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Miscellaneous

Assignment of the absolute configuration at the sulfur atom of thioridazine metabolites by the analysis of their chiroptical properties: The case of thioridazine 2-sulfoxide

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ABSTRACT

Thioridazine (THD) is a commonly prescribed phenotiazine neuroleptic drug, which is extensively biotransformed in the organism producing as main metabolites sulfoxides and a sulfone by sulfur oxidation. Significant differences have been observed in the activity of the THD enantiomers as well as for its main metabolites, and enantioselectivity phenomena have been proved in the metabolic pathway. Here the assignment of the absolute configuration at the sulfur atom of enantiomeric THD-2-sulfoxide (THD-2-SO) has been carried out by circular dichroism (CD) spectroscopy. The stereoisomers were separated by HPLC on Chiralpak AS column, recording the CD spectra for the two collected enantiomeric fractions. The theoretical electronic CD spectrum has been obtained by the TDDFT/B3LYP/6-31G*, as Boltzmann averaging of the contributions calculated for the most stable conformations of the drug. The comparison of the simulated and experimental spectra allowed the absolute configuration at the sulfur atom of the four THD-2-SO stereoisomers to be assigned. The developed method should be useful for a reliable correlation between stereochemistry and activity and/or toxicity.

1. Introduction

Thioridazine (THD), a commonly prescribed phenotiazine neuroleptic drug, used for the treatment of schizophrenia and other psychiatric disorders, is extensively biotransformed in the organism producing as main metabolites the sulfoxides at positions 2 and 5, and the sulfone derivate at position 2 [1,2].

THD is currently administered as a racemate, although significant differences have been observed for the activity of this drug depending on the stereochemistry; the antipsychotic effect of racemic THD is mainly associated with (R)-THD [2]. In the metabolic process a new stereocenter is formed at the sulfur atom in thioridazine-2-sulfoxide (THD-2-SO) and thioridazine-5-sulfoxide (THD-5-SO) metabolites (Fig. 1). So for each of these two compounds two pairs of enantiomers are possible: (Rc, Rs) (Sc, Ss) and (Rc, Ss) (Sc, Rs), the first and second pairs are diastereoisomers. The (R) and (S) configurations of THD and their metabolites are related to the chiral carbon at position 2 in the piperidyl ring (c) and to the sulfur atom of the sulfoxide metabolites (s).

Considering that THD-2-SO and THD-2-SO_2 metabolites are also therapeutically active [3–5], but with undesirable side effects that can be attributed to specific stereoisomers, the assignment of the absolute configuration to each of these streoisomeric forms of the drug and of its metabolites should allow reliable information on the relationship structure/activity in clinical and/or toxicological studies. In addition, THD-5-SO metabolite contributes to the cardio toxicity of the drug [6,7].

We report here the determination of the absolute configuration at the sulfur atom of THD-2-SO. The diastereoisomers were separated by non-chiral HPLC and referred as fast-eluting (FE) THD-2-SO and slow-eluting (SE) THD-2-SO, based on their chromatographic behaviour [1,2]. The enantiomers of the fast-eluted couple (FE) have been separated by HPLC on Chiralpack AS, recording the circular dichroism (CD) spectra for the two collected enantiomeric fractions.

To simulate the experimental electronic CD spectrum of THD 2-SO, we arbitrarily assumed S absolute configuration for the sulfur atom and removed the chain linked to the *N* atom to reduce the numbers of conformers, without neglecting significant chromophores. Two stable conformers have been found in the gas phase: using the above two conformers as input geometries and the TDDFT/B3LYP/6-31G* method [8–10] the theoretical CD

