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Orginal article

Synthesis and evaluation of analogues of congo red as potential compounds against transmissible spongiform encephalopathies

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Abstract

The synthesis of analogues of the amyloid stain Congo red (**1**) as potential compounds against transmissible spongiform encephalopathies (TSEs) is reported. Using the direct method, aniline (**2**) or diamines such as 4,4′-diaminodiphenylsulfone (dapsone, **9**), 3,3′-diaminodiphenylsulfone (**10**), benzidine (**11**), 3,3′-dimethoxybenzidine (**12**) or 3,3′-dichlorobenzidine (**13**) were diazotised to afford the corresponding diazonium salts, which without isolation, were directly used for coupling with a range of aromatic sulfonic or carboxylic acids to provide the corresponding truncated dyes analogues of Congo red, **4**, **6**, **8**, and the symmetrical bis azoic dyes **14**–**19**, **21**–**22**, **24** and **26**–**29** as their sodium salts. Compounds were assayed in a cellular model of scrapie, a sheep TSE. Some of the compounds were shown to have similar activity to the lead compound Congo red. Molecular modelling was carried out to investigate potential structure-activity relationships (SARs) relating to the size and shape of Congo Red analogues. Within the range of compounds tested no discernible SARs were found.

Keywords: Prions; Azo dyes; TSE

1. Introduction

Transmissible spongiform encephalopathies (TSEs or prion diseases) are rare progressive diseases giving rise to degeneration of the brain and are fatal with no known method of treatment [1–3]. The diseases can be sporadic and are naturally occurring in some organisms and are linked more or less strongly to genetic mutations. TSEs can also be caused by infection. Thus inoculation of the infectious agent into the brain can give rise to disease. Examples of TSEs include scrapie in sheep; chronic waste disease in elk and deer; bovine spongiform encephalopathy in cattle; fatal familial insomnia, Creutzfeld Jakob disease and Kuru in humans [4]. The diseases exhibit a long asymptomatic incubation period, leading inevitably to a fatal neurological illness.

Transmission of disease within a single species occurs much more readily than inter-species transmission. The infectious agent either requires or is an abnormally folded prion protein. The normal prion protein (PrP^c), occurs in many tissue types, but especially in the central nervous system on the outer surface of neurons and glial cells. The exact function of PrP is nonetheless poorly understood [5,6]. The lack of prion protein has been reported to change the circadian activity rhythms [7] and sleep in mice. The prion protein has an effect on the intracellular free calcium levels [8], and has also been linked to super-oxide dismutase activity [5].

In spongiform encephalopathies, the PrP^c undergoes a conformational change leading to the abnormal form or abnormal prion protein (PrP^{res}) [9]. The abnormal isoform of the prion protein (PrP^{res}) differs from the normal cellular form (PrP^c) in that it has a much higher resistance to proteinase K and has much lower solubility. The normal cellular protein PrP^c has been shown to

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have a α-helical structure (42%) with little β-sheets (3%). In contrast the PrP^{res} has a high β-sheet (43%) content and a slightly reduced α-helical structure (30%) [10]. Both isoforms of PrP appear to have the same amino acid sequence, and the same covalent modifications, including a single disulfide bond, two complex-type N-linked oligosaccharide chains and a C-terminal glycophosphatidyl-inositol (GPI) anchor [11]. During the course of TSEs, the PrP^{res} forms amyloid plaques. It is uncertain if these plaques themselves have a neurotoxic effect or are a result of the disease.

Several classes of compounds including azo dyes, sulphated polysaccharides, porphyrins and polyene antibiotics [12–15] have been shown to have activity against models of prion diseases [16] and on models of other amyloid diseases such as Alzheimer's disease [12,14]. We were particularly interested by the azo dyes, as these may represent small molecules, which may be amenable to structure activity studies. In particular Congo red (**1**, Fig. 1) is an important lead molecule. It has been shown to 'cure' persistently scrapie-infected cells in cell culture. The amyloid-binding dye Congo red has been reported to prolong the incubation period in scrapie infected hamsters [17] when inoculated intracerebrally, presenting modest therapeutic anti-scrapie activity in vivo. The effect of Congo red is similar to the one observed with sulphated glycans [13,15], as it prevents new accumulation of PrP^{res}; furthermore **1** also inhibits the replication of scrapie infectivity.

2. Chemistry

Although Congo red (**1**) shows anti-TSE activity, it is not a good drug candidate because of non-specific binding, toxicity [18–20] and poor penetration of the blood–brain barrier. The inherent carcinogenicity of **1** is probably due to the degradation into benzidine [21] (**11**) in the digestive system and further accumulation in the bladder, leading to bladder cancer [22] if large doses are ingested. We proposed to carry out structure-activity studies in order to identify important features of the dye for activity, to circumvent these problems (selectivity, membrane permeability) and to increase activity. During the course of this present work, a paper was published by Demaimay et al. [23], which discusses some structure-activity relationship (SAR) studies on Congo red. This present study extends their work and presents additional results and conclusions. We thus were interested in the synthesis of analogues of Congo red (**1**) and several modifications are reported. The synthesis of azoic compounds is widely described [24] and the first step of the synthesis involved the formation of the diazonium salt then coupling with aromatic compounds. The procedures used depend on the nature of the arylamine. We mainly used the direct method, which consisted in adding a solution of a metallic nitrite to a cold solution of the arylamine in aqueous mineral acid (the arylamine may be partly in suspension in the form of a salt). We studied then the coupling of several diazonium salts, obtained from either monoamines or diamines, with a range of sulfonic or carboxylic acids derivatives.

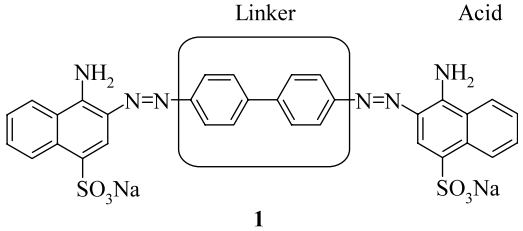

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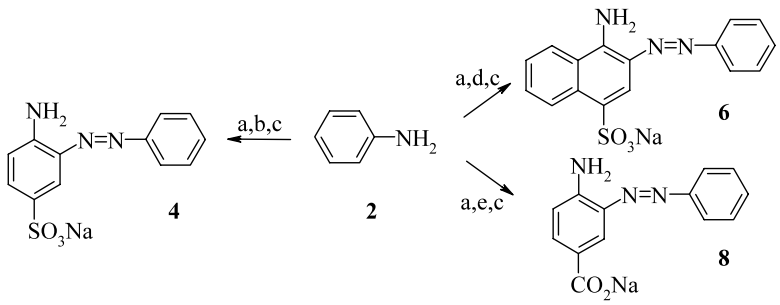
Fig. 1. Congo red.

Congo red (**1**) is a symmetric bis-azo dye and therefore we decided to synthesise firstly the truncated molecules (**4**), (**6**) [25] and (**8**) (Scheme 1) corresponding to only half of the molecule. These compounds provided some information regarding the necessity of the whole molecule for activity, as well as the importance of features such as the nature of the aromatic core (from naphthyl ring to the phenyl ring) or the possibilities of substitution of the sulfonic acid by the corresponding carboxylic derivative. The diazonium salt of aniline (**2**) was obtained according to literature [26] and coupled, without isolation of the diazonium salt, to either sulfanilic acid (**3**), 4-amino-naphthalenesulfonic acid (**5**), or 4-aminobenzoic acid (**7**), commercially available as either their free acids or as their sodium salts. At completion of coupling, as indicated by analysing a sample by mass spectroscopy, addition of 20% sodium chloride solution to the reaction mixture afforded after filtration the corresponding dyes as their sodium salts together with inorganic salts. Purification was obtained either by preparative TLC (1-butanol:ethanol:ammonium hydroxide:pyridine / 4:1:2:3) [27] to yield the dyes (**4**), (**6**) and (**8**) as their pure sodium salts or ion exchange chromatography (Dowex 50WX8-200) in the case of **4** and **6** to give the free acids.

As the anti-TSE activity of such truncated molecules turned out to be significantly decreased [28] (Table 1) in comparison to **1**, indicating the importance of the whole molecule, we decided to synthesise some close analogues of Congo red (**1**) by changing either the nature of the biphenyl spacer originally observed in **1** (length, presence of substituents) or the nature of the acid.

