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NMR study on the stabilization and chiral discrimination of sulforaphane enantiomers and analogues by cyclodextrins

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ABSTRACT

Sulforaphane (SFN), a phytochemical isolated from broccoli, is an important antitumoral compound with additional beneficial effect on other important diseases. However, the chemical instability of SFN has hampered its clinical use. In order to circumvent this problem, we report the first comparative study on the inclusion complexes of SFN and SFN homologues with different cyclodextrins by NMR spectroscopy. From this study it has been shown that α-CD is the most indicated cyclodextrin for the stabilization of SFN and SFN homologues, and that the highest affinity constant is that of the isothiocyanate obtained from the wasabi. Furthermore, the study of the inclusion complexes of α-CD and the non-natural SFN and analogues with *S* absolute configuration at sulfur shows for the first time that α-CD is able to discriminate between the two enantiomers, with the natural *R* enantiomers forming the inclusion complexes with higher affinity.

1. Introduction

Considered by the FDA as one of the most significant anticancer products, sulforaphane [(*R*)-1-isothiocyanato-4-(methylsulfinyl)butane)] a phytochemical product, (Lenzi, Fimognari, & Hrelia, 2014) has received increasing attention in recent years (Zhang, Zhang, Talay, Cho, & Posner, 1992). Isolated in 1992 from broccoli, it was immediately proved to be the most effective inducer of the so-called phase-2 cytoprotective, antioxidant/detoxification system (Dinkova-Kostova & Talalay, 2008), itself regulated by the Keap1/Nrf2 pathway/Are pathway (Kensler et al., 2013). Sulforaphane belongs to a family of phytochemicals, containing an isothiocyante group, present in large quantities in cruciferous such as broccoli, cauliflower, watercress, Brussels sprouts, and cabbage. These organic isothiocyanates are present in the plants in the form of their biologically inactive precursors, the glucosinolates (Grubb & Abel, 2006; Halkier & Gershenzon, 2006). These inactive precursors are converted to their cognate, biologically active isothiocyanates, by the enzymatic action of myrosinase upon mastication of the plant tissue by humans, pathogens, and predators and upon food preparation (Kliebenstein, Kroymann, & Olds, 2005). Myrosinase, a thioglycosidase, catalyses the hydrolysis of the thioglycosidic bond of the glucosinolates such as **glucoraphanin 1**, leading to unstable intermediates, which at physiological pH, predominantly undergo Lossen rearrangements to the corresponding isothiocyanates, such as **2** (Kliebenstein, Kroymann, & Olds, 2005) (Scheme 1).

Animal studies on rats have established the chemopreventive activity of sulforaphane (SFN) against colon cancer (Chung, Conaway, Rao, & Reddy, 2000; Gamet-Payrastre et al., 2000), and in women at risk for breast cancer (Comblatt et al., 2007). In addition to increasing cellular capacity for detoxifying electrophiles and oxidants, SFN has been shown to induce apoptosis, and to inhibit cell cycle progression and angiogenesis (Clarke, Dashwood, & Ho, 2008; Juge, Mithen, & Traka, 2007; Singh et al., 2009). Further, SFN can cross the blood-brain barrier and can exert its protective effects in the central nervous system (Carrasco-Pozo, & Borges, 2015; Kim et al., 2016; Tozzi et al., 2013). It has potent and selective antibiotic activity, in particular, against *Helicobacter pylori*, a risk for gastric cancer (Fahey et al., 2002; Yanaka et al., 2009). SFN has also been shown to induce anti-proliferative effects via epigenetic mechanisms (Tortorella, Royce, Licciardi, & Karagiannis, 2015), namely acting as dietary histone deacetylase inhibitor through its metabolite SFN-cysteine (Clarke, Hsu, Yu, Dashwood, & Ho, 2011; Meeran, Patel, & Tollefsbol, 2010; Xu, Parmigiani, & Marks, 2007), and to suppress DNA methylation (Hsu et al., 2011). More recently, it has also been shown that SFN serves for the treatment of autism spectrum disorder (Singh et al., 2014), and enhances progerin clearance in Hutchinson–Gilford progeria fibroblasts

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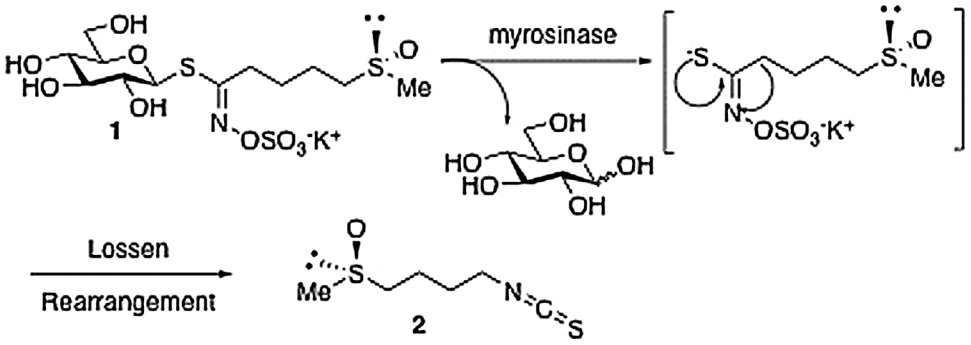


Scheme 1. Biosynthetic pathway to (*R*)-sulforaphane **2**: Lossen rearrangement through myrosinase deglycosylation of glucoraphanin **1**.

(Gabriel, Roedl, Gordon, & Djabali, 2015).