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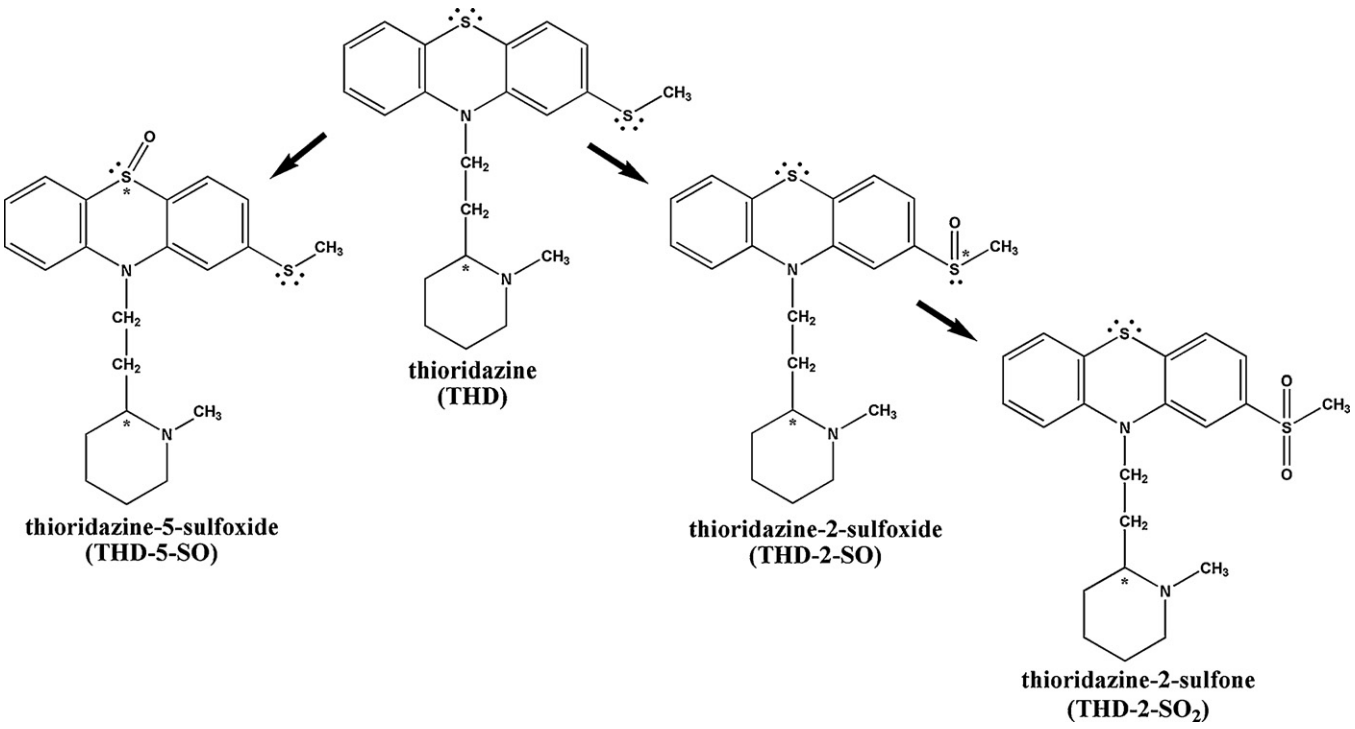


Fig. 1. *rac*-Thioridazine metabolic profile in human.

spectrum (after Boltzmann averaging) was obtained, with the simulated curve (S) AC of the sulfur atom that corresponds to the first eluted peak.

2. Materials and methods

2.1. Chemicals

*r*ac-Thioridazine, thioridazine-2-sulfone and thioridazine-2-sulfoxide, were kindly supplied by Novartis Pharma AG (Basel, Switzerland). The THD-2-SO standard sample consisted mainly of the racemic mixture of the enantiomers of the fast-eluted (FE) diastereoisomer, according to the separation procedure reported in the literature [1]. Stock standard solutions were prepared in methanol at concentrations of 200 and 400 μg mL^{-1} and were stored at −20 °C in the absence of light. HPLC-grade hexane, methanol, diethyl ether were purchased from Mallinckrodt Baker Inc. (Paris, France) and ethanol from Merck (Darmstadt, Germany). All other chemicals were of analytical-grade in the highest purity available.

2.2. Instruments and analytical methods

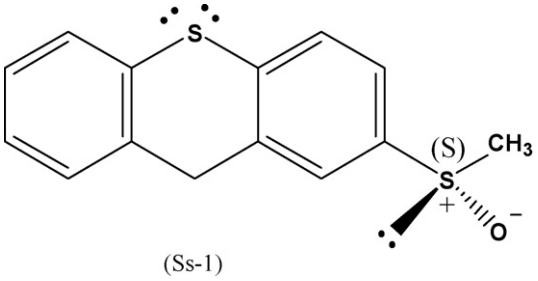
CD spectra were measured using a JASCO spectropolarimeter (model J-810), equipped with an internal temperature control apparatus JASCO PTC-423S, using a quartz cell with a 1.0 cm optical path at 25 °C. Subtractions of base line (mobile phase or methanol) were carried out for all analysis. The standard scanning conditions were at a rate of 50 nm min^{-1} mode continuous, spectral bandwidth 1 nm, and a response of 1 s. Spectra were taken in the range from 200 to 400 nm.