2.1. Variation of spacer

Commercially available diamines (Fig. 2), 4,4′-diamino-diphenylsulfone (dapsone, **9**), 3,3′-diamino-diphenylsulfone (**10**), benzidine (**11**), 3,3′-dimethoxybenzidine



Scheme 1. (a) HCl 10%, 0 °C, $NaNO_2$ 1.05 equiv.; (b) sulfanilic acid (**3**), Na_2CO_3, 0 °C→r.t.; (c) NaCl; (d) 4-amino-naphthalenesulfonic acid (**5**), Na_2CO_3, 0 °C→r.t.; (e) 4-amino-benzoic acid (**7**), Na_2CO_3, 0 °C→r.t.

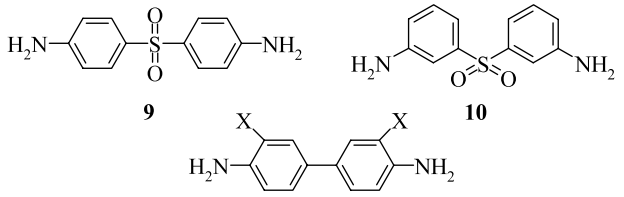


Fig. 2. Linkers.

(**12**) and 3,3′-dichlorobenzidine (**13**) were selected. This should give some variation in the length and also in the electronic and steric properties of the linker. In addition the sulfones **9** and **10** should give rise to different dihedral angles between the two aromatic rings of the linker, alter the electronic properties of the linker and on metabolism give rise to less toxic breakdown products. The 3,3′-dimethoxybenzidine (**12**) and 3,3′-dichlorobenzidine (**13**) were selected as they should cause minor alterations to the steric and electronic properties of the linker.

These linkers were diazotised according to literature precedent [24,26] and coupled with either sulfanilic acid (**3**) or 4-amino-naphthalenesulfonic acid (**5**) to give

Table 1
Dihedral angle between phenyl groups and distance between acid residues

Compd.	Number as in Ref. [28]	PrP^{res} (% of controls)		Distance acid in Å			Angle		
		100 μM	1 μM	Method 1	Method 2	Method 3	Method 1	Method 2	Method 3
1	CR	5.5	53.3	20.34	21.07	20.24 [a]	40.1	46.6	57
4	IV	29.2	99.5						
6	III	63	129.2						
8	V	26.7	99.1						
14	XI	62.7	86.7	19.75	21.35	8.60 [b]	[c]		
15	IX	8.5	117.3	17.49	20.60	5.68 [b]	[c]		
16	X	23.7	142.8	8.31	12.71	9.10 [b]	[c]		
17	VII	5.7	184.6	20.08	20.99	20.43 [a]	40.7	47.8	55.2
18	VI	2.9	218	20.04	21.07	20.94 [a]	41.8	47.1	55.6
19	XII	4.6	187.1	20.36	20.29	20.18 [a]	40.6	46.4	56.7
21	XIV	10.5	148.5	20.45	20.46	20.28 [a]	43.6	46.9	55.2
22	XIII	3.6	223.7	20.30	21.40	20.24 [a]	41.7	45.9	56.8
24	XV	5.4	218.3	14.86	14.91	14.10	43.3	48.4	57.2
26	XXI	46.8	103.3	21.46	20.42	8.9 [a,d]			
						17.27		[c]	
27	XXII	56.4	72.7	16.63	18.71	8.2 [a,d]			
						18.99		[c]	
28	XIX	2.5	107.2	20.20	20.20	19.84	39.7	47.2	54.9
29	XX	23.5	115.4	20.15	20.44	19.79	43.5	48.3	54.7

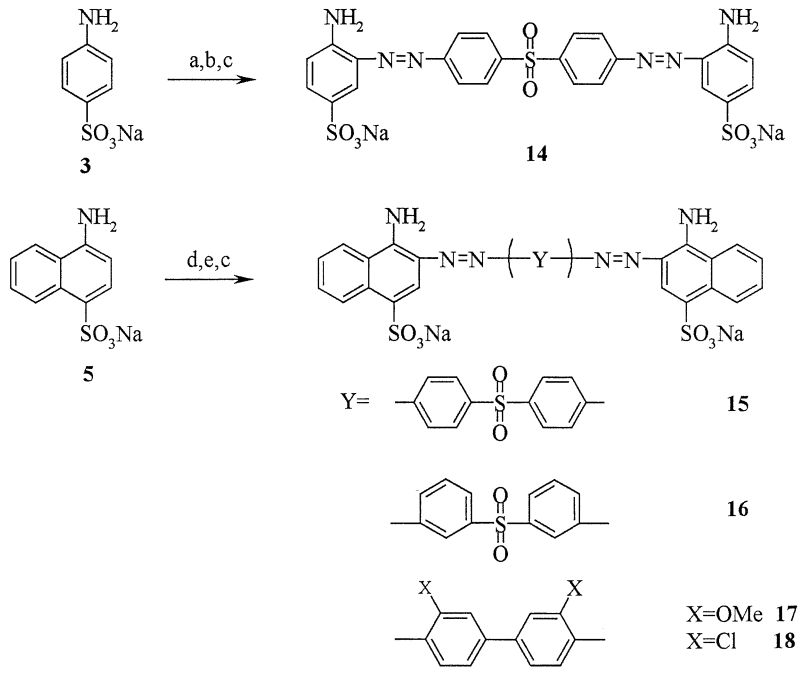
Method 1: Monte-Carlo conformational search. *Method 2:* quantum mechanics. *Method 3:* simulated annealing.

[a] H-bonding between either the amino or the hydroxyl and the azo bonds.

[b] Population of conformers with strong intramolecular H-bonding between the amino or the hydroxyl and the sulfonic group on the other naphtyl or phenyl unit.

[c] Sulfones angle not defined.

[d] Second population of conformers within a range of 3–5 kcal from the population of conformers of lowest energy. The azo bonds present a staggered conformation.



Scheme 2. (a) 4,4′-Diaminodiphenyl sulfone **9**, HCl, $NaNO_2$, 0 °C; (b) Na_2CO_3, 0 °C → r.t., then reflux 1 h; (c) 10% NaCl; (d) linkers **9**, **10**, **12** or **13**, HCl, $NaNO_2$, 0 °C; (e) Na_2CO_3, 0 °C → r.t. then 50 °C for **12** and **13**.

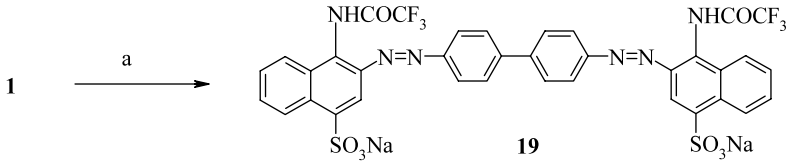
compounds **14**–**18** (Scheme 2). The products were contaminated with sodium chloride and then purified by soxhlet extraction followed by preparative TLC. The structure of each compound was determined by one and or two dimensional ^1H and ^{13}C-NMR experiments. Compound **15** [29] was prepared according to the general procedure. The procedure for the preparation of compound **14** was slightly modified as the reaction did not go to completion when sulfanilic acid (**3**) was coupled to the diazonium salt of 4,4′-diaminodiphenylsulfone (**9**). Compound **14** was prepared as a bright yellow solid after a 1 h reflux of the aqueous solution of the reaction mixture followed by cooling and addition of 10% sodium chloride solution. The dye was obtained with a low yield, probably resulting from the degradation of the diazonium salt through the heating. The formation of compounds **17** [30] and **18** [31] resulting from the coupling of 4-amino-naphthalenesulfonic acid (**5**) with the diazonium salts of 3,3′-dimethoxybenzidine (**12**) and 3,3′-dichlorobenzidine (**13**) required also further heating of the reaction mixture. In order to prevent decomposition of the diazonium salt, we decided not to heat the aqueous solution higher than 50 °C. This temperature was sufficient to obtain the dyes with good yield after 4–5 h.

