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Short communication

Phenylsulfonylnitromethanes as potent irreversible inhibitors of aldose reductase

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Abstract – Aldose reductase (AR) inhibition provides a viable pharmacologically direct mode for the treatment of diabetic complications. We have synthesized a series of N-4 substituted analogues (**15–21**) of the known aldose reductase inhibitor phenyl-sulfonylnitromethane. The compounds are potent inhibitors of AR with IC_{50}s between 0.01 and 0.19 μM. Some of the compounds are also potent affinity labels for AR. Compound **19** exhibits the highest and almost complete irreversible inhibition of AR known to date. © 1999 Éditions scientifiques et médicales Elsevier SAS

aldose reductase / diabetic complication / phenylsulphonyl-nitromethane / irreversible inhibitors / aldose reductase inhibitors

1. Introduction

Over 50% of diabetics develop tissue-damaging complications [1]. These complications develop in tissues capable of insulin-independent glucose uptake and result in retinopathy, nephropathy, cataract, keratopathy, neuropathy and angiopathy. Results of the recent Diabetes Control and Complications Trial suggest that tight control of blood sugar levels can reduce the incidence and severity of diabetic complications [1]; however, on a practical basis, tight control is difficult to maintain. This has spurred efforts toward the development of alternative treatments with agents acting by mechanisms independent of the control of blood glucose. Aldose reductase inhibitors (ARIs) have provided therapeutically useful agents [2], which in long-term animal studies demonstrate beneficial prevention or delay in the onset and progression of diabetic complications with no significant adverse effects [3]. The search for clinically useful ARIs has been an on-going process since the late 1960s. This effort has led to the discovery of a number of structurally diverse compounds as ARIs *(figure 1)*.

Kinetic studies suggest that despite their structural variations, ARIs exhibit either uncompetitive or noncompetitive inhibition and bind to a site on the enzyme which is independent of the substrate and NADPH cofactor binding sites [4–6]. Using affinity-labelling studies, Kador et al. [7] demonstrated that there are three distinct binding sites on the AR enzyme, namely, the substrate site, cofactor site and inhibitor site. This observation is, however, at odds with recent X-ray crystallographic studies involving the ternary complex between AR, NADPH and zopolrestat in which the inhibitor was found completely sequestered into the substrate site [8]. In an effort to study the nature of the inhibitor binding site of AR, we previously reported a series of affinity labels (**7–10**) *(table I)* based on the reversible inhibitor alrestatin [9]. Based on the remarkable irreversible inhibitory activity of 5-iodoacetamidoalrestatin (**10**) in our previous studies [7, 9] and the possibility of using affinity labels to locate the binding site(s) of ARIs on AR, we synthesized affinity labels (**15–17**) and (**19–21**) along with their known acetylated derivatives (**14** and **18**) *(table I)*, derived from a new class (sulfonylnitromethanes) of potent AR inhibitors, and examined the compounds for AR inhibitory activity.

2. Chemistry

Sulfonylnitromethanes have been synthesized by three principal synthetic routes. These include the sulfonation

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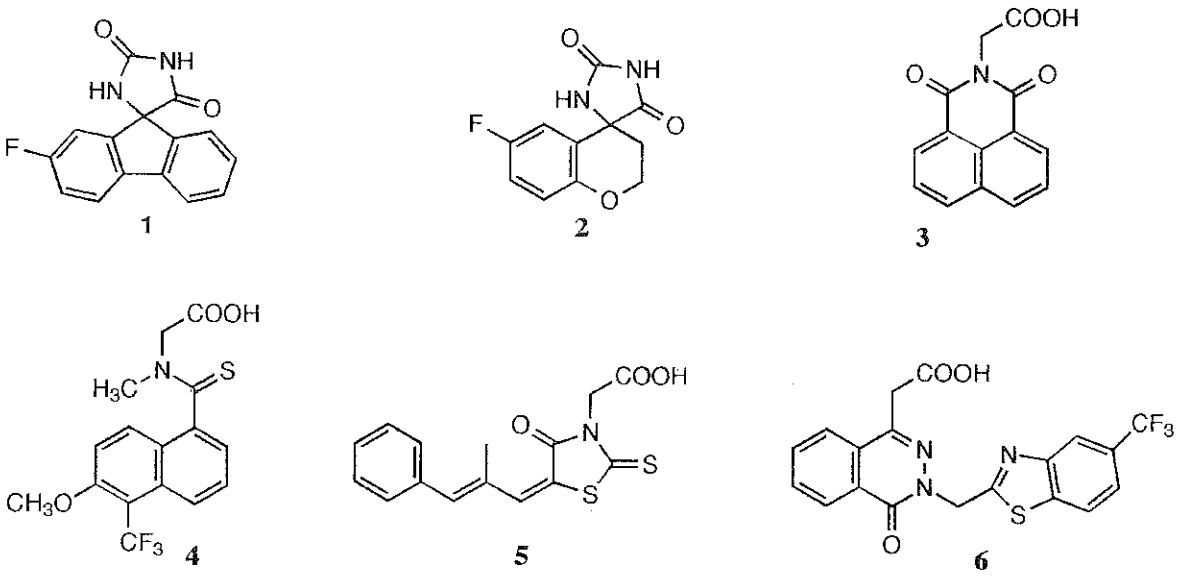


