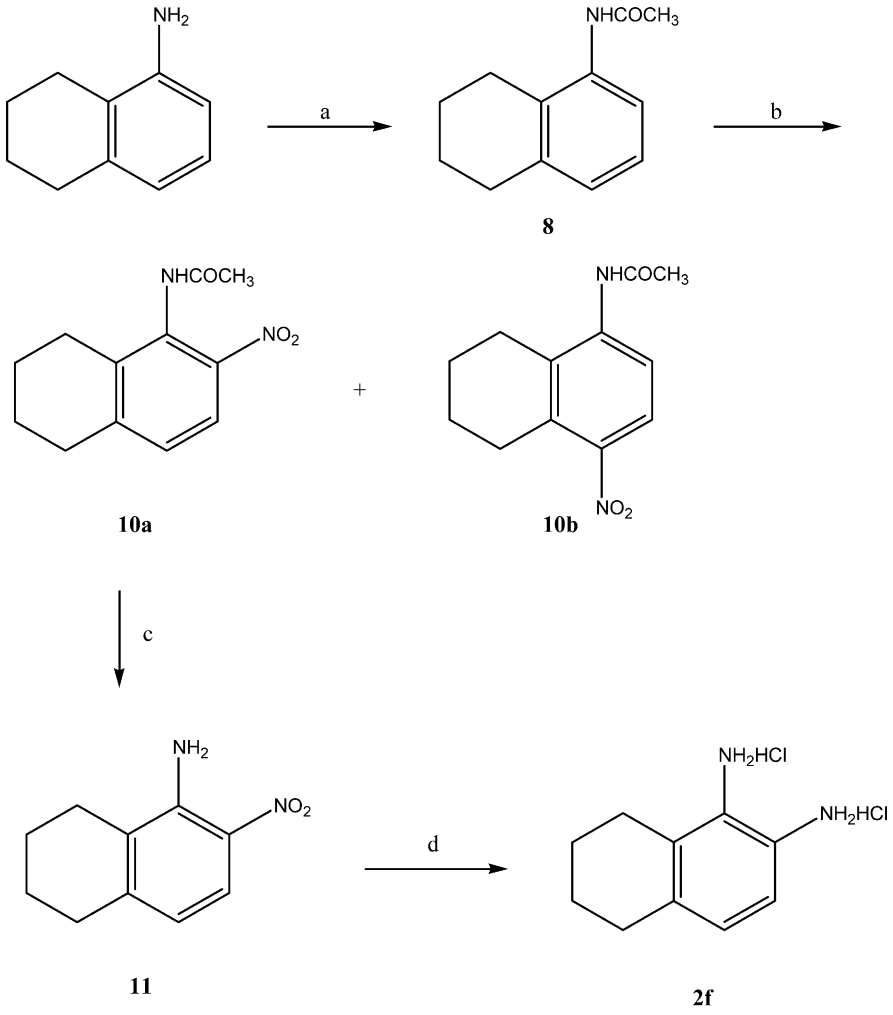
*4.1.13. 1-Acetylamino-4-solphonylchloride-5,6,7,8-tetrahydro-naphthalene (**9**)*

To cooled chloro-sulphonic acid (3.5 ml, 53 mmol) **8** (2 g, 10.56 mmol) was added with caution keeping the temperature at 10–15 °C. After heating at 60 °C for 2 h, then cooling at r.t. the reaction mixture was poured onto ice and the insoluble was filtered off and repeatedly washed with water to give **9** (2.3 g 76.6%). M.p. 184–



a) NH_2OH HCl, KOH, EtOH, Δ. b) H_2, 10%Pd/ C; EtOH, HCl

Scheme 3.



a) $(CH_3CO)_2O$, CH_3COOH, Δ; b) HNO_3, CH_3COOH, Δ; c) 6N HCl, Δ;
d) H_2, 10%Pd/C, EtOH.

Scheme 4.