2.2. Variation of the terminal substituents

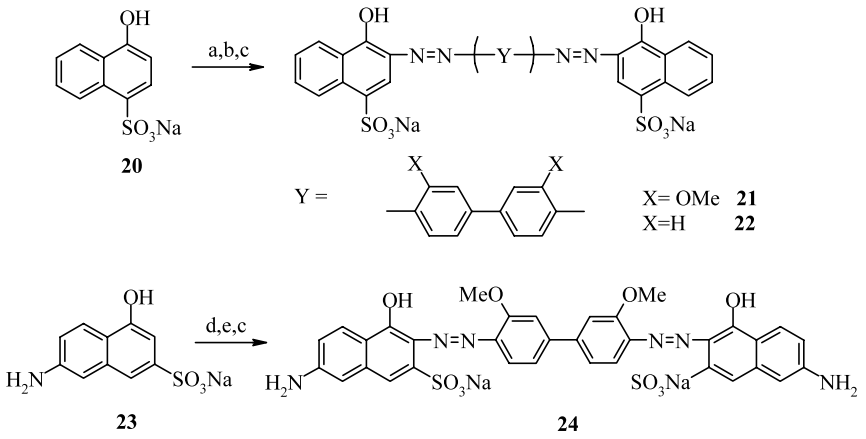
Systematic variation of the terminal substituents was undertaken. Firstly the necessity of the amino functionality was probed, and then variation of the sulfonic acid was carried out.

2.2.1. Variation of the amino functionality

The first variation attempted was to acetylate the amino functionality in Congo red. Reacting Congo red with acetic anhydride did not yield the desired *N*-acetylated derivative. However, reaction with trifluoroacetic anhydride led to conversion to the trifluoroacetamide derivative (**19**) (Scheme 3). Thus a solution of Congo red (**1**) in anhydrous *N*,*N*′-dimethylformamide was treated with an excess of trifluoroacetic anhydride at 0 °C for 1 h to give the N-protected compound. The solvents were removed and the product washed several times with methanol to give **19**, as indicated by ^{19}F-NMR experiment and mass spectroscopy.



Scheme 3. (a) $(CF_3CO)_2O$, DMF, 0 °C → r.t., 1 h.



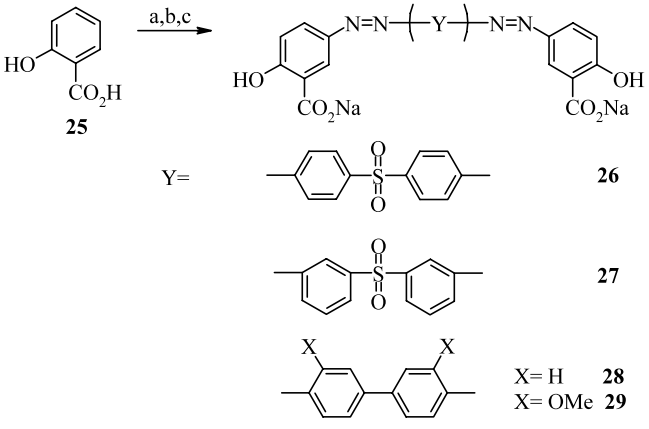
Scheme 4. (a) Linkers **9**, **11** or **12**, HCl, $NaNO_2$, 0 °C; (b) Na_2CO_3 0 °C → r.t., then 50 °C;(c) 10% NaCl. (d) Linker **12**, HCl, $NaNO_2$, 0 °C; (e) Na_2CO_3 0 °C → r.t., then 50 °C.

A series of compounds were also prepared in which the amino functionality was replaced by a hydroxyl group (Scheme 4). Benzidine (**11**) and 3,3′-dimethoxybenzidine (**12**) were diazotised according to literature [24] and the corresponding diazonium salts were coupled at 0 °C with 4-hydroxynaphthalene sulfonic acid (**20**) to give diazo compounds **21** [32] and **22** [33]. As previously discussed those were contaminated with sodium chloride. The structure of each compound was determined by one and or two dimensional ^1H and ^{13}C-NMR experiments. The synthesis of compounds **21** and **22** required further heating of the aqueous solution at 50 °C in order to obtain the corresponding dyes. This temperature was sufficient to permit the completion of the reaction and prevent degradation of the diazonium salt.

One further variation was attempted by coupling 6-amino-1-naphthol-3-sulfonic acid (**23**) (Scheme 4) to the diazonium salt of 3,3′-dimethoxybenzidine (**12**) which should give further variation in the terminal region. The orientation of the coupling can be controlled as the coupling is reported to be pH dependent [34,35] this controlled reaction was used in the manufacture of direct dyes to obtain bis-azo dyes. During this coupling reaction, the pH was controlled at >7–8 as at pH <3–4 the coupling occurred on the ring bearing the amino functionality. In basic conditions, the coupling occur in *ortho* of the hydroxyl moiety giving rise to the desired compound. No starting material **23** was detected by mass spectroscopy after completion of the reaction and the experimental ^1H and ^{13}C-NMR of the product **24** are in agreement with the proposed structure.

2.2.2. Variation in the nature of the acid

The replacement of the sulfonate group with less acidic groups, which should improve pharmacokinetic properties and selectivity, was then studied. This has precedence from the work on Alzheimer's disease where it has been shown that replacing the sulfonate with carboxylate groups as with Chrysamine G (**28**) leads to compounds with improved binding to β-amyloid [12,36] and better pharmacokinetic properties. We thus studied the coupling (Scheme 5) of the different diamines, 4,4′-diaminodiphenylsulfone (**9**), 3,3′-diaminodiphenylsulfone (**10**), benzidine (**11**) and 3,3′-dimethoxybenzidine (**12**) with salicylic acid (**25**). Chrysamine G (**28**) was previously synthesised [37,38] according to a similar procedure. All reactions proceeded at room temperature (r.t.), once the diazonium solution was added at 0 °C. The evolution of the coupling was monitored by mass spectroscopy. Addition of the solution of the diazonium salts of **9**–**13** obtained according to literature [24] to a cold solution of **25** in Na_2CO_3 was followed after completion of coupling by addition of 10% NaCl solution to give the diazo compounds **26**–**29** [39] as dark brown solids, which were then purified by soxhlet extraction using 95% ethanol (compounds **26**, **27**, **29**) or preparative TLC using 1-butanol:ethanol:ammonium hydroxide:pyridine / 4:1:2:3 for compound **28** [27].



Scheme 5. (a) Linkers **9**, **10**, **11** or **12**, HCl, $NaNO_2$, 0 °C; Na_2CO_3; (b) Na_2CO_3 0 °C → r.t.; (c) 10% NaCl.

3. Modelling studies

Studies in models of Alzheimer's disease [12] and Demaimay's study [23] of prion diseases have indicated the importance of the dihedral angle between the two phenyl rings of the benzidine for activity. In these studies, active compounds were calculated to have a dihedral angle of about 35°. When the dihedral angle was significantly increased (for example by 2,2′-substitution) activity decreased.

In this study, three methods were investigated to calculate the dihedral angle. In addition, we decided to measure the distance between the acidic groups to see if this led to any SARs. There are several difficulties that need to be taken into account with the modelling studies:

a) The 'active' conformation, presumably bound to the abnormal or PrP^c, may not be in the lowest energy.
b) The molecules will be solvated, which will have an effect on the conformation.
c) There may be several low energy conformations.
d) The dyes may exist as a supramolecule [40–42] in solution depending on the concentration of dye.

3.1. Method 1: Monte-Carlo conformational search

We carried out molecular modelling on O2 Silicon Graphics workstations for each compound using Macromodel® 6.0 and performing a Monte-Carlo conformational search with the Amber force field and the GB/SA solvation model for water as implemented in Macromodel. The structures were drawn in the 'draw' mode with the double bond *trans* and the sulfonate and carboxylate groups represented in the anionic form. The dihedral angle and the intra-molecular distance between either the two sulfonates or the two carboxylate groups of each dye were measured and the set of results obtained for each compound is shown in Table 1.

3.2. Method 2: quantum mechanics

Similar data was obtained using quantum mechanics calculations (in vacuum) by minimising the compounds in Sybyl (using Geisteiger–Hückel charges, the tripos forcefield, distance dependent dielectric constant to help mimic the shield effects of solvent and steepest descent minimization utilising an energy gradient cut-off at 0.001 kcal mol^{-1}) and subsequent loading of the minimised structures in Mopac (PM3 hamiltonian with full geometry optimization in internal coordinate space). This predicted dihedral values larger by about 5–6° and similar bond length values.