The HPLC analyses were carried out using a Shimadzu (Kyoto, Japan) HPLC system, consisting of a LC 10 AD_{VP} model solvent pump, system controller SCL $10A_{VB}$, a column oven CTO-$10AS_{VP}$, a Rheodyne model 7125 injector with a 20 μL loop, a SPD-$M10A_{VP}$ diode array detector operating at 262 nm and a software Class VP for data acquisition.

A Chiralpak AD column (250 mm × 4.6 mm I.D., 10 μm particle size, Chiral Technologies, Exton, PA, USA) was used for the resolution of THD enantiomers. The analytical column was protected by a RP-8 endcapped guard column (4 mm × 4 mm, Merck, Darmstadt, Germany), hexane:ethanol:diethylamine (99:1:0.1, v/v/v, respectively) was used as mobile phase, at the flow rate of 1 mL min^{-1} and UV detection was performed at 262 nm [11]. THD-2-SO_2 enantiomers were resolved using a Chiralcel OD-H column (150 mm × 4.6 mm I.D., 5 μm particle size, Chiral Technologies, Exton, PA, USA), protected by a RP-8 endcapped guard column (4 mm × 4 mm, Merck Darmstadt, Germany). Hexane:2-propanol:diethylamine (90:10:0.1, v/v/v, respectively) was used as mobile phase at the flow rate of 1 mL min^{-1} and UV detection was carried out at 262 nm [11]. The resolution of THD-2-SO stereoisomers was performed on a Chiralpak AS column (250 mm × 4.6 mm, I.D., 10 μm particle size, Chiral Technologies, Exton, PA, USA), protected by a RP-8 endcapped guard column (4 mm × 4 mm, Merck, Darmstadt, Germany), using a mobile phase consisting of hexane:ethanol:methanol:diethylamine (92:6:2:0.5, v/v/v/v, respectively) at a flow rate of 1.4 mL min^{-1} [12]. The analyses were carried out in an acclimatized room with the temperature set at 23 (±2) °C. The chromatographic retention was analysed as the capacity factor (k), where k is defined as $(t_{drug} - t_0)/t_0$ (t_{drug} = retention of the solute; t_0 = retention of a non-retained solute), and as the enantioselectivity (α), where $\alpha = k_2/k_1$ (k_1 and k_2 are the capacity factors of the first and second eluted enantiomers, respectively). The resolution R was determined as: $R = 2[(t_R)_B - (t_R)_A]/W_A + W_B$, where $(t_R)_B$ and $(t_R)_A$ are the retention times of the analytes A and B respectively, and W_A and W_B are the peak widths.

2.3. Semi-preparative enantioselective HPLC

The single enantiomers of THD, THD-2-SO and THD-2-SO_2 were obtained by repetitive semi-preparative HPLC resolution using the chiral stationary phases described in Section 2.2. The standard solutions of THD, THD-2-SO and of THD-2-SO_2 (25 μL of a solution of 200 μg mL^{-1} in methanol) were dissolved in 1 mL of methanol



(Ss-1)

Fig. 2. Simplified sulfoxide structure (Ss)-1, employed in ECD calculations.

resulting in concentration of 5 μg mL^{-1}. The enantiomeric fractions were manually collected after repetitive injections.

2.4. Absolute configuration assignment

All calculations have been carried out on a simple PC endowed with a single Pentium IV 3.16 GHz processor. The preliminary conformational distribution search has been performed by Spartan02 package [13] using the MMFF94s molecular mechanics force field, using the simplified structure with (S)-absolute configuration at the sulfur atom, as reported in Fig. 2. The "Systematic" option was used and search of all possible conformers has been performed, considering the degrees of freedom of system and retaining only the structures with an energy of not more than 2 kcal/mole above the most stable conformer. This analysis provided for the simplified structure only two conformers. These geometries have been afterwards optimised by DFT/B3LYP/6-31G* of Gaussian03 [10]. All conformers are real minima, no imaginary vibrational frequencies have been found. The CD calculations have been carried out by means of time-dependent DFT methods using the hybrid B3LYP functional and the 6-31G* as available within Gaussian03. Both velocity and length formalism have been used in CD calculations.