Based on these data, it is therefore not surprising that there is currently a great interest in the use of sulforaphane in the clinic and as food supplement (Houghton, Fasset, & Coombes, 2013). However, up to now there is no marketed drug with sulforaphane, being the entire SFN-related market based on broccoli extracts, anti-aging creams, diet concentrates and other herbal products. In this sense, it should be pointed out that broccoli supplements normally contain glucoraphanin, but not myrosinase, which makes questionable their ability to provide sulforaphane, since although the glucopharanin can be converted to SFN by microbiota (Bheemreddy & Jeffery, 2007), the bioavailability of SFN in this case is six times lower than when the conversion to SFN is prior to ingestion (Shapiro, Fahey, Wade, Stephenson, & Talalay, 2001). However, several clinical trials are being carried out to treat different pathologies including prostate and breast cancer, autism (both in phase II), schizophrenia, or diabetes type 2 among others (https://clinicaltrials.gov/ct2/results?term=Sulforaphane&Search=Search, 1st of June 2017). One of the main reasons that no pharmaceutical preparation applicable to any particular disease is currently commercialized is the instability of the SFN molecule, since it is susceptible to degradation by the action of oxygen, heat and even at physiological conditions, consequence of its chemical structure (Jin, Wang, Rosen, & Ho, 1999). SFN is a small molecule with two reactive functional groups, a dialkyl sulfoxide and an isothiocyante. At high temperature, the sulfoxide group undergoes a *syn* β-elimination affording butenyl isothiocyanate and methyl sulfenic acid, a transient molecule, which evolves to other volatile sulfur derivatives (Cubbage, Guo, McCulla, & Jenks, 2001). The high reactivity of the isothiocyanate group, which is at the basis of its biological activity, is also responsible for its degradation in water and in biological media leading to non-volatile, inactive organic compounds (Dubuois, Marchal, Lacroix, & Cabou, 2012).

Thus, beside the active research carried out to find chemically stable and biologically more active analogues, there is a great interest in developing effective ways to stabilize natural sulforaphane in particular and natural isothiocyanates in general. Two approximations have been reported, using either cyclodextrins (Dagan et al. 2008; Fahey et al., 2017), mainly hydroxypropyl cyclodextrin (Wu, Liang, Yuan, Wang, & Yan, 2010; Wu, Mao, Mei, & Liu, 2013), or spray dry microencapsulation into maltodextrin (Wu, Zou, Mao, Huang, & Liu, 2014).

Cyclodextrins (CDs) are macrocyclic oligosaccharides obtained from the enzymatic degradation of starch, composed by α-D-glucopyranose units (α − 1 → 4)-linked (Szejtli, 1998). The most relevant members of this family are constituted by 6, 7 and 8 units of glucose, denominated α-, β- and γ-CD, respectively, Fig. 1. They have truncated cone geometry, with the secondary hydroxyls (OH-2 and OH-3) at the wider edge of the cone and the primary hydroxyls (OH-6) of the sugar residues at the narrow edge. The skeletal carbons, the ethereal oxygens and the H-3 and H-5 protons, form the internal cavity, which gives it a hydrophobic character. This unique structure makes CDs behave like water-soluble nanocontainers for hydrophobic substances of appropriate size. Additionally, CDs are biocompatible, do not elicit immune response, and have low toxicities in animals and humans. Thus, since Freudenberg registered the first patent on the use of CDs in the formulation of drugs (Freudenberg, Cramer, & Plieninger, 1953), their properties have found many applications in pharmaceutical, cosmetic, agrochemical and food industry, among others (Davis & Brewster, 2004). From a more fundamental perspective, cyclodextrins have found applications in the design of molecular machines (Wenz, Han, & Muller, 2006), nanoparticles (Mejia-Ariza, Graña-Suárez, Verboom, & Huskens, 2017), catalysts (Vriezema et al., 2005) or artificial enzymes (Villalonga, Cao, & Fragoso, 2007). The molecular inclusion properties of the CDs have been exploited, for example, to improve the bioavailability of poorly soluble drugs and to protect the active molecules from degradation processes or prevent them from reacting with other components. Additionally, some unpleasant side effects, such as tissue irritation, bad taste or bad odour, can also be avoided by using CDs as transporters. Surprisingly, CDs have been scarcely used in the formulation of sulfinyl isothiocyanates, with only a patent work disclosing the use of α-CD (Dagan et al. 2008) which has recently been included in a preclinical study (Fahey et al., 2017) and two papers using the second-generation HP-β-CD.

However, there is no data on the strength of the inclusion complexes of SFN with cyclodextrins, that due to their different size cavities, Fig. 1, one expects that they could have different association constants. Additionally, for a given cyclodextrin the association constant may be different for each of the SFN enantiomers, used usually in racemic form in a number of studies.

Based on these premises, and within our interest in the asymmetric synthesis of SFN analogues (Elhalem et al.,2014; Khiar et al., 2009, 2013), in the present work we report a comparative study of the inclusion of (*R*)-1-isothiocyanato-6-(methylsulfinyl)hexane **4**-*R*, an isothiocyanate found in the wasabi with α-, β-, and hydroxypropyl-β-cyclodextrin, Fig. 1. A comparative study for the inclusion of each enantiomer of sulforaphane **2** (**2**-*R* and **2**-*S*), and each enantiomer of its homologues 1-isothiocyanato-5-(methylsulfinyl)pentane **3** (**3**-*R* and **3**-*S*), and 1-isothiocyanato-6-(methylsulfinyl)hexane (**4**-*R* and **4**-*S*) with α-cyclodextrin, revealed for the first time an interesting chiral discrimination phenomena, Fig. 2. We also report a degradation study of the SFN analogues in aqueous solution, over time, at room temperature in the presence and absence of cyclodextrins.

2. Results and discussions

We have recently reported an enantiodivergent approach for the synthesis of both enantiomers of sulforaphane and sulforaphane homologues with different chain length between the sulfinyl sulfur and the isothiocyanate groups and different substituents on the sulfinyl sulfur, Scheme 2 (Khiar et al., 2009).