3.3. Method 3: simulated annealing

Another set of results was obtained by loading in Sybyl the minimised structures previously obtained in Mopac followed by simulated annealing (using Geisteiger–Hückel charges, the tripos forcefield and distance dependent dielectric constant to help mimic the shield effects of solvent) for each compound by heating at 1000 K during 1000 fs and then cooling to 0 K for 2000 fs. This cycle was repeated for 100 times. We selected then for each compound the conformations of lowest energy obtained. The predicted dihedral values are reported in Table 1 and generally are larger by 15–17°. Interestingly strong intramolecular hydrogen bonding was observed (Table 1). Insertion of a sulfone (compounds **14**, **15** and **16**) within the biphenyl system induced strong intramolecular hydrogen bonding between one part of the molecule, bearing the sulfonic moiety, with the amino moiety of the other part, thus forcing the molecule to loose its symmetry and then adopting a 'cage-like' conformation (Fig. 4). The set of results obtained for compounds **14** and **15** bearing a sulfone in position 4,4′- is different using this last method in comparison to the previous data. This may be due to insufficient searching of conformational space in methods 1 and 2, when compared to simulated annealing.

4. Biological data

The compounds synthesised were assayed for their ability to inhibit the conversion of PrP^c to its proteinase-resistant conformer (PrP^{res}) in scrapie-infected SMB cells. Compounds were tested at two different concentrations, 100 and 1 μM, using Congo red (**1**) as reference. This evaluation has been published in detail elsewhere [28] but the results are presented in Fig. 3 to facilitate cross-referencing and discussion of structure-biological activity relationships.

5. Discussion

The aim of this work was the synthesis and evaluation of some analogues of Congo red (**1**) as potential anti-scrapie agents with persistently scrapie-infected SMB cells as a model for TSEs. Systematic variation was carried out on a number of different features of the molecule.

5.1. The symmetry

Congo red is a symmetrical molecule. Our data suggests that the whole molecule is required for full

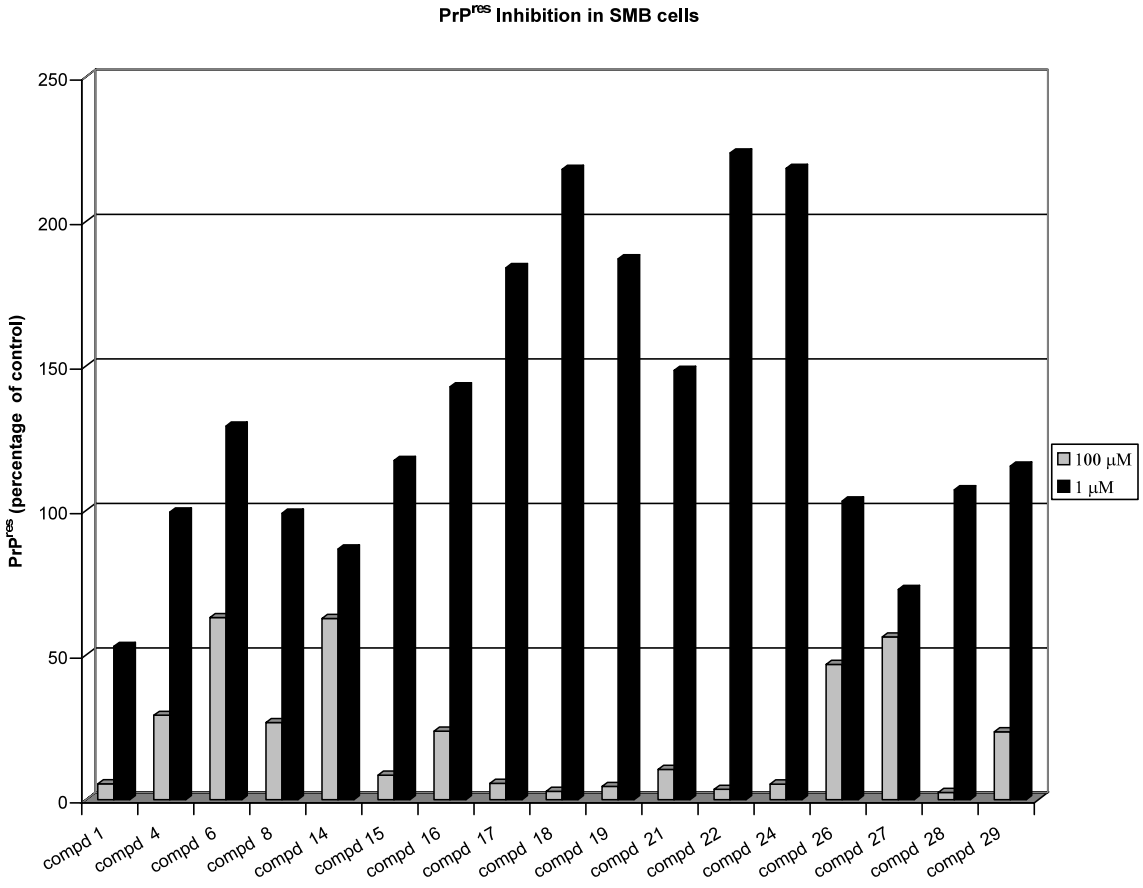


Fig. 3. Biological activity.

activity (compare compounds **4**, **6** and **8** with compound **1**).

5.2. The spacer

It is possible to make some changes in the central spacer without a major effect on activity at 100 μM, though there is some loss of activity at 1 μM (compounds **14**–**18**). Thus compounds **14**, **15** and **16** have sulfones in the molecule. Compounds **17** and **18** have substituents on the 3,3′-positions of the benzidine ring. These do not seem to have a large effect on the dihedral angle of the molecule and hence not on the conformation of the molecule.

5.3. The amino group

Acetylation of the amino groups with trifluoroacetic anhydride (compound **19**) did not have a large effect on activity at 100μM. Similarly replacement of the amino group with a hydroxyl group (compounds **21**, **22** and **24**) did not seem to have a marked effect on activity at 100 μM. This means that it is not necessary to have a positive charge at this position, although the necessity of having an H-bond donor at this position cannot be ruled out.

5.4. The acid group

Congo red (**1**) has a sulfonic acid group. At physiological pH this group is completely ionised and will probably prevent uptake across the blood–brain barrier [12]. Compounds **26**–**29** represent compounds in which the sulfonate has been replaced by a carboxylate, which should have greater permeability across the blood–brain barrier. With the benzidine-derived compounds **28**–**29**, there was little effect on activity compared to Congo red (**1**) at 100μM. However with compounds **26** and **27**, the sulfone analogues, there was lower activity.

Modelling was undertaken with three different methods to see if there were any major differences. The features that have been mentioned as possibly being important for activity are the distance between the sulfonic acid moieties and the dihedral angle across the benzidine unit [23]. For most of the compounds described here, the three modelling methods predicted relatively similar distances and dihedral angles (where these could be determined) (Table 1).

5.5. Benzidine derived analogues (compounds 1, 17 and 18)

In the examples described here there did not seem to be a very wide variation in distance and angle and there was no discernible relationships between activity and either distance or dihedral angle. Demaimay et al. [23] reported some compounds (2,2′-substituted benzidines) which had a significantly larger dihedral angle, than those 3,3′-substituted molecules; these compounds appeared to have lower activity.

5.6. Sulfone analogues (14–16 and 26–27)

An interesting variation described here is the sulfones. Two types of sulfone analogues were synthesised with the sulfone either in position 1,1′- (**14**, **15** and **26**) or in position 2,2′- (**16** and **27**). The sulfones introduce a bend into the molecule, destroying the planarity of the linker unit. A small decrease in activity was observed at 100 μM when modifying the position of the sulfone from 1,1′- (**15**) to 2,2′-(**16**). Compound **15** had thus similar activities to the benzidine derived analogues (**1**, **17** and **18**). The exceptions to this were compounds **14**, **26** and **27**. The differences in activity observed for **14** may be related to the modification of the aromatic ring from a naphthyl unit as in **1** to a phenyl unit, rather than the presence of a sulfone. Compounds **26** and **27** showed decreased activity at 100 μM compared to their benzidine analogues **28** and **29**. Interestingly, two populations of conformers were observed (within 3 kcal mol^{-1}) when using simulated annealing. The population of conformers of lowest energy presented strong intramolecular H-bonding between the hydroxyl moiety and the azo bond (Fig. 4). This decrease in activity could be due to the coexistence of those two populations. When compounds **14**–**16** were studied by simulated annealing, the lowest energy conformations predicted strong local H-hydrogen bonding (Table 1, Fig. 4) which brought the two sulfonic acid groups closer to each other, as well as intramolecular π-stacking. It is interesting that some of the sulfones have activity despite having a bend in the molecule. This bend could reduce the possibility of intermolecular p-stacking, which has been speculated to be important in the mechanism of these types of compounds [40–42].