2.5. Stereochemical stability of THD-2-SO upon UV irradiation

The slow eluted (SE) diastereoisomer of THD-2-SO was obtained by UV irradiation of the FE diastereoisomer at 254 nm for 4 h. The four stereoisomers, two enantiomers for each diastereoisomer, were separated by preparative HPLC and characterized by CD spectroscopy for assigning their absolute stereochemistry.

3. Results and discussion

3.1. Stereochemical characterization of THD and of its metabolites by enantioselective HPLC and circular dichroism

Enantioselective HPLC was successfully applied for the stereoisomeric resolution of THD and of its main metabolites. In particular, enantiomeric fractions of THD, THD-2SO$_2$ and of both the diastereoisomers of THD-2SO were obtained at preparative level, in order to carrying out the CD spectra of each fraction, and then to establish the relationship between sign of the CD spectra and absolute configuration. Thus different chiral stationary phases were used in order to have the enantioselectivity and the resolution useful for preparative application.

3.1.1. THD

Separation of the THD enantiomers was obtained on a Chiralpak AD column (Table 1, Fig. 1a-SM). The enantioselective HPLC method was applied to prepare the single enantiomers for carrying out their CD spectra. Methanol solutions of the two fractions were obtained after evaporation under nitrogen of the mobile phase after their collection. The absolute configuration of the two enantiomeric fractions from Chiralpak AD column is (S) and (R) for the first and second eluted enantiomers, respectively, as already reported in the literature on the basis of the chromatographic behaviour of defined stereochemistry standards [1], and from the behaviour of *rac*-THD in racemization and degradation studies [11]. The CD spectrum carried out between 350 and 200 nm (Fig. 2a-SM) shows at least two low intensity bands centred at about 260 [positive for (S)-THD] and 235 nm [negative for (S)-THD].

Table 1
Chromatographic parameters for the chiral separation of THD and of its metabolites.

	csp	k_1	α	Rs	e.o.
THD	A	1.35	1.35	1.45	(S)/(R)
THD-2SO$_2$	B	4.35	1.12	1.37	(R)/(S)
THD-2SO (FE)	C	9.70	2.19	5.87	(Sc, Ss)/(Rc, Rs)
THD-2SO (SE)	C	10.67	1.58	4.68	(Rc, Ss)/(Sc,Rs)

csp, chiral stationary phase: A, Chiralpak AD, mobile phase hexane/ethanol/diethylamine 99/1/0.1, v/v/v[11] respectively, 1 ml/min, t_0 = 3.50 min; B, Chiralcel OD-H, mobile phase hexane/2-propanol/diethylamine 90/10/0.1, v/v/v respectively, 1 ml/min[11], t_0 = 2.00 min; C, Chiralpak AS, mobile phase hexane/ethanol/methanol/diethylamine 92/6/2/0.5, v/v/v/v respectively, 1.4 ml/min[12], t_0 = 1.94 min; k_1, capacity factor of the first eluted enantiomer; α, enantioselectivity; Rs, resolution; e.o. elution order.

3.1.2. THD-2-SO$_2$

The single enantiomers of THD-2-SO$_2$ were obtained by preparative enantioselective HPLC (Fig. 1b-SM) on a Chiralcel OD-H column. A relatively low enantioselectivity was obtained in the enantioresolution of the metabolite (Table 1), in agreement to literature data (11). However an almost base-line separation (Rs 1.37), allowed a preparative collection of the two enantiomers. Methanol solutions of the two fractions were obtained upon evaporation under nitrogen of the mobile phase after their collection. The absolute configuration of the two enantiomeric fractions from Chiralcel OD-H column is (R) and (S) for the first and second eluted enantiomers (Table 1), respectively, as already reported in the literature on the basis of the chromatographic behaviour of defined stereochemistry standards [1,14]. The CD spectrum carried out between 350 and 200 nm (Fig. 2b-SM) shows at least two low intensity bands centred at about 260 [positive for (S)-THD-2SO$_2$] and 235 nm [negative for (S)-THD-2SO$_2$]. The same relationship between sign of the CD and absolute configuration was observed for both the drug and its metabolite (Fig. 2-SM).