The key step of the approach is the diastereoselective synthesis of both sulfinate esters epimer at sulfur (**8**-*R*, **9**-*R*, **10**-*R*, and **8**-*S*, **9**-*S*, **10**-*S*) from racemic sulfinyl chlorides **5–7** using as single chiral auxiliary the sugar derived diacetone-D-glucose (DAGOH) (Fernández & Khiar, 2003), by a simple change of the achiral base used to catalyse the reaction (Fernández & Khiar, 2003). The rest of the process consisted of

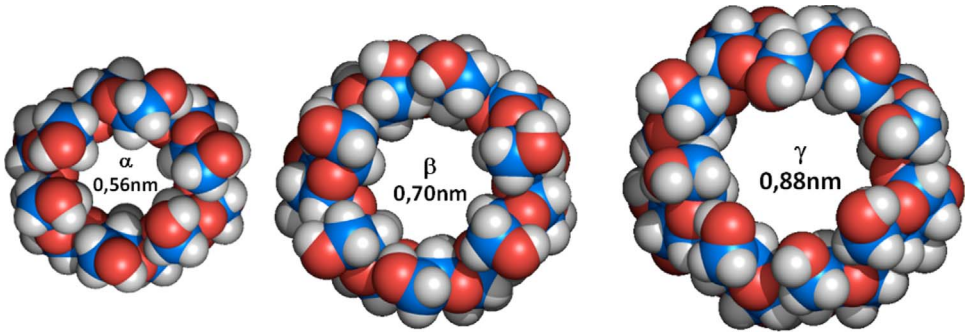


Fig. 1. Size of the internal cavities ofα, β and γ-Cyclodextrins.

the condensation of methyl magnesium bromide on the sulfinate esters, followed by a two-step one pot approach consisting of a Staudinger reaction with triphenylphosphine and subsequent aza Wittig-type condensation of the resulting iminophopsphorane with carbon disulphide. Following this approximation, both enantiomers of sulforaphane (**2**-*R* and **2**-*S*), 1-isothiocyanato-5-(methylsulfinyl)pentane (**3**-*R* and **3**-*S*), and 1-isothiocyanato-6-(methylsulfinyl)hexane (**4**-*R* and **4**-*S*) found in Japanese horseradish, wasabi (*Wasabia japonica*) (Morimitsu et al. 2002), were obtained in good chemical yields and in enantiopure forms.

Among the different methods used for the study of weak and moderate strength inclusion complexes (Thordarson, 2011), NMR spectroscopy is well suited as both guest and host molecules can be simultaneously observed at the atomic level (Fielding, 2000). The success of NMR spectroscopy in this field is due to its ability to study complex chemical systems, to determine complex stoichiometries, association constants, and conformations and to obtain information on their symmetry and dynamics. Since the rates of complex formation and decomposition are usually faster than the chemical shift time scale (often misleadingly named NMR time scale), the observed chemical shifts are the mole fraction weighted averages of the chemical shifts existing in the free and complexed molecules (Schneider, Hacket, Rüdiger, & Takeda, 1998).

Thus, the formation of the inclusion complexes of SFN and SFN-analogues used as guests with CDs used as hosts, their stoichiometry, association constants (K_a) and degree of degradation have been determined by NMR spectroscopy. In a first place and in order to determine the best cyclodextrin for the inclusion of SFN and SFN analogues, we used (R)-1-isothiocyanato-6-(methylsulfinyl)hexane **4**-R_S as guest and α-, β-, and HP-βCD as hosts. After assigning all the protons of the compounds using homonuclear 1D and 2D NMR experiments, Fig. 3A, we carried out the titration experiments. In the case of β−CD, we prepared 15 assays in which the concentration of the guest was maintained constant, while the concentration of the host was increased from 0 mM to 9.93 mM, Fig. 3C. A down-field shift was observed for the resonances of all the three groups of protons used as diagnostic signals for the titration. These resonances shift from 1.6653 ppm in the absence of CD to 1.7419 ppm at a maximum CD concentration (9.93 mM) in the case of the two protons belonging to the C5 ($CH_2C\mathbf{H_2}CH_2$), from 2.8192 to 2.8572 ppm for the methylene in α to the sulfinyl group ($C\mathbf{H_2}S$) and from 2.6276 to 2.6611 ppm for the three protons of the methylsulfinyl group ($SC\mathbf{H_3}$), Fig. 3C. The registered variation of the chemical shifts between the complexed and free guest for three types of protons, Fig. 3B, was plotted against an increasing concentration of the host, and allowed the determination of the association constant of the inclusion complex using iterative least squares fitting procedure on the basis of a 1:1 stoichiometry model. The excellent fitting obtained was supportive of the stoichiometry of the inclusion complex, and allowed us to determine the association constant (K_a). As is usual with the experimental measurements, three different values have been obtained for the K_a, being the value calculated with the $C\mathbf{H_2}S$ chemical shift data significantly higher (504 M^{-1}) than the other two (with an approximate value of 200 M^{-1}). It was assumed that the $C\mathbf{H_2}S$ protons, the ones experiencing the shortest shift and therefore least sensitive to molecular inclusion, may be affected by some other factor that distorts the measurement, so we consider the average value of the other two constants, whose values are relatively close. Therefore the value of K_a at equilibrium for the complex formed by **4**-*R* and β-CD is 192 ± 53 M^{-1} (Table 1, entry 1). Regardless of the dispersion of the calculated constants, which is not uncommon with CDs (Connors, 1997; Rekharsky & Inoue, 1998), the value of K_a is moderate, probably because the cavity of the β-CD is too large in comparison with the size of the guest to induce a tight fitting. For this reason, we performed the following tests with other cyclodextrins, α-CD and HP-β-CD, to find the best coupling between host and guest.

In this case, beside the three groups of protons used in the titration of β−CD and *R*-6HITC, we have also registered the chemical shift variation of the two protons corresponding to the carbon attached directly to the isothiocyanate group ($C\mathbf{H_2}NCS$), Fig. 3D. Following the same approximation than before, in the case of HP-β-CD we have had discordance with only one of the signals corresponding to the protons of