Therefore in summary, a number of azo dyes related to Congo red have been prepared and then screened against a persistently scrapie-infected cell line. It proved possible to make modifications to the structure of Congo red to either increase its lipophilicity or to remove the central benzidine unit without having a large reduction in activity against the scrapie model of infection. According to the modelling, most of the compounds contained similar dihedral angles at the biphenyl ring and a relatively similar distance between the charged sulfonic acid/carboxylic acid groups.

6. Experimental part

All solvents and chemicals were purchased either from Fluka, Aldrich or Jansen. Water refers to deionised water. Preparative TLC were carried out either on precoated silica plates (Kieselgel 60 F_{254} BDH) or on precoated silica plates made from silica (Silica gel GF_{254}) with visualisation via UV light and/or $KMnO_4$ solution. All ^{1}H, ^{13}C and ^{19}F-NMR spectra were recorded on a

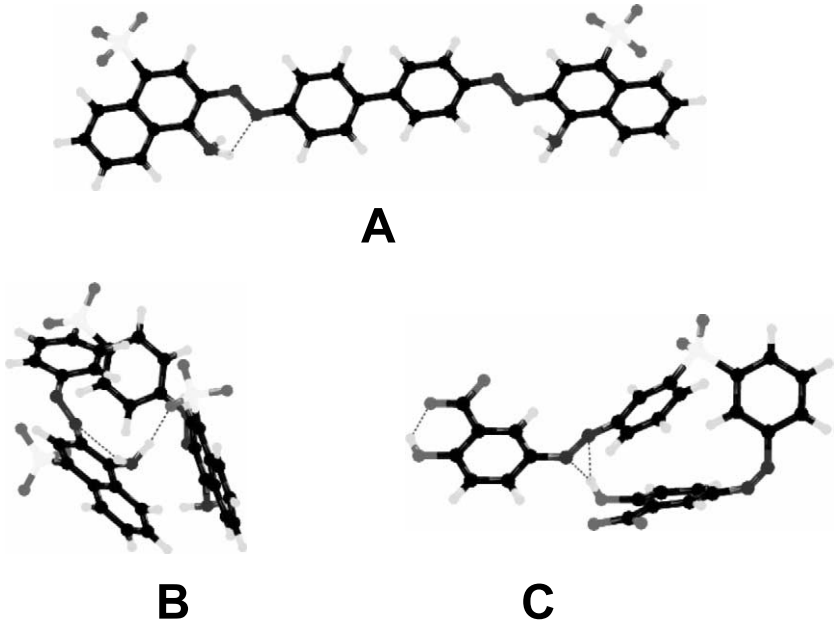


Fig. 4. Lowest energy conformations from simulated annealing (method 3); (A) congo red, compound **1**; (B) compound **15**; (C) compound **27**.

Bruker Advance DPX300 spectrometer at 300, 75 and 282.38 MHz, respectively. Chemical shifts are reported downfield in parts per million using $CDCl_3$ as an internal reference at 7.27 ppm unless otherwise stated and all coupling constants (J values) are in hertz. Low-resolution mass spectra were recorded on a Fisons VG platform Electrospray mass spectrometer. High-resolution mass spectra were recorded by the EPSRC National Mass Spectrometry Service Centre (Chemistry department, Swansea). The name of the different compounds was given according to the IUPAC rules and we decided to number the compounds as indicated on Fig. 5 for the purpose of simplification of the ^1H and ^{13}C-NMR spectra. Molecular modelling was carried out on O2 Silicon Graphics workstations. Appropriate safety measures were used for compounds **11**, **12** and **13**, which are known to be carcinogenic [21].

6.1. 4-Amino-3- (2-phenyl-1-diazenyl)-1-benzenesulfonic acid, sodium salt (*4*)

Aniline (**2**) was diazotised by adding 1 equiv. of 4 N $NaNO_2$ (5.5 mL) to a solution of the amine (2 mL; 21.47 mmol) in a 2% HCl solution (100 mL) at 0–5 °C. After completion of diazotisation (~ 2 h), the light orange diazonium solution was divided in two equal parts and directly used. A solution of the diazonium salt (52 mL) was added dropwise to a solution of sulfanilic acid (**3**) (1.869 g; 10.97 mmol) in 5% Na_2CO_3 (60 mL). The light brown-orange residue obtained after filtration was dried to give the dye as its sodium salt with inorganic salts. A sample (208 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 159 mg of a yellow dark solid. A sample was then purified by preparative TLC (1-butanol:ethanol:ammonium hydroxide:pyridine / 4:1:2:3) to give the pure compound. ^1H-NMR (300 MHz, DMSO-d^6) δ_H 7.63 (d, 2H, $J = 8.5$ Hz, H-3 and H-5), 7.46 (d, 1H, $J = 6.8$ Hz, H-8), 7.44 (d, 1H, $J = 6.8$ Hz, H-11), 7.40 (td, 3H, $J = 2.6$ and 8.5 Hz, H-1, H-2 and H-6), 7.17 (t, 1H, $J = 6.8$ Hz, H-12); HR-ES$^-$-MS Calc. for $C_{12}H_{10}N_3O_3SNa$ [M-Na^+]$^-$ 276.0443 Found: 276.0443.

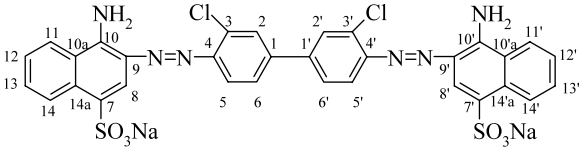


Fig. 5. Example of NMR numbering.

6.2. 4-Amino-3-(2-phenyl-1-diazenyl)-1-naphthalenesulfonic acid, sodium salt (*6*) [25]

A solution of the diazonium salt of aniline (52 mL) was added dropwise at 0 °C to a solution of 4-amino-naphthalenesulfonic acid sodium salt (as its bis-hydrate) (**5**) (2.63 g; 10.97 mmol) in 5% Na_2CO_3 (60 mL). The red-orange residue obtained after filtration was dried to give the dye as its sodium salt with inorganic salts. A sample (200 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 157 mg of a yellow dark solid. 95 mg of compound **6** were also purified directly by preparative TLC (1-butanol:ethanol:ammonium hydroxide:pyridine / 4:1:2:3) to yield 71 mg as the pure compound. Treatment of another sample by ion exchange resin (Dowex 50WX8-200) gave the corresponding free acid. ^1H-NMR (300 MHz, CD_3OD) δ_H 8.78 (d, 1H, $J = 8.3$ Hz, H-14), 8.62 (s, 1H, H-8), 8.31 (d, 1H, $J = 8.2$ Hz, H-11), 7.89 (AB, 2H, H-3, H-5), 7.65 (dd, 1H, $J = 1.2$ and 8.3 Hz, H-13), 7.65–7.48 (m, 3H, H-1, H-2, H-6), 7.41 (d, 1H, $J = 1.2$ Hz, H-12); ^{13}C-NMR (75 MHz, CD_3OD) δ_C 151.63 (C-4), 145.46 (C-10), 131.56 (C-9), 129.26 (C-14a), 129.22 (C-7), 125.09 (C-10a), 122.23 (C-8); 129.83, 129.33, 128.99, 127.49, 125.09, 123.65, 123.07 (ring); HR-ES$^-$-MS Calc. for $C_{16}H_{12}N_3O_3SNa$ [M-Na^+]$^-$ 326.0599 Found: 326.0600.

6.3. 4-Amino-3-(2-phenyl-1-diazenyl)-1-benzoic acid, sodium salt (*8*)

Aniline (**2**) was diazotised at 0 °C by addition of a 4 N $NaNO_2$ solution (2.75 mL; 757 mg) to a solution of the amine (1 mL; 10.97 mmol) in 2% HCl (20 mL). After completion of diazotisation (~ 2 h), the diazonium solution (~ 11 mL; 5.48 mmol) was then added dropwise to a solution of 4-amino benzoic acid (**7**) (1 equiv.; 950 mg) in 5% Na_2CO_3 (50 mL). After 2 days stirring at r.t., the reaction was stopped by adding 10% NaCl solution (100 mL) and the reaction mixture filtered to give 2.257 g of the dried dye with inorganic salts (156% yield). ^1H-NMR (300 MHz, DMSO-d^6) δ_H 7.93 (d, 2H, $J = 8.3$ Hz, H-3 and H-5), 7.53–7.32 (m, 5H, H-1, H-2, H-6, H-8 and H-11), 7.22 (t, 1H, $J = 7$ Hz, H-12); ^{13}C-NMR (75 MHz, CD_3OD) δ_C 168.1(CO_2Na); C-IV: 148.21, 146.55, 126.16; ring: 131.85, 130.17, 127.31, 125.47, 120.22, 118.21 and 116.1. HR-ES$^-$-MS Calc. for $C_{13}H_3N_2O_{10}Na$ [M-Na^+]$^-$ 240.0773 Found: 240.0773.