Figure 1. Aldose reductase inhibitors.

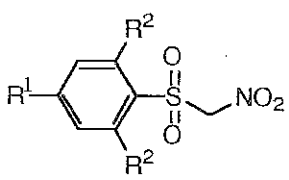
of α-halo nitro compounds [10], the oxidation of α-nitro sulfide [11] and the alkaline nitration of sulfones [12]. Alkaline nitration, which is based on a free radical chain process rather than nucleophilic displacement of iodide by the nitro anion, was the most fruitful method for our synthesis. The synthesis of affinity labels **15–17** and **19–21** was achieved as depicted in *figure 2*. The amino moiety of dimethylaniline (**22**) was protected by treatment with acetic anhydride to give **23,** which was reacted with chlorosulfonic acid to yield the corresponding sulfonyl chloride **25**. Compound **25** as well as the commercially available N-acetylsulfonyl chloride (**24**) were reduced using sodium sulfite in the presence of sodium bicarbonate buffer to give the corresponding sulfinic acids **26** and **27** [13]. The acids were converted to their sodium salts and then reacted with two-fold excess of nitromethane in the presence of iodine and sodium methoxide at 0 °C to give the corresponding N-(acetylphenyl)sulfonyl nitromethane derivatives **14** and **18** [13, 14]. Basic hydrolysis of the acetyl group afforded **12** and **13**. The latter compounds were treated with thiophosgene in acetone to give the corresponding isothiocyanate analogues **15** and **19**, respectively. Treatment of **12** or **13** with chloro- or iodoacetic anhydride afforded the corresponding chloro- or iodoacetamido derivatives **16** and **20** or **17** and **21**, respectively.

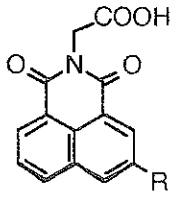
3. Results and discussion

The sulfonylnitromethane analogues were found to be potent inhibitors of recombinant rat lens AR with IC_{50}s ranging between 0.01 to 0.19 μM *(table I)*. Subsequent gel filtration studies for irreversible binding also indicated that compounds **15–17** and **19–21** are potent affinity labels for AR *(table I)*. Comparison of the activities of compounds **15–17** with that of compounds **19–21** indicates that *ortho* dimethyl substitution potentiates irreversible inhibition of AR in this class of compounds. In contrast to the affinity labels (**7–10**, *table I*) derived from alrestatin, the irreversible inhibitory activity for the present compounds is not directly linked to the chemical reactivity of their electrophilic groups. Irreversible inhibitory activity for both sets of compounds decreased in order: isothiocyanato analogues > iodoacetamido analogues > chloroacetamido analogues. No correlation between reversible and irreversible inhibitory activities was observed.

Results from mass spectrometry and molecular modelling studies [7], carboxymethylation studies [15], site directed mutagenesis studies [16], as well as kinetic data [4–6] suggest the possibility of multiple inhibitor binding sites on AR which are distinct from the substrate binding site. Structurally diverse, selective affinity labels

Table I. Reversible (IC_{50}) and irreversible (%) inhibition of recombinant rat lens AR.