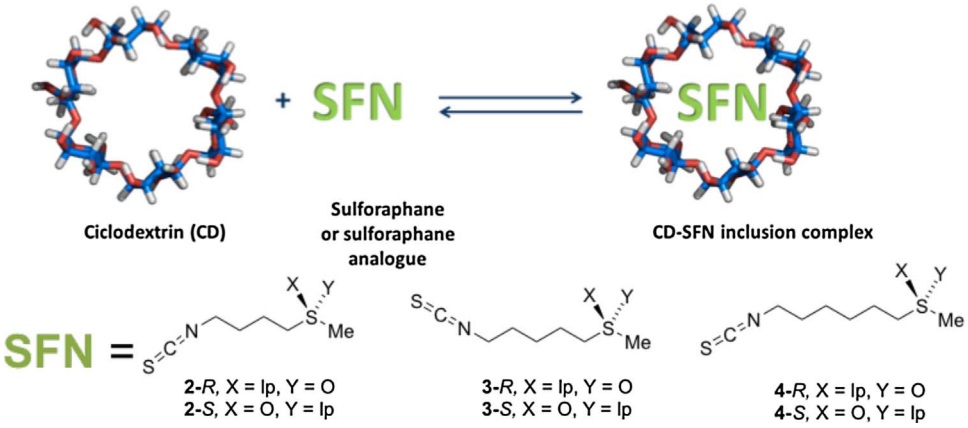
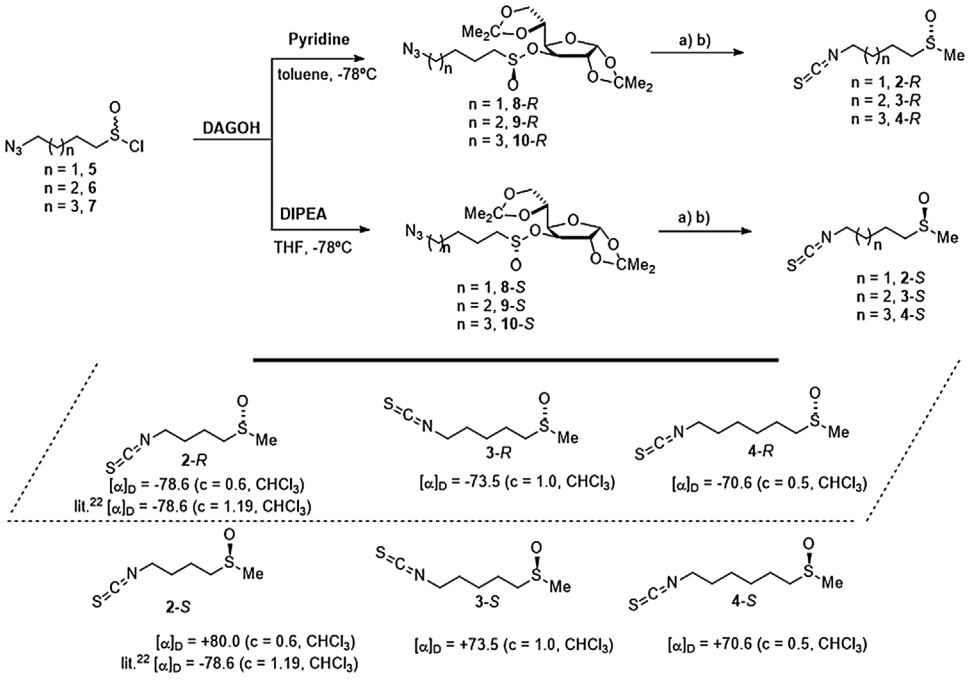


Fig. 2. Structure of both enantiomers of sulforaphane (**2**-*R*, **2**-*S*) and homologues (**3**-*R*, **3**-*S*, **4**-*R*, and **4**-*S*) used in this study to form inclusion complexes with cyclodextrins. The acronym lp stands for electron lone pair.



Scheme 2. Enantiodivergent synthesis of both enantiomers of sulforaphane **2**-*R*/**2**-*S* and homologues **3**-*R*/**3**-*S* and **4**-*R*/**4**-*S*: a) CH_3MgBr, toluene b) (i) PPh_3, Et_2O, reflux, (ii) CS_2, reflux.

a central methylene group in the aliphatic chain, which has resulted in a K_a significantly inferior to the rest, therefore, we have not taken it into account. The calculated value of the K_a (Table 1, entry 2) is 135 ± 24.8 M^{-1}, indicating that the interaction between HP-β-CD and the **4**-*R* analogue is weaker than in the case of β-CD. However, the analysis of the complexation properties of α-CD towards the same guest **4**-*R* presents some significant differences with respect to the other cyclodextrins. While the obtained values are also compatible with the existence of a complex with a 1:1 stoichiometry, the association constant K_a is significantly higher. In this case, the calculated association constant K_a (Table 1, entry 3) is 1560 ± 105 M^{-1}, that is 8 times larger than that obtained with β-CD, and more than one order of magnitude than that of HP-β-CD, the most widely used cyclodextrin for the stabilization of SFN. Based on this result, which highlights the complementarity of the smaller size cavity of α-CD to host the SFN analogue **4**-*R*, we conducted a study in order to unravel its affinity to others isothiocyanates including sulforaphane and, most importantly, to study the chiral discrimination between both enantiomers of each isothiocyanate, if there is any. In the case of natural sulforaphane [(*R*)-1-isothiocyanato-4-(methylsulfinyl)butane)] **2**-*R*, the titration has been carried out using the chemical shift change of the methylsulfinyl group, and three methylene groups of the internal aliphatic chain (CH_2 α to the