6.4. bis-(4-(1-Amino-4-sulfo-2-phenylazo)-phenyl)-sulfone, di-sodium salt (*14*)

Dapsone (**9**) was tetrazotised by adding 2 mL of 4 N $NaNO_2$ to the diamine (1 g; 4.027 mmol) in 2% HCl (35 mL). The solution of the diazonium salt (2.013 mmol) was added dropwise at 0 °C to a solution of sulfanilic

acid (**3**) (697 mg; 2 equiv.) in 5% Na_2CO_3 (60 mL). After 2 h at r.t., only a small amount of the dye could be detected by low resolution [ES^--MS $(M\text{-}2Na^+)^-$ 616.63; 307.2 $((M\text{-}2Na^+)^{2-}$]. The crude mixture was triturated with small pieces of cotton [43] clothes and heated at reflux for 3 h. The remaining solution was concentrated and the water was removed by evaporation to give the 1.04 g dye (**14**). ^{1}H-NMR (300 MHz, DMSO-d^6): δ_H 7.93 (dd, 2H, $J = 2.7$ and 8.7 Hz, H-12 and H-12′), 7.86 (d, 4H, $J = 8.8$ Hz, H-3, H-3′, H-5 and H-5′), 7.60–7.56 (m, 8H, H-2, H-2′, H-6, H-6′, H-8, H-8′, H-11 and H-11′); ^{13}C-NMR (75 MHz, DMSO-d^6) δ_C C-IV: 147.26, 146.77, 145.47, 134.87, 134.59; ring: 128.49, 128.32, 128.25, 127.89, 126.10, 118.19, 117.91; HR-ES^--MS Calc. for $C_{24}H_{18}N_6O_8S_3Na_2$ $[M\text{-}2Na^+]^{2-}$ 307.0174 Found: 307.0174.

6.5. bis-(4-(1-Amino-4-sulfo-2-naphtylazo)-phenyl)-sulfone, di-sodium salt (15) [29]

A solution of the diazonium salt of Dapsone (**9**) (2.013 mmol) was added at 0 °C dropwise to a solution of 4-amino-naphthalenesulfonic acid sodium salt (**5**) (as its bis-hydrate) (987 mg; 2 equiv.) in 5% Na_2CO_3 (60 mL). 2.74 g of dye was obtained after filtration as its sodium salt with inorganic salts. A sample (345 mg) of the crude mixture was purified by ion exchange chromatography to give 83 mg of the dye as its free acid form. A sample (47 mg) was also purified using by preparative TLC (1-butanol:ethanol:ammonium hydroxide:pyridine / 4:1:2:3) to give 24 mg of the pure dye (**15**). ^{1}H-NMR (300 MHz, CD_3OD) δ_H 8.75 (d, 2H, $J =$ 8.1 Hz, H-14 and H-14′), 8.55 (s, 2H, H-8 and H-8′), 8.32 (d, 2H, $J = 8.4$ Hz, H-11 and H-11′), 7.89 (AB, 8H, $J = 8.7$ Hz, H-1, H-1′, H-2, H-2′, H-3, H-3′, H-4 and H-4′); 7.65 (ddd, 2H, $J = 1.2$, 8.4 Hz, H-13 and H-13′), 7.54 (ddd, 2H, H-12 and H-12′); ^{13}C-NMR (75 MHz, CD_3OD) δ_C 154.82 (C-4), 150.80 (C-1), 144.82 (C-10), 139.54, 132.56, 130.34, 130.23, 130.15 (C_{IV}), 129.69, 128.18, 126.55, 125.49, 124.47 (ring) 123.51 (C-8). HR-ES^--MS Calc. for $C_{32}H_{22}N_6O_8S_3Na_2$ $[M\text{-}2Na^+]^{2-}$ 357.0331 Found: 357.0333.

6.6. bis-(3-(1-Amino-4-sulfo-2-naphtylazo)-phenyl)-sulfone, di-sodium salt (16)

3,3′-Diamino biphenyl sulfone (**10**) was tetrazotised by adding 4 mL of 4 N $NaNO_2$ to the diamine (2 g; 8.05 mmol) in 2% HCl (50 mL). The solution of diazonium salt (2.685 mmol) was added dropwise to a solution of **5** (1.316 g; 2 equiv.) in 5% Na_2CO_3 (50 mL). The reaction mixture was stirred for 2 days at r.t., then a 10% NaCl solution (30 mL) was added and 3.257 g of dye was obtained after filtration as its sodium salt with inorganic salts. A sample (70 mg) was purified by preparative chromatography (1-butanol:ethanol:ammonium hydroxide:pyridine / 4:1:2:3) to yield two fractions of **16**, respectively 35 and 16 mg, the latter being of a lesser degree of purity. ^{1}H-NMR (300 MHz, DMSO-d^6) δ_H 8.73 (d, 2H, $J = 8.3$ Hz, H-14 and H-14′), 8.62 (s, 2H, H-8 and H-8′), 8.55 (d, 2H, $J = 8.3$ Hz, H-11 and H-11′), 8.31 (s, 2H, H-2 and H-2′), 8.28 (d, 2H, $J = 8.1$ Hz, H-4 and H-4′), 8.10 (s, NH_2), 8.08 (d, 2H, $J = 8.1$ Hz, H-6 and H-6′), 7.77 (t, 2H, $J = 8.1$ Hz, H-5 and H-5′), 7.61 (t, 2H, $J = 8.3$ Hz H-13 and H-13′), 7.49 (t, 2H, $J = 8.3$ Hz, H-14 and H-14′); ^{13}C-NMR (75 MHz, DMSO-d^6) δ_C C-IV: 153.56, 146.95, 142.68, 132.69, 132.36, 129.04, 124.53; ring: 131.11, 128.95, 128.46, 127.76, 127.59, 125.40, 124.46, 120.62, 118.37. HR-ES^--MS Calc. for $C_{32}H_{22}N_6O_8S_3Na_2$ $[M\text{-}2Na^+]^{2-}$ 357.0331 Found: 357.0334.

6.7. 4,4′-Diamino-3,3′-(3,3′-dimethoxy-biphenyl-4,4′-diyl-bis-azo)-bis-naphthalene-1-sulfonic acid, di-sodium salt (17) [30]

3,3′-Dimethoxybenzidine (**11**) was purchased as its free base; a 4 N $NaNO_2$ solution (2 equiv.; 1.2 mL) was added at 0 °C under vigorous stirring to a solution of the diamine (576 mg; 2.36 mmol) in 2% HCl solution. The flask was protected from the light, as the starting material is light sensitive. The evolution of the tetrazotisation was followed by MS and the reaction went to completion after 3 h. The light brown-orange solution of diazonium salt (2.36 mmol) was added dropwise at 0 °C through a canular to a solution of 4-amino naphthalene sulfonic acid sodium salt (**5**) (1.156 g; 2 equiv.) in Na_2CO_3 (30 mL). The evolution of the reaction was monitored by MS and no coupling was observed after 2 h. The reaction mixture was stirred overnight at r.t., and then heated for 6 h at 50 °C for completion of the coupling. The reaction mixture was cooled down and a 20% NaCl solution (100 mL) was then added; after filtration, the dye (**17**) was obtained as a dark red solid (1.64 g) with inorganic salts. A sample (200 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 126 mg as the pure compound **17**. ^{1}H-NMR (300 MHz, CD_3OD) δ_H 8.74 (d, 2H, $J = 8$ Hz, H-14 and H-14′); 8.61 (s, 2H, H-8 and H-8′); 8.27 (d, 2H, $J = 8.2$ Hz, H-5 and H-5′); 7.62–7.35 (m, 8H, H-2, H-2′, H-6, H-6′, H-12, H-12′, H-13 and H-13′); 4.13 (s, 6H, OCH_3); HR-ES^--MS Calc. for $C_{34}H_{26}N_6O_8S_2Na_2$ $[M\text{-}2Na^+]^{2-}$ 355.0627 Found: 355.0628.