#	R^1	R^2	R[a]	IC_{50} (μM)	% Relative irreversibility at 10^{-4}M
7			NCS	ND[b]	56.0
8			$NHCOCH_2Cl$	0.55 ± 0.04	0.0
9			$NHCOCH_2Br$	0.60 ± 0.05	46.0
10			$NHCOCH_2I$	0.40 ± 0.02	89.0
11	H	CH_3		ND[b]	ND[b]
12	NH_2	H		0.19 ± 0.66[c]	0.0
13	NH_2	CH_3		0.13 ± 0.04[c]	0.0
14	$NHCOCH_3$	H		0.09 ± 0.09	0.0
15	NCS	H		0.05 ± 0.05	80.7
16	$NHCOCH_2Cl$	H		0.06 ± 0.07	58.0
17	$NHCOCH_2I$	H		0.09 ± 0.03	73.8
18	$NHCOCH_3$	CH_3		0.09 ± 0.09	0.0
19	NCS	CH_3		0.13 ± 0.03	99.5
20	$NHCOCH_2Cl$	CH_3		0.01 ± 0.04	86.6
21	$NHCOCH_2I$	CH_3		0.03 ± 0.001	96.8

[a] The alrestatin derivatives were previously reported [9].
[b] ND, not determined.
[c] Reported values from [13, 14].

can be useful tools for investigating the possibility of multiple inhibitor sites on AR. The present results indicate that compounds **19** and **21** are more potent affinity labels than 5-iodoacetamidoalrestatin (**10**) and that apparently complete irreversible inhibition of AR can be achieved with compound **19**. Future determination of the exact site(s) of interaction with the enzyme by these compounds and identification of the reactive nucleophilic residue(s) that bind to these compounds will provide insight into the possibility of multiple inhibitor binding sites on AR.

4. Experimental protocols

4.1. Chemistry

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin Elmer System 2000 FT-IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AX 300 spectrometer. Chemical shift values are reported in parts per million (δ) relative to tetramethylsilane (TMS) as an internal standard. Spectral data are consistent with assigned structures. Elemental analyses were performed by Atlantic Microlab Inc., Norcross GA, and experimentally determined values are within ± 0.4% of the theoretical values. Routine thin-layer chromatography (TLC) was performed on silica gel GHIF plates (Analtech Inc., Newark DE). Flash chromatography was performed on silica gel (Merck, grade 60, 230–400 mesh, 60 Å). Acetonitrile (MeCN) was dried by distillation, dimethylformamide (DMF) was dried by distillation from P_2O_5. All solvents (except anhydrous MeCN and DMF) were stored over 3 or 4 Å molecular sieves.

4.1.1. N-Acetyl-3,5-dimethylaniline **23**

Acetic anhydride (60 mL) was added slowly to 3,5-dimethylaniline (40 mL, 0.32 mol). The hot reaction mixture was allowed to cool to room temperature to give 42 g (80%) of **23** as a white solid: m.p. 137–139 °C (Lit. [13] 138 °C). IR (KBr, cm^{-1}) 1 663 (C=O). ^{1}H NMR ($CDCl_3$) δ 7.12 (s, 2H, aromatic), 6.75 (s, 1H, aromatic), 2.28 (s, 6H, $2CH_3$), 2.15 (s, 3H, $COCH_3$), 1.63 (br, 1H, NHCO).