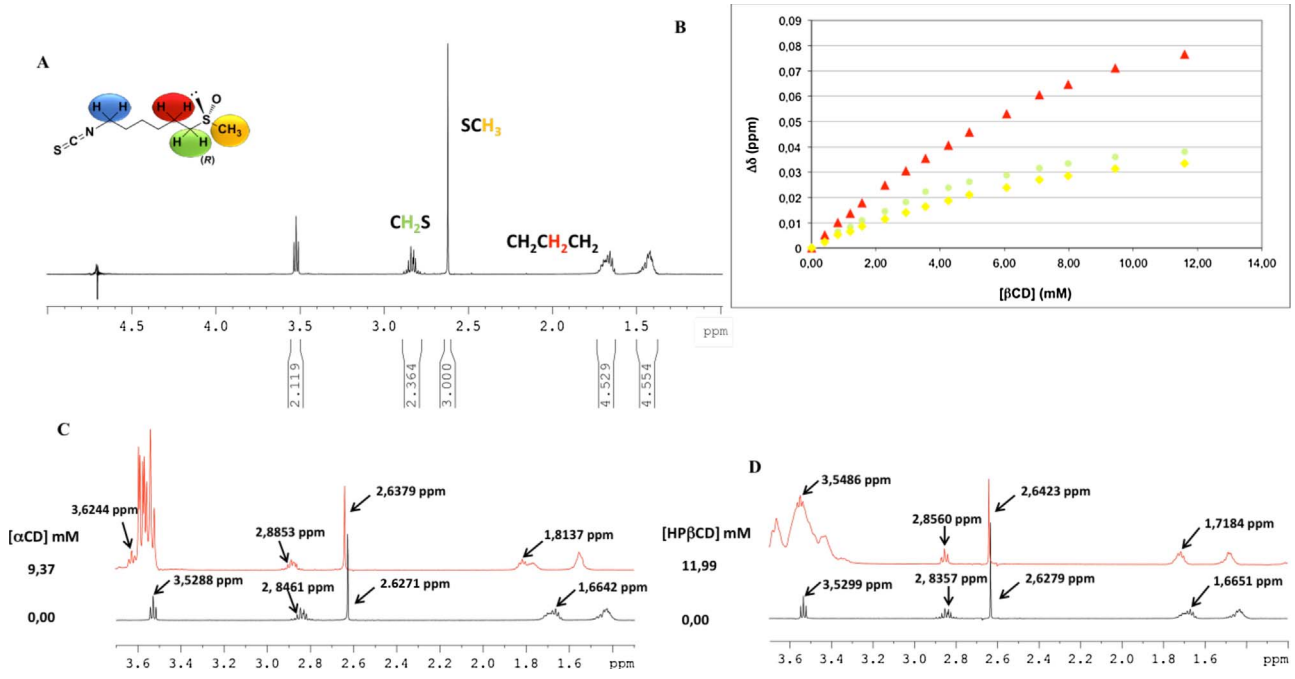
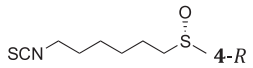
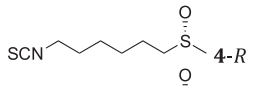
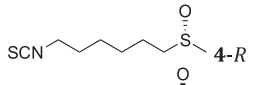
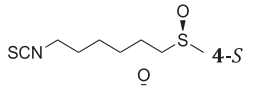
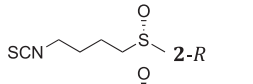
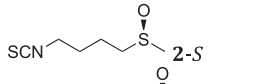
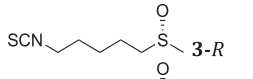
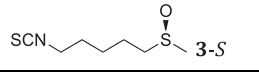


Fig. 3. ^{1}HNMR titration experiments of R_S-1-isothiocyanato-6-(methylsulfinyl)hexane **4**-*R* with α- and β-CDs: (A) ^{1}HNMR spectra of pure **4**-*R*. (B) Chemical shift changes of reporters protons upon adding increasing amount of β-CD host. (C) Portion of ^{1}HNMR spectra of **4**-*R* alone and in the presence of 9.37 mM of α-CD. (D) Portion of the ^{1}HNMR spectra of **4**-*R* alone and in the presence of 11.99 mM of HP-β-CD.

Table 1
Association constants of both enantiomers of sulforaphane **2**-*R*/**2**-*S* and homologues **3**-*R*/**3**-*S* and **4**-*R*/**4**-*S*.

Entry	Compound	Cyclodextrin	Association Constant (m^{-1})
1	 **4**-*R*	HP-β-CD	135 ± 25
2	 **4**-*R*	β-CD	192 ± 53
3	 **4**-*R*	α-CD	1560 ± 105
4	 **4**-*S*	α-CD	887 ± 203
5	 **2**-*R*	α-CD	519 ± 56
6	 **2**-*S*	α-CD	176 ± 36
7	 **3**-*R*	α-CD	1229 ± 127
8	 **3**-*S*	α-CD	715 ± 132

sulfinyl, and CH_2 α and β to the isothiocyanate group, see supporting information). In this case, the four association constants obtained were in the same range (463 M^{-1} for CH_2 α to the isothiocyanate, 526 M^{-1} for CH_2 β to the isothiocyanate, 533 M^{-1} for CH_2 α to the sulfoxide group, and 611 M^{-1} for the methyl group), the average value being 519 ± 55.8 (Table 1, entry 5), a value lower than that of **4**-*R* [(R)-1-isothiocyanato-6-(methylsulfinyl)hexane)]. In the case of the analogue (*R*)-1-isothiocyanato-5-(methylsulfinyl)pentane **3**-*R*, the titration has been carried out using the chemical shift change of the methyl sulfinyl group, and four methylene groups of the internal aliphatic chain (CH_2 α and β to the sulfinyl, and the CH_2 α and β to the isothiocyanate group, see supporting information). In this case, the association constants measured for each of the five proton signals were also in the same range (1180 M^{-1} for CH_2 α to the isothiocyanate, 1130 M^{-1} for CH_2 β to the isothiocyanate, 1250 M^{-1} for CH_2 α to the sulfoxide group, 1260 M^{-1} for CH_2 β to the sulfoxide group and 1660 M^{-1} for the methyl group), the average value being 1229 ± 127 M^{-1}, a value lower than that of **4**-*R*, and higher than that of natural sulforaphane. The results obtained so far indicate a clear relationship between the length of the aliphatic chain and the affinity towards α-cyclodextrin. The highest affinity constant being that of the compound with the longest aliphatic chain between the sulfinyl and isothiocyanate groups, *ie* **4**-*R* [(*R*)-1-isothiocyanato-6-(methylsulfinyl)hexane)].

These results encourage us to undergo a study on the chiral discrimination of α-CD between the two enantiomers of a given sulfinylisothiocyanate molecule. In this sense, due to the presence of D-glucose units, CDs are chiral molecules and as such can form disatereoisomers with other molecules having a chiral centre. The capacity of α-cyclodextrin to discriminate between two enantiomers of a chiral guest was firstly reported in the late fifties of the last century (Cramer & Dietsche, 1959). The discovery of the crucial importance of chirality on the biological activity of drugs has deeply changed the legislation on their commercialization by the drug agencies worldwide (Nguyen, He, & Pham-Huy, 2006). Thus, the preparation and characterization of single enantiomers has been a standing area of interest in the last three decades (Borman, 2001). In this sense, CDs have been widely studied in chiral resolution of racemates (Kano, 1997), as chiral reactors for enantioselective transformations (Macaev & Boldescu, 2015) and in chiral discrimination of enantiomers in solution (Shahgaldian & Pieles, 2006). Thus, our next objective was the study of the inclusion complexes of α-CD and the non-natural SFN and analogues, in order to determine the possible ability of α-CD to discriminate between (*R*) and (*S*)-enantiomers of SFN and SFN analogues guests. This study is important taking into account the future evolution of sulforaphane and sulforaphane analogues market. In this sense, most of the medical applications either use broccoli extracts rich in SFN that is the (*R*)-enantiomer, while others use racemic mixtures obtained from non-asymmetric synthesis. For industrial production of SFN, it is predicted that the racemic mixture will be preferred, consequence of the low cost of non-enantioselective chemical synthesis.