6.8. 4,4′-Diamino-3,3′-(3,3′-dimethoxy-biphenyl-4,4′-diyl-bis-azo)-bis-naphthalene-1-sulfonic acid, di-sodium salt (18) [31]

3,3′-Dichlorobenzidine (**13**) was purchased as its chlorhydrate salt and was delivered in isopac bottle containing 990–999 mg of material. 2% HCl (50 mL) was added. A second aliquot of 30 mL was then added

due to poor solubility. The solution was transferred through a canular in a flask and a 4 N $NaNO_2$ solution (2 equiv.; 1.55 mL) was added at 0 °C under vigorous stirring. The evolution of the tetrazotisation was followed by MS and the reaction went to completion after 2 h. A solution of diazonium salt (1.022 mmol) was added dropwise at 0 °C to a solution of 4-amino naphthalene sulfonic acid, sodium salt (**5**) (501 mg; 2 equiv.) in Na_2CO_3 solution (30 mL). The reaction mixture was stirred overnight at r.t., after which a sample was analysed by MS indicating the completion of the coupling. A 20% NaCl solution (50 mL) was then added and after filtration, the dye was obtained as a dark red solid (586 mg) with inorganic salts. A sample (70 mg) was purified by preparative TLC (1-butanol:ethanol:ammonium hydroxide:pyridine / 4:1:2:3) to give 66 mg of the pure dye **18**. ^{1}H-NMR (300 MHz, CD_3OD) δ_H 8.91 (broad s, NH_2), 8.76 (d, 2H, $J = 8.3$ Hz, H-14 and H-14′), 8.53 (d, 2H, $J = 8.3$ Hz, H-11 and H-11′), 8.33 (s, 2H, H-8 and H-8′), 8.15 (d, 2H, $J = 1.9$ Hz, H-2 and H-2′), 8.11 (d, 2H, $J = 8.7$ Hz, H-5 and H-5′), 7.93 (dd, 2H, $J = 1.9$ Hz and 8.7 Hz, H-6 and H-6′), 7.64 (t, 2H, $J = 8.5$ Hz, H-13 and H-13′), 7.52 (t, 2H, $J = 8.5$ Hz H-12 and H-12′); ^{13}C-NMR (75 MHz, DMSO-d^6) δ_C C-IV: 147.51, 143.93, 139.11, 132.77, 132.27, 131.50, 129.15; ring: 128.66, 127.95, 126.10, 124.91, 123.98, 122.87, 122.80, 117.50. HR-ES$^-$-MS Calc. for $C_{32}H_{20}N_6O_6S_2Cl_2Na_2$ $[M\text{-}2Na^+]^{2-}$ 359.0132 Found: 359.0133.

6.9. 4,4′-Di-(2,2,2-trifluoroacetyl)-amino-3,3′-(biphenyl-4,4′-diyl-bis-azo)-bis-naphthalene-1-sulfonic acid, di-sodium salt (*19*)

A large excess of trifluoroacetic anhydride (0.5 mL) was added at 0 °C to a solution of Congo red (**1**) (100 mg; 0.143 mL) in dry DMF (2.5 mL). The reaction mixture was stirred and monitored by MS. The reaction went to completion after 1 h. The solution was washed several times with MeOH to eliminate the excess of trifluoroacetic anhydride and the solvents were removed under vacuum to give 124 mg of the desired compound as a light brown-orange material. ^{1}H-NMR (300 MHz, CD_3OD) δ_H 9.02 (dd, 2H, $J = 8.4$ and 2.1 Hz, H-14 and H-14′), 8.79 (s, 2H, H-8 and H-8′), 8.22 (dd, 2H, $J = 8.4$ and 2.1 Hz, H-11 and H-11′), 8.14 (d, 4H, $J = 8.5$ Hz, H-5, H-5′, H-3 and H-3′), 8.03 (d, 4H, $J = 8.5$ Hz, H-2, H-2′, H-6 and H-6′), 7.80 (ddd, 2H, $J = 1.8$ Hz, 6.6 and 8.6 Hz, H-13 and H-13′), 7.77 (ddd, 2H, $J = 1.8$, 6.6 and 8.6 Hz, H-12 and H-12′); ^{13}C-NMR (75 MHz, CD_3OD) δ_C 152.81, 143.52, 143.37, 142.70, 133.71, 131.38, 131.18, 128.82, 128.16, 127.86, 123.91, 123.75, 114.51. ^{19}F-NMR δ_F −73.54. HR-ES$^-$-MS Calc. for $C_{36}H_{20}N_6O_8S_2F_6Na_2$ $[M\text{-}2Na^+]^{2-}$ 421.0344 Found: 421.0348.

6.10. 4,4′-Dihydroxy-3,3′-(3,3′-dimethoxy-biphenyl-4,4′-diyl-bis-azo)-bis-naphthalene-1-sulfonic acid, di-sodium salt (*21*) [32]

3,3′-Dimethoxybenzidine (**11**) was tetrazotised by adding 4 N $NaNO_2$ solution (2 equiv.; 0.65 mL) to a solution of the diamine (300 mg; 1.228 mmol) in 2% HCl solution (20 mL). The light brown-orange solution of diazonium salt (0.409 mmol) was then added dropwise through a canular to a solution of 4-hydroxy naphthalene sulfonic acid (**20**) (70% pure; 288 mg; 2 equiv.) in Na_2CO_3 (30 mL). The evolution of the reaction was monitored by MS and no coupling was observed after 2 h. The reaction mixture was stirred overnight at r.t., after which a sample was analysed by MS indicating the completion of the coupling. A 10% NaCl solution (100 mL) was then added and after filtration, the dye (**21**) was obtained as a dark red solid (424 mg) with inorganic salts. A sample (209 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 126 mg as the pure compound **21**. ^{1}H-NMR (300 MHz, DMSO-d^6) 8.51 (d, 2H, $J = 8$ Hz, H-14 and H-14′), 8.43 (d, 2H, $J =$ 7.9 Hz, H-11 and H-11′), 8.16 (s, 2H, H-8 and H-8′) 7.72–7.84 (m, 2H, H-5 and H-5′), 7.50–7.32 (m, 6H, H-2, H-2′, H-6, H-6′, H-13 and H-13′), 7.24–7.32 (m, 2H, H-12 and H-12′); 4.12 (s, 6H, OCH_3); HR-ES$^-$-MS Calc. for $C_{34}H_{24}N_4O_{10}S_2Na_2$ $[M\text{-}2Na^+]^{2-}$ 356.0467 Found: 356.0467.

6.11. 4,4′-Dihydroxy-3,3′-(biphenyl-4,4′-diyl-bis-azo)-bis-naphthalene-1-sulfonic acid, di-sodium salt (*22*) [33]

Benzidine (**11**) [*Caution:* benzidine is carcinogenic] was tetrazotised at 0–5 °C, by adding a 4 N $NaNO_2$ solution (2.05 equiv.; 0.39 mL) to a solution of the diamine (140 mg; 0.76 mmol) in HCl N (5 mL). The evolution of the tetrazotisation was followed by MS and the reaction went to completion after 90 min. The diazonium solution was then added dropwise through a canular at 0 °C to a solution of 4-hydroxy naphthalene sulfonic acid (**20**) (70% pure; 535 mg; 2 equiv.) in saturated Na_2CO_3 (10 mL). The reaction mixture was stirred overnight at r.t., after which a sample was analysed by MS indicating the completion of the coupling. A 20% NaCl solution (50 mL) was then added and after filtration, the dye was obtained as a dark red solid (424 mg) with inorganic salts. A sample (223 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 188 mg as the compound **22**. ^{1}H-NMR (300 MHz, DMSO-d^6) δ_H 8.51 (d, 2H, $J = 8$ Hz, H-14 and H-14′), 8.43 (d, 2H, $J = 7.9$ Hz, H-11 and H-11′), 8.16 (s, 2H, H-8 and H-8′) 7.80–7.72 (m, 2H, H-5 and H-5′), 7.54–7.38 (m, 6H, H-2, H-2′, H-6, H-6′, H-13 and H-13′), 7.24–7.32 (m, 2H, H-12 and H-12′); HR-ES$^-$-MS Calc. for $C_{32}H_{20}N_4O_8S_3Na_2$ $[M\text{-}2Na^+]^{2-}$ 326.0362 Found: 326.0364.