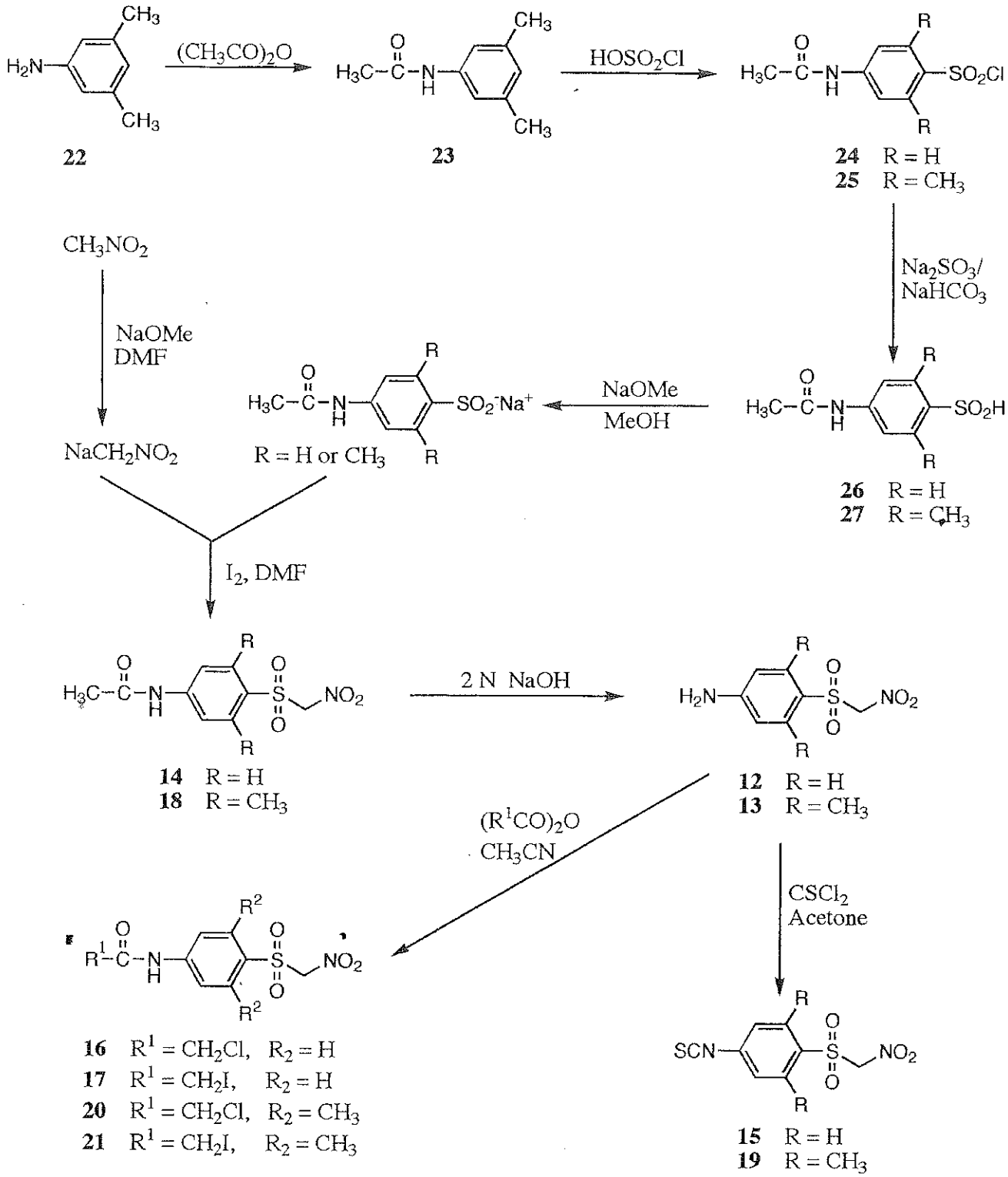


Figure 2. Synthetic route to phenylsulfonylnitromethanes.

4.1.2. (4-Acetamido-2,6-dimethylphenyl)sulfonyl chloride **25**

In a three-necked round bottom flask fitted with a mechanical stirrer was placed chlorosulfonic acid (100 g, 0.85 mol). The flask was cooled (12–15 °C) by means of an ice-bath and N-acetyl-3,5-dimethylaniline (28.07 g, 0.172 mol) was added slowly over a 15 min period. After the addition was complete, the mixture was allowed to warm to room temperature and then was heated to 60 °C with stirring. After 2.5 h, the mixture was added slowly to ice-water mixture (330 mL) with stirring. The off-white precipitate that formed was filtered and dried in a desiccator under vacuum overnight to give 22 g of crude product. An analytical sample was recrystallized from benzene: m.p. 157–159 °C. IR (KBr, cm^{-1}) 3 322 (NH), 1 683 (C=O), 1 360 (SO_2) and 1 172 (SO_2). ^1H NMR (CDCl3) δ 7.65 (br, 1H, NH), 7.42 (s, 2H, aromatic), 2.72 (s, 6H, $2CH_3$), 2.22 (s, 3H, $COCH_3$).