187 °C $C_{12}H_{14}ClNO_3S$ (287.76). ^1H NMR: 9.25 (s, 1H exchangeable with D_2O $NHCOCH_3$); 7.6 (d, 1H, H–Ar); 7.2 (d, 1H, H–Ar); 2.90–3.00 (m, 2H, H-5, H-8); 2.43–2.59 (m, 2H, H-5, H-8); 2.18 (s, 3H CH_3); 1.68–1.95 (m, 4H, 2H-6, 2H-7).

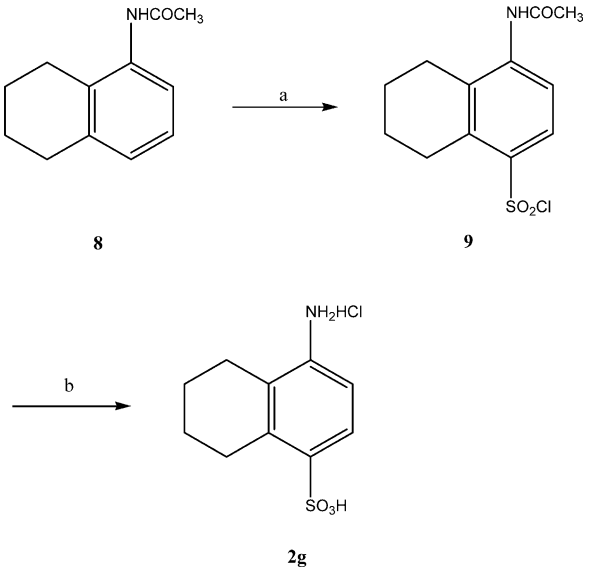
*4.1.14. 1-Amino-4-sulphonic acid-5,6,7,8-tetrahydro-naphthalene (**2g**)*

A solution of **9** (2 g, 6.9 mmol) in 6 N HCl (20 ml) was refluxed for 1 h. After cooling the precipitate was collected by filtration, washed with water and dried in vacuo to give **2g** (1.23 g, 68%). M.p. 201–203 °C $C_{10}H_{14}ClNO_3S$ (262.61) ^1H NMR: 8.8–10.0 (br s, 4H exchangeable with D_2O NH_2HCl, SO_3H); 7.7 (d, 1H, H–Ar); 7.15 (d, 1H, H–Ar); 3.10–3.3 (m, 2H, H-5, H-8); 2.6–2.8 (m, 2H, H-5, H-8); 1.60–2.00 (m, 4H, 2H-6, 2H-7).

4.2. Pharmacology

4.2.1. Purification of PrP^{res}

PrP^{res} was partially purified from brains of Syrian hamster euthanized in the terminal stage after infection with 263k scrapie strain, as previously described [15]; 500 mg of brain was homogenized in 4.5 ml of lysis buffer (100 mmol NaCl, 10 mmol EDTA, 0.5% NP_4O, 0.5% NaDOC, 10 mmol Tris–HCl pH 4) and centrifuged at 6,700 rpm for 15 min at 4 °C. The supernatant was supplemented with an equal volume of sarcosyl 20% and centrifuged at 14.000 rpm for 45 min at 4 °C. Tris-buffered saline containing 0.1% *N*-tetradecyl-*N*,*N*-di-



a) HSO_3Cl, Δ; b) 6N HCl, Δ;

Scheme 5.

methyl-3-ammonium-1-propanesulfonate (TBS+0.1% SB3-14) was added to the supernatant in 1/3 v/v proportion. After centrifugation (55,000 rpm, 4 °C, 2 h) in a TL100 ultracentrifuge (Beckman), the PrP^{res}—containing pellet was re-suspended in TBS (10% NaCl+ 0.1% SB3-14) by sonication and re-centrifuged in a sucrose cushion under the same conditions.

The final pellet was re-suspended by sonication in TBS 0.1% SB3-14 and stored at −20 °C.

4.2.2. PrP^{res} treatment with CR and its derivatives

Samples containing equal amounts of PrP^{res} were incubated at 37 °C for 1 h in the absence or presence of CR or its derivatives, at increasing concentration (25, 50, 250, 500 and 750 μg/ml final).