3.1.3. THD-2-SO

The enantioresolution of THD-2-SO FE diastereoisomer was obtained by enantioselective HPLC on a Chiralpak AS column (Fig. 3a), according to the method reported in the literature [12]. A quite high enantioselectivity factor (α = 2.19, Table 1) allowed an easily application of the HPLC method for the collection of the single enantiomers of THD-2SO FE. Methanol solutions of the two fractions were obtained after evaporation under nitrogen of the mobile phase after their collection. The absolute configuration of the two enantiomeric fractions from Chiralpak AS column is (S) and (R) at the methylpiperidin moiety [FE diastereoisomer] for the first and second eluted enantiomers, respectively (Table 1), as already reported in the literature on the basis of the chromatographic elution order previously determined by following the metabolism of the single enantiomers of THD [1] and chemical correlation [14]. The CD spectrum carried out between 400 and 200 nm (Fig. 4) shows at least three bands centred at about 270, 240, and 210 nm. A low intensity CD band was also observed at wavelengths longer than 300 nm (Fig. 4). The CD spectrum is dominated by the contribution arising from the chirality of the sulfoxide moiety, and a ScSs and RcRs absolute configuration was assigned to the first and to second eluted enantiomers, respectively, on the basis of the com-

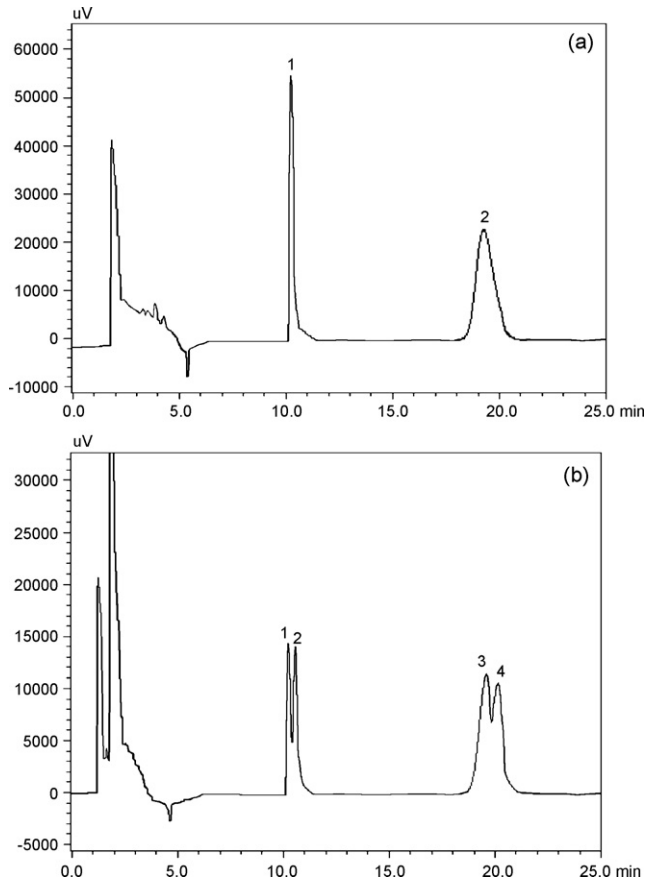


Fig. 3. (a) Resolution of (1) (Sc, Ss) and (2) (Rc, Rs) THD-2SO FE enantiomers; (b) resolution of (1) (Sc, Ss), (2) (Rc, Ss), (3) (Sc, Rs) and (4) (Rc, Rs) THD-2SO. Chiralpak AS column, mobile phase hexane:ethanol:methanol:diethylamine (92:6:2:0.5, v/v/v/v respectively), flow rate 1.4 mL min^{-1} [12].