4.1.3. (4-Acetamidophenyl)sulfinic acid **26**

N-Acetylsulfanilyl chloride (11.9 g, 0.05 mol) was added to a vigorously stirred solution of sodium bicarbonate (10 g, 0.119 mol) and sodium sulfite (14.29 g, 0.113 mol) in water (60 mL) at 70–80 °C. The mixture was heated for 1 h and allowed to cool to room temperature. The white precipitate that separated out was redissolved in water and acidified with 60% sulfuric acid until a white precipitate appeared. The suspension was left in a refrigerator overnight. The solid was recovered by filtration and recrystallized from water to give 7.23 g (72%) of **26** as white needle-like crystals: m.p. 149–151 °C. IR (KBr, cm^{-1}) 3 321 (NH), 1 668 (C=O), 1 320 (SO_2), 1 084 (SO_2). ^{1}HNMR (DMSO-d_6) δ 10.20 (s, 1H, NHCO), 7.73 (d, 2H, aromatic), 7.58 (d, 2H, aromatic), 2.06 (s, 3H, $COCH_3$).

4.1.4. (4-Acetamido-2,6-dimethylphenyl)sulfinic acid **27**

Sulfonylchloride **25** (22 g, 0.084 mol) was transformed to sulfinic acid **27** as described above for the synthesis of **26**. The crude product was recrystallized from water to give 5.5 g (29%) of **27** as white crystals: m.p. 158–159 °C. IR (KBr, cm^{-1}) 3 321 (NH), 1 668 (C=O), 1 320 (SO_2), 1 084 (SO_2). ^1H NMR ($CDCl_3$) δ 7.31 (s, 2H, aromatic), 2.59 (s, 6H, $2CH_3$), 2.10 (s, 3H, $COCH_3$).

4.1.5. (4-Acetamidophenyl)sulfonylnitromethane **14**

Sodium metal (0.62 g, 0.027 mol) was dissolved in dry MeOH and **26** (5.5 g, 0.027 mol) was added and the mixture was stirred overnight. The solvent was removed under reduced pressure and the residue was kept in a desiccator. Another sample of sodium metal (1.09 g, 0.047 mol) was dissolved in dry MeOH, the solvent was stripped off under reduced pressure, and the residue was dissolved in dry DMF (15 mL) and cooled to 0 °C. Nitromethane (3.22 g, 0.052 mol) was dissolved in dry DMF (15 mL) and added slowly to the cooled NaOMe solution. The reaction mixture was stirred at 0 °C for 15 min during which period a bright yellow precipitate separated out. The previously prepared sodium salt of **26** was dissolved in dry DMF, cooled to 0 °C, and added to the bright yellow mixture followed immediately by the addition of iodine (6.08 g, 0.024 mol). The reaction was stirred at 0 °C for 4 h and then allowed to warm to room temperature over 30 min. The dark solution was poured into ice-water and decolourized with sodium sulfite. It was acidified slowly to pH 1.5 with 2 N HCl and the resulting suspension was stored in a refrigerator overnight followed by filtration to recover the product which was dried and recrystallized from MeOH to give a 63% yield of **14**: m.p. 220–222 °C (dec.) (Lit. [14] 228–229 °C). IR (KBr, cm^{-1}) 3 259 (NH), 1 674 (C=O), 1 590 (NO_2), 1 553 (NO_2). ^1H NMR (DMSO-d_6) δ 10.54 (s, 1H, NHCO), 7.90 (s, 4H, aromatic), 6.57 (s, 2H, CH_2NO_2), 2.13 (s, 3H, $COCH_3$).

4.1.6. (4-Acetamido-2,6-dimethylphenyl)sulfonylnitromethane **18**

Compound **27** (2 g, 8.8 mmol) was transformed into **18** as described above for the synthesis of **14**. The off-white solid obtained was purified by column chromatography over silica gel with hexane/ethyl acetate (1:2) as the eluant followed by recrystallization from ethanol to yield white crystals: m.p. 177–179 °C (Lit. [13] 179–180 °C). IR (KBr, cm^{-1}) 3 308 (NH), 1 677 (C=O), 1 534 (NO_2). ^1H NMR (acetone-d_6) δ 9.50 (s, 1H, NHCO), 7.59 (s, 2H, aromatic), 6.15 (s, 2H, CH_2NO_2), 2.60 (s, 6H, $2CH_3$), 2.05 (s, 3H, $COCH_3$).