Following the same approximation used for the determination of the affinity constants explained before, we determine the association constants of α-CD with the (*S*)-enantiomer of SFN **2**-*S*, with (*S*)-1-isothiocyanato-6-(methylsulfinyl)hexane **4**-*S*, and with (*S*)-1-isothiocyanato-5-(methylsulfinyl)pentane **3**-*S*. In the case of the non natural (*S*)-SFN **2**-*S*, the average of 4 association constants, obtained through the titration of the methylsulfinyl group, and three methylene groups of the internal aliphatic chain, gave a value of 176 ± 36 M^{-1}. Taking into account that the association constant of α-CD and natural SFN is 519 ± 56 M^{-1}, quasi three times higher than that with the (*S*)-enantiomer, we can conclude that there is an important chiral discrimination between the two enantiomers of sulforaphane by α-CD. The same trend has been obtained with **3**-*S* and **4**-*S* analogues. The average values obtained for the former is 715 ± 132 M^{-1} (Table 1, entry 8) and 887 ± 203 M^{-1} (Table 1, entry 4) for the later, quasi twice lower than those obtained with the corresponding enantiomers **3**-*R* and **4**-*R* respectively. These results indicate that the chiral discrimination of α-CD between the two enantiomers of sulfinyl-alkane-isothiocyanates is general, being the inclusion complex with the natural (*R*)-enantiomer the most favoured one. The magnitude of the chemical shift changes in the inclusion complexes of α-CD for each of the enantiomers, allows the determination of the topology of the supramolecular complexes. In this sense, the analyses of the six inclusion complexes show that the methylene groups of the aliphatic chain, between the two functionalities are all affected, but at different extents. The major chemical shift difference is observed for the methylene γ to the sulfinyl group, and the minor one is the methylene α- to the sulfoxide. The methylene near the isothiocyanate are also affected by the cyclodextrin effect, but at a lesser extent than the central ones. At the contrary, the chemical shift of the methyl substituent of the sulfinyl sulfur is practically the same in the inclusion complex than in the free compounds. All these data indicate that the inclusion of SFN and analogues consists in the incorporation of the aliphatic chain and the isothiocyanate moiety into the α-CD cavity. The resulting complex can be further stabilized by hydrogen bonding between the basic sufinyl oxygen and the secondary hydroxy groups situated at the wider edge of truncated cone-shaped cyclodextrin. The observed stereospecificity of inclusion of SFN and SFN analogues into α-CD, can thus be explained by the interaction between the methyl substituent of the sulfoxide group and the cyclodextrin substituent, favouring the enantiomers with the *R* absolute configuration at the sulfinyl sulfur, Fig. 4.

The proposed model, can adequately explain the reported stabilization of cyclodextrin. Indeed, as it was indicated before, the high aqueous instability of sulforaphane is a consequence of the high reactivity of the isothiocyanate group, which in presence of water leads to the free amine, followed by reaction of the amine with an isothiocyanate of other molecules affording the dimeric thiourea compound. In the inclusion complex the isothiocyanate group is incorporated into the hydrophobic cavity of the cyclodextrin and is thus less accessible to the water molecules. Based on this assumption we conducted a comparative stability study of **4**-*R* (*R*-1-isothiocyanato-6-(methylsulfinyl)hexane) in aqueous solution at room temperature in the absence, Fig. 5 and in the presence of HP-β-CD and α-CD, Fig. 6.

The study has bee conducted on a 3 mM solution of the SFN analogue **4**-***R*** in D_2O, followed by recording successive ^1H NMR spectra at different time lapses. Degradation was monitored by the decrease of

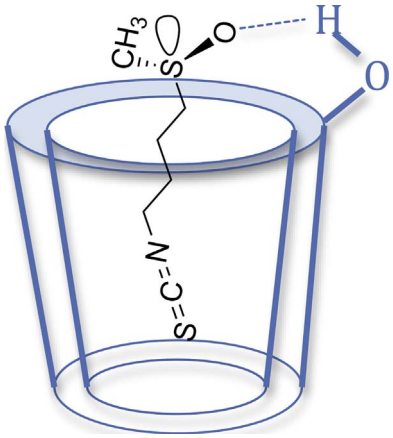


Fig. 4. Explanative model for the observed inclusion stereospecificity of sulfinyl isothiocyanate into α-CD.

relative intensity of C*H*$_2$NCS resonance and C*H*$_3$S splitting. At time 0 (Fig. 5A) signals appear as triplet and singlet, respectively, with the expected 2/3-relative intensity. Successive experiments revealed the decrease of C*H*$_2$NCS resonance intensity (ca. 50% in 4.5 months at rt, Fig. 5B) and the parallel splitting of C*H*$_3$S signal. In parallel, two other aliquots of the starting **4**-*R* solution were added α- or HPβCD to a **4**-***R***:CD molar ratio of 1:3. Despite the overlapping with CD signals, which prevented the quantification of C*H*$_2$NCS resonance intensity decrease, the larger splitting of the methyl signal resulted a good alternative in these cases (Fig. 6).

The integrity of the analogue was monitored for a similar period of time (4.5 months), after which degradation proved to be much slower: approximately 16% and 36% for the α- and HPβCD formulations, respectively, as determined by separate integration of the splitted methyl signals. This result is in agreement with the lower concentration of unprotected (CD-uncomplexed) **4**-*R* in the equilibrium estimated in ca. 10% and 40%, respectively for α- and HPβCD according to the measured K_{as} (Table 1). This observation is further supported by the overall intensity increase of the signal in the range 2.95–2.80 ppm attributed to *N*-linked methylene groups arising from isothiocyanate degradation into amine and ulterior thiourea formation (Fig. 6B and D).