After incubation, all samples were treated with proteinase K (PK, Boehring Manheim) (300 μg/ml final concentration, 37 °C, 1 h) and digestion was stopped by the addition of PMSF (phenylmethylsulfonyl fluoride, Sigma), 1 mM final concentration. Half of each PK-digested incubation product was treated with 3 M guanidine thiocyanate (pH 2.5) for 10 min and bovine serum albumin (BSA) (200 μg/ml final concentration)

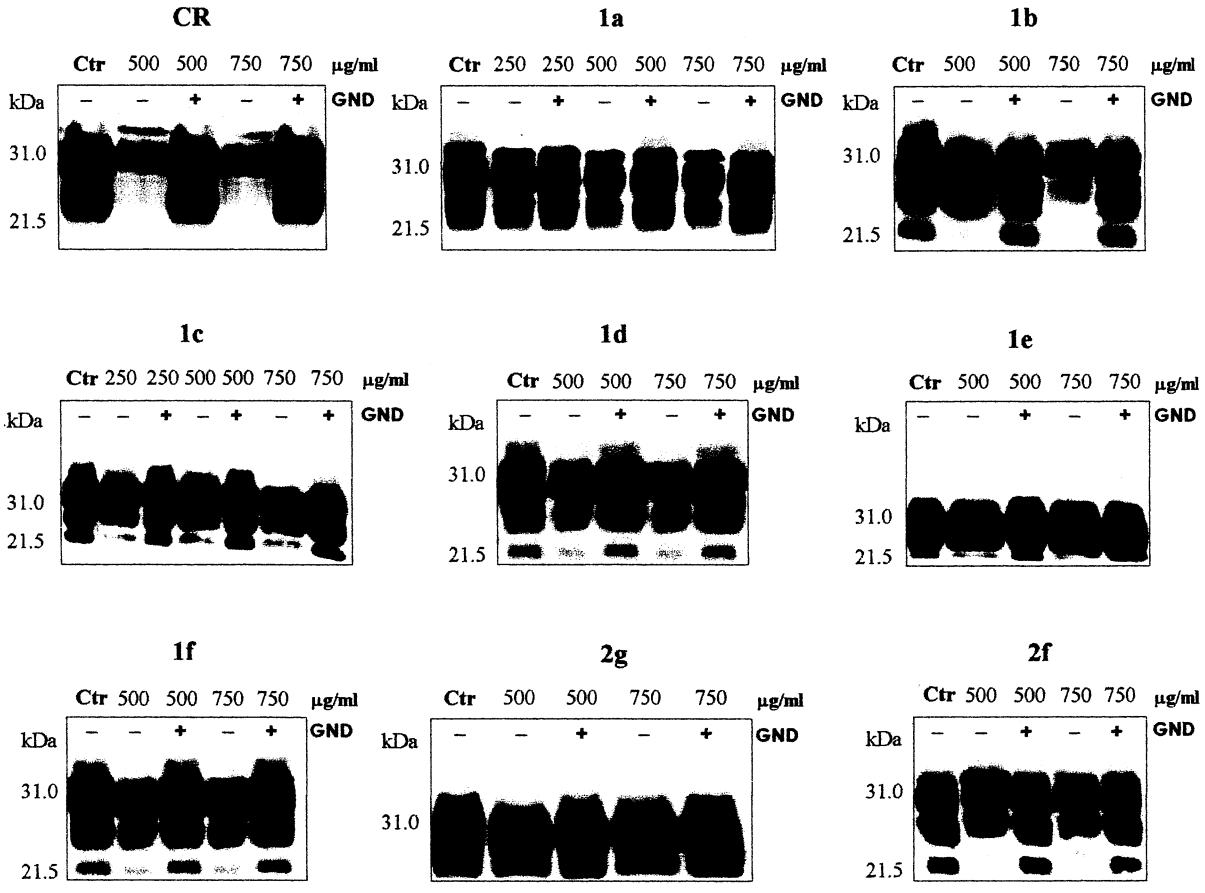


Fig. 1. Western blot analysis: samples containing equal amounts of PrP^{res} were incubated at 37 °C for 1 h alone (Ctr) or with increasing concentrations of Congo red and its derivatives. All samples were evaluated both after digestion with PK, and after treatment with 3 M guanidine thiocyanate (GND+). Above each image the name and concentration of tested compound are reported. The result of **1a** was not significant because the compound was not stable in incubation medium.