4.1.7. (4-Aminophenyl)sulfonylnitromethane **12**

A solution of **14** (0.2 g, 0.77 mmol) in 2 N NaOH (2 mL) was heated for 1 h at 80 °C. The mixture was then poured into ice-water mixture (10 mL) containing acetic acid (0.3 mL) to yield a solid which was extracted with EtOAc, dried ($MgSO_4$), and evaporated under reduced pressure. The residue was purified by column chromatography over silica gel with hexane/EtOAc (1:1) as the eluant followed by recrystallization from EtOH to give a 78% yield of **12**: m.p. 128–130 °C. IR (KBr, cm^{-1}) 3 396 (NH_2), 1 554 (NO_2). ^1H NMR (DMSO-d_6) δ 7.51 (s, 2H, aromatic), 6.67 (s, 2H, aromatic), 6.44 (s, 2H, CH_2NO_2), 6.30 (s, 2H, Ar-NH_2).

4.1.8. (4-Amino-2,6-dimethylphenyl)sulfonylnitromethane **13**

Compound **18** (0.146 g, 0.51 mmol) was transformed into **13** in 81% yield as described above for the synthesis of **12**: m.p. 131–133 °C (Lit. [13] 132–133 °C). IR (KBr,

cm^{-1}) 3 474 (NH_2), 3 376 (NH_2), 1 551 (NO_2). ^1H NMR (DMSO-d_6) δ 7.54 (s, 2H, aromatic), 6.40 (s, 2H, CH_2NO_2), 2.62 (s, 6H, $2CH_3$), 6.32 (s, 2H, Ar-NH_2).

4.1.9. (4-Isothiocyanatophenyl)sulfonylnitromethane **15**

Thiophosgene (12 drops) was added to a solution of **12** (0.06 g, 0.28 mmol) in dry acetone (4.5 mL) and the mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography over silica gel with hexane/EtOAc (1:1) as the eluant to yield 0.06 g (87%) of **15** as a bright yellow solid: m.p. 159–160 °C. IR (KBr, cm^{-1}) 2 131 (NCS). ^1H NMR (acetone-d_6) δ 8.09 (d, 2H, aromatic), 7.72 (d, 2H, aromatic), 6.35 (s, 2H, CH_2NO_2). MS (EI, m/z) 258^+. Anal. $C_8H_6N_2S_2O_4$ (C, H, N, O, S).

4.1.10. (4-Isothiocyanato-2,6-dimethylphenyl)sulfonylnitromethane **19**

Thiophosgene (8 drops) was added to a solution of **13** (0.06 g, 2.4 mmol) in dry acetone (3 mL) and the mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography over silica gel with hexane/EtOAc (1:1) as the eluant to yield 0.065 g (93%) of **19** as a white solid: m.p. 110–112 °C. IR (KBr, cm^{-1}) 2 009 (NCS). ^1H NMR (acetone-d_6) δ 7.35 (s, 2H, aromatic), 6.27 (s, 2H, CH_2NO_2), 2.68 (s, 6H, $2CH_3$). MS (EI, m/z) 286^+. Anal. $C_{10}H_{10}N_2S_2O_4$ (C, H, N, O, S).

4.1.11. (4-Chloroacetamidophenyl)sulfonylnitromethane **16**

Chloroacetic anhydride (73 mg, 0.43 mmol) was added to a solution of **12** (0.06 g, 0.27 mmol) in dry CH_3CN (4 mL) and the mixture was stirred at room temperature for 18 h. The solvent was concentrated to precipitate pure **16** in 0.044 g (55%) yield as a white solid: m.p. 212 °C. IR (KBr, cm^{-1}) 3 279 (NH), 1 684 (C=O). ^1H NMR (DMSO-d_6) δ 10.86 (s, 1H, NHCO), 7.92 (d, 4H, aromatic), 6.60 (s, 2H, $CH2NO_2$), 4.34 (s, 2H, CH_2Cl). MS (EI, m/z) 292^+. Anal. $C_9H_9N_2SO_5Cl$ (C, H, N, O, S, Cl).

4.1.12. (4-Chloroacetamido-2,6-dimethylphenyl)sulfonylnitromethane **20**

Compound **13** (0.05 g, 2.0 mmol) was treated as described for the synthesis of **16** to yield 0.06 g (87%) of **20** as a bright yellow solid: m.p. 160–161 °C. IR (KBr, cm^{-1}) 3 366 (NH), 1 723 (C=O). ^1H NMR (DMSO-d_6) δ 10.66 (s, 1H, NHCO), 7.53 (s, 2H, aromatic), 6.50 (s, 2H, CH_2NO_2), 4.31 (s, 2H, CH_2Cl), 2.56 (s, 6H, $2CH_3$). MS (EI, m/z) 320^+. Anal. $C_{11}H_{13}N_2SO_5Cl$ (C, H, N, O, S, Cl).