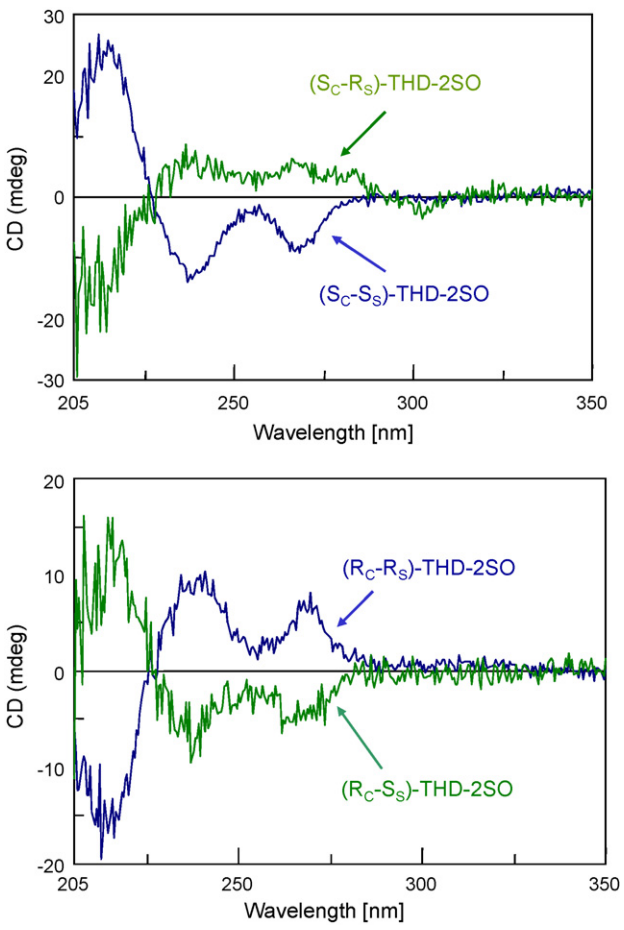


Fig. 5. (a) CD spectra of (Sc, Ss) and (Sc, Rs) THD-2SO diastereoisomers and (b) CD spectra of (Rc, Rs) and (Rc, Ss) THD-2SO diastereoisomers.

parison of the experimental CD spectra with the simulated CD curve of the ScSs enantiomer, as obtained by the TDDFT/B3LYP/6-31G* (see Section 3.1.4).

The other stereoisomers of THD-2-SO (i.e. the pair of enantiomers of the SE diastereoisomer) were obtained by irradiation of the solution of THD-2-SO (FE) by UV light (254 nm) for 4 h [15]. The stereoisomeric mixture was analysed by using the same enantioselective HPLC method upon Chiralpack AS already successfully employed for the enantiomeric resolution of the FE diastereoisomer. The observed four chromatographic peaks (Fig. 3b) were assigned to the ScSs (first eluted), RcSs (second eluted), ScRs (third eluted) and RcRs (fourth eluted) stereoisomers, on the basis of the methods reported and functional tests [1,12,16]. Each fraction was collected and the CD spectra recorded between 400 and 200 nm. The results obtained are in agreement to the prevalence of the sulfoxide moiety contribution to the CD signal. The epimerisation of ScSs stereoisomer to ScRs determines an inversion of the CD sign (Fig. 5a), as well in the case of the epimerisation of the RcRs stereoisomer to RcSs one (Fig. 5b).

Thus CD resulted very efficient to the reliable determination of the enantioselectivity phenomena involved in the metabolism of THD. This is particularly important taking into account the already demonstrated relationship between absolute configuration and pharmacological activity of THD.

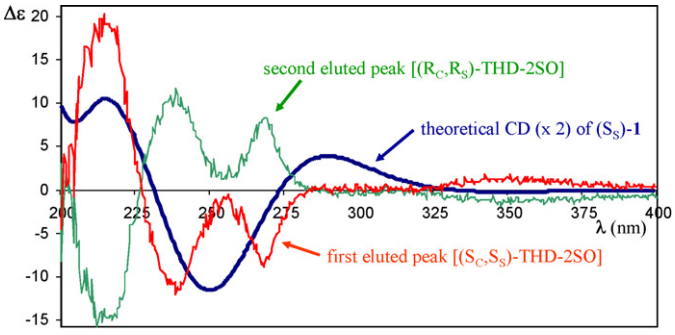


Fig. 4. Experimental CD spectra of THD-2SO [(S_CS_S), first eluted peak; (R_CR_S), second eluted peak] and theoretical (TDDFT/B3LYP/6-31G*, dipole velocity formalism, 40 states) CD spectrum (20 nm blue shifted) of the model compound (Ss)-**1** of THD-2SO.