3. Conclusions

In summary, we have reported in this study the first comparative study on the strength of the inclusion complexes of SFN and SFN homologues with different cyclodextrins by NMR spectroscopy. From this study it has been shown that despite of being widely used in the stabilisation of sulforaphane, HP-β-CD is the least indicated guest for this process as it forms the inclusion complex with SFN homologue **4**-*R* with the lesser stability, being α-CD the most indicated cyclodextrin.

The study of inclusion complexes of α-CD with natural SFN and natural SFN homologues shows that the highest affinity constant is that of the compound with the lengest aliphatic chain between the sulfinyl and isothiocyanate groups, *ie* (*R*)-1-isothiocyanato-6-(methylsulfinyl) hexane **4**-*R*, an important isothiocyanate obtained from the wasabi. The study of the inclusion complexes of α-CD and the non-natural SFN and analogues with *S* absolute configuration at sulfur shows for the first time that α-CD is able to discriminate between the two enantiomers, with the natural *R* enantiomers forming the inclusion complexes with higher affinity. Based on the obtained experimental results, an explanative model on the topology of the inclusion complexes of isothiocyanates and α-CD as well as on the stereospecificity observed has been proposed. The NMR study on the aqueous stabilization of isothiocyanate by cyclodextrins, confirms the titration studies and shows that the protecting capacity of the cyclodextrins as hosts is directly related to the stability of the inclusion complex with isothiocyanates guests, being the α-CD having the smallest internal hydrophobic cavity the better host.

4. Experimental section

4.1. General experimental methods

^{1}H (and ^{13}C NMR) spectra were recorded at 500 (125.7 MHz) with a Bruker Avance DRX500. 2D COSY and HMQC experiments were used to assist on NMR assignments. Chemical shifts are reported in ppm, and coupling constants are reported in Hz. Routine spectra were referenced to the residual proton or carbon signals of the solvent. High-resolution mass spectra were recorded on a Kratos M(S)-80RFA 241-MC apparatus. Optical rotations were measured at 20 °C in a 1-dm glass tube. α-, β-, and HP**β**CDs were purchased from Aldrich and used without purification. SFN **2**-*R*, isothiocyanates **3**-*R* and **4**-*R* as well as their enantiomers **2**-*S*, **3**-*S* and **4**-*S* used in this study were obtained following the recently reported enantiodivergent method based on the DAG-methodology.

4.2. (-)-(R)-1-Isothiocyanato-4-(methylsulfinyl)butane (2-R)

Colourless liquid. R_f = 0.15 (EtOAc / MeOH, 9:1); $[\alpha]_D$ = −78.6 (c = 0.6, CHCl3); ^{1}H NMR (500 MHz, CDCl3) δ 3.62 (t, 2H, *J* = 10.5 Hz), 2.84–2.67(m, 2H), 2.61 (s, 3H), 2.02–1.86 (m, 4H); ^{13}C NMR (125 MHz, CDCl3) δ 53.5, 44.6, 38.7, 29.0, 20.1; HRMS *m/e* calcd. for $C_6H_{11}NOS_2$ $(M+H)^+$: 178.0360, found: 178.0367.

4.3. (+)-(S)-1-Isothiocyanato-4-(methylsulfinyl)butane (2-S)

Colourless liquid. R_f = 0.30 (EtOAc / MeOH, 9:1); $[\alpha]_D$ = + 80.00 (c = 0.8, $CHCl_3$); Spectroscopical data identical to those of the **2**-***R*** enantiomer. HRMS *m/e* calcd. for $C_6H_{12}NOS_2$ $(M+H)^+$: 178.0360, found: 178.0358.

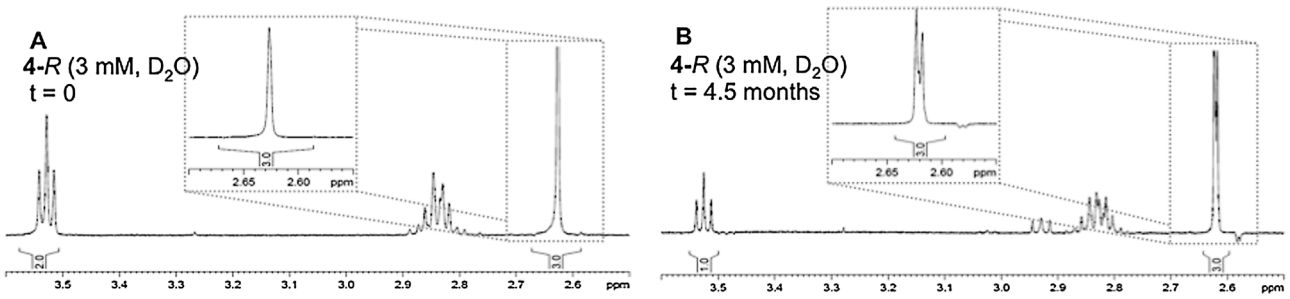


Fig. 5. Section of the ^{1}H NMR spectra of **4**-***R*** (500 MHz, 3 mM, D_2O) at (A) time 0 and (B) after 4.5 months at room temperature. The inbox highlights the C*H*$_3$ signal splitting upon degradation, which is quantified on the basis of the decrease of intensity of C*H*$_2$NCS resonance (3.5 ppm) vs. C*H*$_3$ signal (2.6 ppm) in ca. 50%.