4.1.13. (4-Iodoacetamidophenyl)sulfonylnitromethane **17**

Iodoacetic anhydride (0.106 g, 0.29 mmol) was added to a solution of **12** (0.05 g, 0.23 mmol) in dry CH_3CN (4 mL) and the mixture was stirred at room temperature for 18 h. The mixture was concentrated and $CHCl_3$ was added and kept in a refrigerator overnight to precipitate pure **17** as pale yellow crystals in 95% yield: m.p. 165 °C. IR (KBr, cm^{-1}) 3 356 (NH), 1 690 (C=O). ^1H NMR (DMSO-d_6) δ 10.88 (s, 1H, NHCO), 7.50 (d, 4H, aromatic), 6.61 (s, 2H, CH_2NO_2), 3.87 (s, 2H, CH_2I). MS (EI, m/z) 384^+. Anal. $C_9H_9N_2SO_5I$ (C, H, N, O, S, I).

4.1.14. (4-Iodoacetamido-2,6-dimethylphenyl)sulfonylnitromethane **21**

This compound was synthesized as described for the synthesis of **17** by reacting iodoacetic anhydride (0.094 g, 0.26 mmol) with **13** (0.05 g, 2.0 mmol). Compound **21** was obtained in 95% yield as white crystals. M.p. 200 °C. IR (KBr, cm^{-1}) 3 366 (NH), 1 723 (C=O). ^1H NMR (DMSO-d_6) δ 10.68 (s, 1H, NHCO), 7.50 (s, 2H, aromatic), 6.49 (s, 2H, CH_2NO_2), 3.84 (s, 2H, CH_2I), 2.51 (s, 6H, $2CH_3$). Anal. $C_{11}H_{13}N_2SO_5I$ (C, H, N, O, S, I).

4.2. Biological studies

4.2.1. Enzyme purification

Recombinant rat lens AR was purified by a series of chromatographic procedures as previously described [17]. Briefly, AR was released from *E. coli* by sonication and the mixture was centrifuged at 10 000 *g* for 10 min. The supernatant was then subjected to gel filtration on a Sephadex G-75 column (2.5 × 90 cm), equilibrated with 10 mM imidazole-HCl buffer, pH 7.5 containing 7 mM 2-mercaptoethanol and eluted with the same imidazole buffer. The eluent was collected in 220-drop aliquots (ca. 10 mL) and fractions containing AR activity were applied to a Matrex Gel Orange A affinity column (2.5 × 15 cm). The affinity column was washed with the imidazole buffer (ca. 500 mL) and the enzyme was eluted with the same imidazole buffer containing 0.1 mM NADPH. Fractions eluted with NADPH were chromatofocused on a Mono P (HR 5/20) column developed at a flow rate of 1 mL/min with Polybuffer 74 (diluted 10-fold and containing 7 mM 2-mercaptoethanol). The protein concentration of the eluent was monitored at 280 nm and peaks containing AR activity were collected and concentrated on Centricon 10 filters.

4.2.2. Enzyme assay

Reductase activity was spectrophotometrically assayed on a Shimadzu UV 2100U spectrophotometer by following the decrease in the absorption of NADPH at 340 nm

over a 4-min period with DL-glyceraldehyde as substrate [9]. Each 1 mL cuvette contained equal units of enzyme, 0.10 M Na, K phosphate buffer, pH 6.2, 0.3 mM NADPH with/without 10 mM substrate and inhibitor. Appropriate controls were employed to negate potential changes in the absorption of nucleotide and/or protein modification reagents or ARIs at 340 nm in the absence of substrate.

4.2.3. Affinity binding

Rat lens AR was passed through NAP-5 desalting columns equilibrated with 0.1 M sodium phosphate buffer, pH 7.6, to remove potentially reactive 2-mercaptoethanol which stabilizes AR [9]. Equal aliquots of enzyme were then combined with either reversible or irreversible inhibitor dissolved in 0.1 M sodium phosphate buffer, pH 7.6, and the reaction was allowed to proceed at room temperature for 15 min. Reversible and unreacted inhibitor were then removed from the protein by gel filtration with a NAP-5 desalting column with 0.1 M phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. Residual AR activity was spectrophotometrically measured. All experiments were conducted at least in triplicate.

Acknowledgements

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