3.1.4. Ab initio CD calculations and absolute configuration determination of THD-2-SO

Nowadays the analysis of CD spectra can be carried out using *ab initio* techniques, leading to safe structural determinations such as the assignment of the molecular absolute configuration [17,18]. We decided then to simulate the experimental CD spectrum of THD 2-SO by means of the Time-Dependent Density Functional Theory (TDDFT) method, using the state-of-the-art B3LYP functional [19], which, with the 6-31G* (or higher) basis sets is increasingly used by the experimental organic chemists to assign the absolute configuration of new synthetic and natural compounds [20], by means of the GAUSSIAN03 package [10]. Theoretical CD spectra were obtained both in the length and velocity representation, using the lowest 40 states, from excitation energies and rotational strengths, as a sum of Gaussian functions centred at the wavelength of each transition, with a parameter σ (width of the band at 1/e height) of 0.2 eV [21].

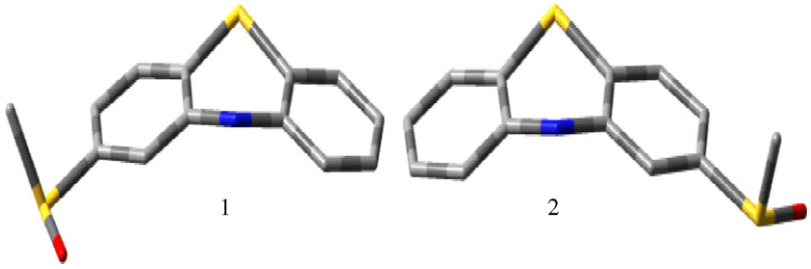


Fig. 6. Conformer 1 (left, $\Delta G = 0.0$ kcal/mol, 81% pop) and conformer 2 (right, $\Delta G = 0.86$ kcal/mol, 19% pop) structures.

The CD spectra were calculated both in the length and velocity formalisms to guarantee origin independence and evaluate the quality of the molecular wave functions employed [22].

In practice, to simulate the experimental CD spectrum of THD-2-SO, we arbitrarily assumed S absolute configuration for the sulfur atom and, to reduce the computational effort, we did our calculations on a simplified structure of THD-2-SO [(S_S)-1, Fig. 2], by removing the aliphatic chain linked to the *N* atom: this fact should reduce the number of conformers, without neglecting significant chromophores.

Conformational analysis (see also Computational Details section) was carried out by initial molecular mechanics calculations using the MMFF94s force field of SPARTAN02 [11], finding two conformers the structure of which was optimised at the DFT/B3LYP/6-31G* level of theory. These optimised structures, reported in Fig. 6 with the corresponding population, have been employed as input geometries of the CD calculations with the TDDFT/B3LYP/6-31G* method [18,19]. In this way we obtained the theoretical CD spectrum (after Boltzmann averaging) reported in Fig. 4, in comparison with the experimental spectra for the two eluted fractions of THD-2SO.

The assumed (S) absolute configuration of the sulfur atom allows a simulation of the experimental CD spectrum of the first eluted peak: in fact the sequence of positive, negative, positive Cotton effects observed for this peak is satisfactorily reproduced by the calculations in position and intensity. In particular, it is important to note that our calculations provided a nice simulation even of the weak, positive, lowest energy Cotton effect (Fig. 4).

4. Conclusions

The Sc, Ss and Rc, Rs absolute configuration was assigned to the first and second eluted fractions of THD-2SO, respectively. The satisfactory simulation of the (Sc, Ss)-THD-2SO CD spectrum by the calculated CD spectrum of (Ss)-**1**, demonstrates that the CD spectrum depends primarily on the absolute configuration at the sulfur atom. This result, which concerns one of the main metabolites of such an important drug, is really relevant for these studies because this information allows a deeper understanding of the stereochemical stability of THD-2SO, and then will permit to propose reliable correlations between stereochemistry and activity and/or toxicity. From a more general point of view, this study shows that the modern computational methods of absolute configurational assignments on the basis of the CD spectra are becoming fundamental research tools in medicinal chemistry as well.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.01.047.

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