Table 2
Microanalyses

	Formula mol. wt.	Calculated (%)	Found (%)
1a	$C_{10}H_9N_2NaO_3S$	C 46.15	C 46.42
	260.25	H 3.49	H 3.51
		N 10.76	N 10.43
		S 12.32	S 12.26
1b	$C_{10}H_{10}N_2O_3S$	C 50.41	C 50.39
	238.26	H 4.23	H 4.22
		N 11.76	N 11.71
		S 13.46	S 13.47
1c	$C_{11}H_{11}ClN_2O_2$	C 55.36	C 55.28
	238.05	H 4.65	H 4.63
		N 11.74	N 11.64
		Cl 14.85	Cl 14.90
1d	$C_{10}H_{11}N_3O_2S$	C 50.62	C 50.56
	237.28	H 4.67	H 4.62
		N 17.71	N 17.81
		S 13.51	S 13.41
1f	$C_{10}H_{12}C_{l2}N_2$	C 51.97	C 51.86%
	231.12	H 5.23	H 5.25
		N 12.12	N 12.14
		Cl 30.68	Cl 30.72
2f	$C_{10}H_{16}Cl_2N_2$	C 51.08	C 51.10
	235.15	H 6.86	H 6.90
		N 11.91	N 11.93
		Cl 30.15	Cl 30.17
2g	$C_{10}H_{14}ClNO_3S$	C 45.54	C 45.56
	263.04	H 5.35	H 5.38
		N 5.31	N 5.24
		Cl 13.44	Cl 13.43
		S 12.16	S 12.25

was added; these last samples were then precipitated with 10 volumes of methanol.

4.2.3. Immunoblotting

All samples were boiled in SDS sample buffer for 10 min, separated on 12% SDS-polyacrylamide gel and transferred electrophoretically to PVDF (polyvinylidene fluoride, Immobilon-P Transfer Membrane, 0.45 μm, Millipore). The membrane was treated in 5% fat-free milk powder (blocking step) in TBST (0.1 Tween 20, 100 mmol NaCl, 10 mmol Tris–HCl; pH 7.8; all reagents purchased by Biorad) and processed with mouse monoclonal anti-PrP antibody 3F4 (diluted 1:50,000) that recognizes the region 109–112 of human and hamster PrP [13]. This antibody was developed by R.J. Kascsak. Horseradish Peroxidase-linked *anti*-mouse Ig (Amersham Pharmacia Biotech), diluted 1:3000, was used in the second staining step, and the reaction developed using ECL (enhanced chemoluminescence, Amersham Pharmacia Biotech) [16].

5. Results and discussion

The data from the biological in vitro assay (see Table 2) showed that **1b** (3,4-diamino-naphthalene-1-sulphonic acid) was comparable to RC in its ability to bind PrP^{res} aggregates. This result seems to support the hypothesis of an in vivo hydrogenolysis of RC by azoreductase to give **1a** as the active moiety, similarly to other pro-drugs (e.g. sulphasalazine). It is to be noted that we were unable to test **1b** as its sodium salt **1a**, having found that it decomposed when kept in the incubation medium at room temperature. Substitution of the SO_3H group of **1b** with –COOH (**1c**) or SO_2NH_2 (**1d**) retained a significant activity, though lower than that of the parent compound **1b**. Unexpectedly, removal of the sulphonic group as in 1,2-diamino-naphthalene (**1f**) led to a still active compound. Interestingly, reduction of the unsubstituted benzenic group of **1f** to **2f** did not affect its activity. On the contrary, the lack of the 2 amino group as in **1e** led to an inactive compound. The importance of this group in eliciting binding properties to PrP^{res}, is also supported by the inactivity of the 4-amino-5,6,7,8-tetrahydro-napthalen-1-sulphonic acid (**2g**).

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