6.12. 4,4′-Dihydroxy-3,3′-(3,3′-dimethoxy-biphenyl-4,4′-diyl-bis-azo)-bis-naphthalene-6-amino-2-sulfonic acid, di-sodium salt (**24**)

3,3′-Dimethoxybenzidine (**12**) was tetrazotised by adding 4 N $NaNO_2$ solution (2 equiv.; 0.65 mL) to a solution of the diamine (300 mg; 1.228 mmol) in 2% HCl solution (20 mL). The light brown-orange solution of diazonium salt (0.409 mmol) was then added dropwise through a canular to a solution of 6-amino-1-naphtol-3-sulfonic acid (**23**) (90% pure; 217 mg; 2 equiv.) in Na_2CO_3 (30 mL). The evolution of the reaction was monitored by MS and no coupling was observed after 2 h. The reaction mixture was stirred overnight at r.t., after which a sample was analysed by MS indicating the reaction was not complete, therefore the reaction mixture was heated first 2 h at 50 °C then 2 days further; the advancement of the reaction was monitored and after 2 days, as no further evolution could be observed a 10% NaCl solution (50 mL) was then added and after filtration, the dye (**24**) was obtained as a dark red solid (901 mg) with inorganic salts and remaining starting material (**23**). A sample (211 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 149 mg as the pure compound **24**. ^{1}H-NMR (300 MHz, DMSO-d^6) δ_H 7.98 (d, 2H, $J = 8.6$ Hz, H-5 and H-5′), 7.93 (d, 2H, $J = 8.5$ Hz, H-10 and H-10′), 7.42 (d, 2H, $J = 8.6$ Hz, H-6 and H-6′), 7.42 (s, 2H, H-14 and H-14′), 7.22 (s, 2H, H-2 and H-2′), 6.65 (d, 2H, $J = 8.4$ Hz, H-11 and H-11′), 6.63 (s, 2H, H-13 and H-13′), 6.41 (broad s, NH_2), 4.07 (s, 6H, OCH_3); HR-ES$^-$-MS Calc. for $C_{34}H_{26}N_6O_{10}S_2Na_2$ $[M\text{-}2Na^+]^{2-}$ 371.0576 Found: 371.0577.

6.13. 6,6′-Dihydroxy-3,3′-(4,4′-sulfonyl-bis-biphenylazo)-di-benzoic acid, di-sodium salt (**26**) [39]

A solution of the diazonium salt of 4,4′-diaminodiphenylsulfone (**9**) (2.01 mmol) was added dropwise to a solution of salicylic acid (**25**) (556 mg; 2 equiv.) in 5% Na_2CO_3 (50 mL). The reaction mixture was stirred overnight at r.t., then heated for 6 h at 50 °C. Addition of 10% NaCl solution to the cold mixture and ensuing filtration gave 1.82 g (153%) of dye as a brown solid as its sodium salt with inorganic salts. A sample (214 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 184 mg as the compound **26**. ^{1}H-NMR (300 MHz, CD_3OD) δ_C 8.53 (d, 2H, $J = 2.5$ Hz, H-8 and H-8′), 8.09 (AB, 8H, $J = 8.6$ Hz, H-2, H-2′, H-3, H-3′, H-5, H-5′, H-6 and H-6′), 7.96 (dd, 2H, $J = 2.5$, 8.8 Hz, H-10 and H-10′), 6.95 (d, 2H, $J = 8.8$ Hz, H-11 and H-11′); ^{13}C-NMR (75 MHz, CD_3OD) δ_C C-IV: 173.46 (CO_2Na), 166.13, 155.38, 144.53, 141.64, 118.99; ring: 128.51, 127.91, 126.13, 122.69, 116.95; HR-ES$^-$-MS Calc. for $C_{26}H_{16}N_4O_8SNa_2$ $[M\text{-}2Na^+]^{2-}$ 272.0345 Found: 272.0343.

6.14. 6,6′-Dihydroxy-3,3′-(3,3′-sulfonyl-bis-biphenylazo)-di-benzoic acid, di-sodium salt (**27**)

A solution of the diazonium salt of 3,3′-diaminodiphenyl sulfone (**10**) (2.215 mmol) was added dropwise at 0 °C to a solution of salicylic acid (**25**) (612 mg; 2 equiv.) in saturated Na_2CO_3 (50 mL). The reaction mixture was stirred overnight at r.t., then heated for 2 h at 50 °C. After addition of 10% NaCl solution to the cold reaction mixture and filtration, 1.376 g of the dye (**26**) was obtained as its sodium salt with inorganic salts. A sample (212 mg) was purified soxhlet extraction to give 207 mg of **26**. ^{1}H-NMR (300 MHz, DMSO-d^6) δ_H 8.29 (d, H, $J = 2.8$ Hz, H-8 and H-8′), 8.28 (d, 2H, $J = 2.1$ Hz, H-2 and H-2′), 8.06 (dd, 4H, $J = 2.1$ and 7.8 Hz, H-4, H-4′, H-6 and H-6′), 7.82 (dd, 2H, $J = 2.8$ and 8.8 Hz, H-10 and H-10′), 7.77 (t, 2H, $J = 7.8$ Hz, H-5 and H-5′), 6.73 (d, 1H, $J = 8.8$ Hz, H-11 and H-11′); HR-ES$^-$-MS Calc. for $C_{26}H_{16}N_4O_8SNa_2$ $[M\text{-}2Na^+]^{2-}$ 272.0345 Found: 272.0342.

6.15. 6,6′-Dihydroxy-3,3′-biphenyl-4,4′-diyl-bis-azo-di-benzoic acid, di-sodium salt (**28**) [37,38]

Benzidine was tetrazotised at 0–5 °C, by adding a 4 N $NaNO_2$ solution (2.05 equiv.; 0.39 mL) to a solution of the diamine (140 mg; 0.76 mmol) in HCl N (5 mL). The reaction mixture was stirred for 90 min. The diazonium solution was then added to a solution of salicylic acid (**25**) (2 equiv.) in saturated Na_2CO_3 solution (10 mL). A 10% NaCl solution (30 mL) was then added. The dye was filtered and dried overnight. A sample was purified by preparative chromatography to give the pure compound **28**. ^{1}H-NMR (300 MHz, CD_3OD) δ_H 8.29 (d, H, $J = 2.6$ Hz, H-8 and H-8′), 7.29 (AB, 8H, H-2, H-2′, H-3, H-3′, H-4, H-4′, H-6 and H-6′), 7.81 (dd, 2H, $J = 2.6$ and 8.7 Hz, H-10 and H-10′), 6.77 (d, 2H, $J = 8.7$ Hz, H-11 and H-11′); HR-ES$^-$-MS Calc. for $C_{26}H_{16}N_4O_6Na_2$ $[M\text{-}2Na^+]^{2-}$ 240.0535 Found: 240.0531.

6.16. 6,6′-Dihydroxy-3,3′-(3,3′-dimethoxy-biphenyl-4,4′-diyl-bis-azo)-dibenzoic acid, di-sodium salt (**29**)

3,3′-Dimethoxybenzidine (**12**) was tetrazotised by adding 4 N $NaNO_2$ solution (2 equiv.; 0.95 mL) to a solution of the diamine (519 mg; 2.124 mmol) in 2% HCl solution (30 mL). The light brown-orange solution of diazonium salt was then added dropwise through a canular to a solution of salicylic acid (**25**) (587 mg; 2 equiv.) in saturated Na_2CO_3 (30 mL). The evolution of the reaction was monitored by MS the reaction mixture was kept overnight under stirring at r.t. A 10% NaCl solution (150 mL) was then added and after filtration, the dye was obtained as a solid (869 mg) with inorganic salts. A sample (221 mg) was treated by soxhlet extraction (200 mL; 95% ethanol) to give 180 mg as

the pure compound **29**. ^{1}H-NMR (300 MHz, d6-DMSO) δ_H 8.26 (d, 2H, $J = 2.6$ Hz, H-8 and H-8′), 7.82–7.77 (m, 2H, $J = 2.6$ and 8.7 Hz, H-10 and H-10′), 7.65–7.54 (m, 2H, H-5 and H-5′), 7.47–7.40 (m, 2H, H-6 and H-6′), 7.36–7.28 (m, 2H, H-2 and H-2′), 6.79 (dd, 2H, $J = 2.5$ and 8.7 Hz, H-11 and H-11′), 4.09 (s, 3H,OMe);HR-ES$^-$-MS Calc. for $C_{28}H_{20}N_4O_8Na_2$ [M-2Na$^+$]$^{2-}$ 270.0641 Found: 270.0640.

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