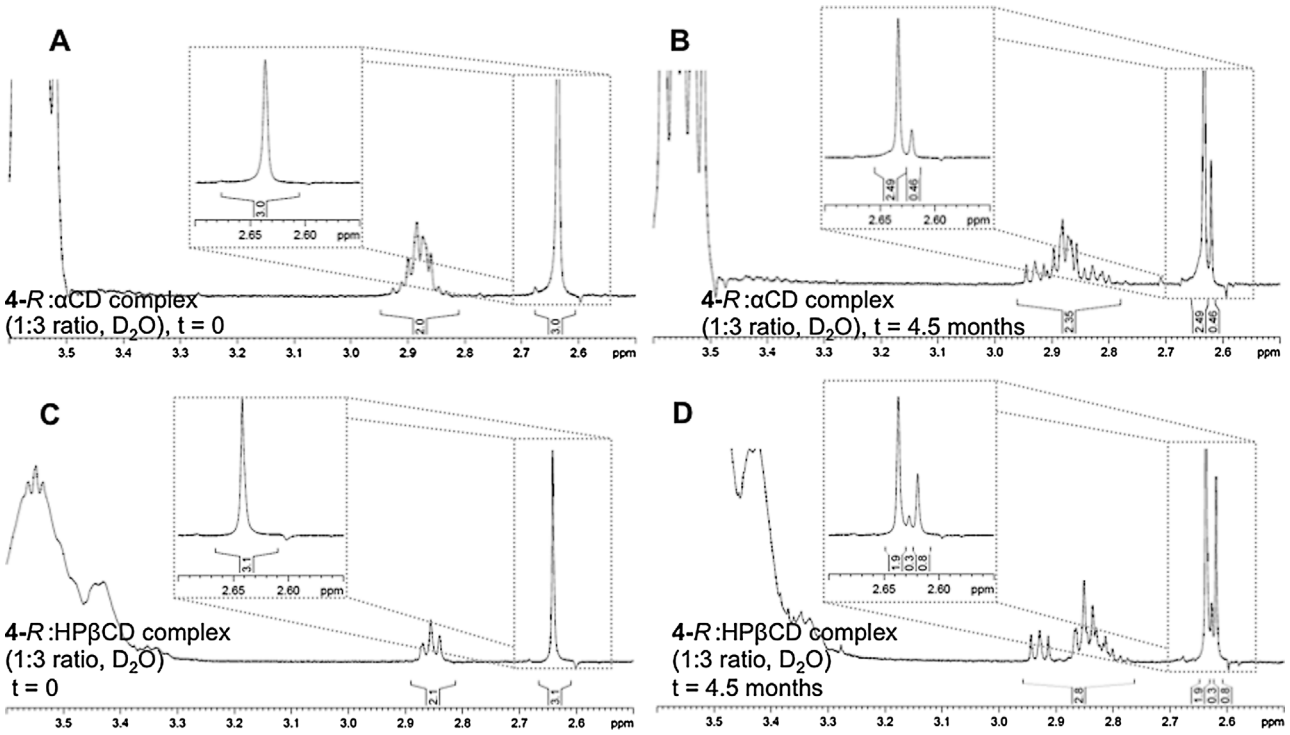


Fig. 6. Section of the ^{1}H NMR spectra of **4-*R*** complexes (500 MHz, 3 mM, D_2O, 1:3 M ratio) with αCD (A and B) or HPβCD (C and D) at time 0 (A and C) and after 4.5 months (b and D) at room temperature. The inbox highlights the CH_3 signal splitting upon degradation (ca. 16% and 36% in 4.5 months for αCD and HPβCD, respectively).

4.4. (-)-(R)-1-isothiocyanato-5-(methylsulfinyl)pentane (4-R)

Ambar syrup. R_f = 0.25 (EtOAc / MeOH, 9:1); $[\alpha]_D$ = −73.48 (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.54 (t, 2H, *J* = 6.5 Hz), 2.76–2.63 (m, 2H), 2.57 (s, 3H), 1.86–1.73 (m, 4H), 1.67–1.55 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 54.2, 44.7, 38.7, 29.6, 25.8, 21.9; HRMS *m/e* calcd. for $C_7H_{13}NONaS_2$ $(M + Na)^+$: 214.0336, found: 214.0339.

4.5. (+)-(S)-1-isothiocyanato-5-(methylsulfinyl)pentane (4-S)

Ambar syrup. The spectroscopic data are identical to those of its enantiomer, **4-*R***. $[\alpha]_D$ = + 69.23 (c = 0.8, $CHCl_3$); HRMS *m/e* calcd. for $C_7H_{14}NOS_2$ $(M+H)^+$: 192.0517, found: 192.0515.

4.6. (R)-(-)-1-isothiocyanato-6-(methylsulfinyl)hexane (5-R)

Ambar syrup. R_f = 0.4 (EtOAc / MeOH, 9:1); $[\alpha]_D$ = −70.62 (c = 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.52 (t, 2H, *J* = 10.5 Hz), 2.77-2.60 (m, 2H), 2.56 (s, 3H), 1.83–1.67 (m, 4H), 1.53-1.46 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 54.4, 44.9, 38.7, 29.6, 27.9, 26.2, 22.4; HRMS *m/e* calcd. for $C_8H_{16}NOS_2$ $(M+H)^+$: 206.0673, found: 206.0669.

4.7. (+)-(S)-1-isothiocyanato-6-(methylsulfinyl)hexane (5-S)

Ambar syrup. The spectroscopic data are identical to those of its enantiomer, **5-*R***. $[\alpha]_D$ = + 68.77 (c = 1.0, $CHCl_3$); HRMS *m/e* calcd. for $C_8H_{16}NOS_2$ $(M+H)^+$: 206.0673, found: 206.0677.

4.7.1. NMR titration experiments

The association constants (K_a) were determined in D_2O at 298 K on a 500 MHz by measuring the proton chemical shift changes of solutions of the SFN and SFN analogues **2–4** upon increasing amounts of host (α-CD, β-CD, or HP-β-CD). In a typical titration experiment, a 3 mM solution of **4-*R*** in D_2O was prepared, a 500-μL aliquot was transferred to a 5-mm NMR tube, and the initial NMR spectrum was recorded. A 21 mM solution of α-CD in the previous host solution was prepared and then sequentially added to the NMR tube containing the guest via microsyringe in 10 μL portions. These amounts were increased until 90–100% complexation of the host. The ^{1}H NMR spectrum of each mixture was recorded after each addition and the chemical shift of the diagnostic signals obtained at 15–19 different host-guest concentration ratios were used in an iterative least-squares fitting procedure (Bisson et al., 2000; Bisson, Hunter, Morales, & Young, 1998).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2017.12.022

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