A Simple ^{13}C NMR Method for the Discrimination of Complex Mixtures of Stereoisomers: All Eight Stereoisomers of α-Tocopherol Resolved

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ABSTRACT A simple one-dimensional ^{13}C NMR method is presented to discriminate between stereoisomers of organic compounds with more than one chiral center. By means of this method it is possible to discriminate between all eight stereoisomers of α-tocopherol. To achieve this the chiral solvating agent (*S*)-(+)-1-(9-anthryl)-2,2,2-trifluoroethanol and the compound of interest were dissolved in high concentrations in chloroform-d, and the nuclear magnetic resonance (NMR) spectrum was recorded at a low temperature. The individual stereoisomers of α-tocopherol were assigned by spikes of the reference compounds. The method was also applied to six other representative examples. *Chirality 27:850–855, 2015.* © 2015 Wiley Periodicals, Inc.

KEY WORDS: enantiomers; diastereoisomers; vitamin E; Pirkle's alcohol; TFAE

Nuclear magnetic resonance (NMR) is a powerful method for the determination of the stereochemical purity of organic compounds.[1] In the case of diastereomers the discrimination between different diastereomers is without effort, provided that the chiral centers are not too far separated. In the case of enantiomers, these enantiomers can be converted into diastereomers with a suitable chiral reagent, or a chiral solvating agent can be used to create a chiral environment. Many chiral solvating agents (CSAs) have been proposed and used with success, and among those CSAs optically pure (*S*)-(+)-1-(9-anthryl)-2,2,2-trifluoroethanol (TFAE, Pirkle's alcohol) is one of the most versatile compounds.[2–4]

Most work with CSAs has been done with ^{1}H NMR, because ^{1}H NMR is sensitive, a high resolution can be obtained, and TFAE induces relatively large chemical shift differences. However, in the case of spectra with broad multiplets, the chemical shift differences between the *R*- and *S*-enantiomer that can be obtained are often not sufficient. This problem becomes even more important when spectra are crowded, exhibiting overlapping multiplets. Most studies with CSAs deal with the discrimination of enantiomeric pairs, and it goes without saying that if the compound studied contains more than one chiral stereogenic center, and if each chiral carbon adopts both possible configurations, this adds to the complexity of the ^{1}H NMR approach.

A simplification of such crowded spectra can be achieved by the homonuclear decoupling (pure shift) approach,[5,6] or as recently proposed[7] by means of ^{13}C NMR. ^{13}C NMR spectroscopy has long been neglected for enantiodiscrimination, and only a few studies exist. This is probably mainly due to the low sensitivity of ^{13}C NMR. It has already been argued,[7] that the sensitivity of ^{13}C NMR has increased tremendously due to the availability of stronger magnetic fields, and due to probes with cryogenically cooled ^{13}C coils, which makes ^{13}C NMR a more suitable technique for enantiodiscrimination than previously. At first sight, ^{13}C NMR seems to be less suitable than ^{1}H NMR for quantitative evaluation of mixtures of stereoisomers, because one has to avoid the nuclear Overhauser effect (NOE) of protons, and long relaxation delays are required to obtain truly quantitative spectra. However, it has been shown convincingly[8] that short relaxation delays and standard broad band decoupling may be used to obtain compound ratios, provided that the compounds have similar structures, and carbon atoms with the same number of attached protons are compared. The latter conditions are fully met when ratios of stereoisomers are determined.

Tocopherols have three chiral carbon atoms, and therefore eight different stereoisomers of α-tocopherol exist (Fig. 1). These eight stereoisomers come as four pairs of enantiomers. The discrimination between all eight stereoisomers of tocopherol is an extremely challenging task.

In the past, several attempts have been undertaken to separate the stereoisomers of (all-*rac*)-α-tocopherol by means of chromatographic techniques. Partial separation was obtained for the four pairs of enantiomers of (all-*rac*)-α-tocopherol as trimethylsilyl (TMS) ether derivative by means of gas chromatography (GC) on an achiral column.[9] Baseline separation of the four pairs of enantiomers was realized by analyzing (all-*rac*)-α-tocopherol as the methyl ether derivative.[10] By means of chiral high-performance liquid chromatography (HPLC) on a Chiralpak OT column, only two peaks were obtained for (all-*rac*)-α-tocopherol as acetyl derivative.[11] Separation of (all-*rac*)-α-tocopherol on a Chiralcel OD-H column also resulted in two peaks: 2*R*-stereoisomers (*RRR*, *RRS*, *RSR*, *RSS*) were separated from 2*S*-stereoisomers (*SRR*, *SRS*, *SSR*, *SSS*).[12] By means of a Chiralpak OP(+) column, the acetyl derivative of (all-*rac*)-α-tocopherol could be separated into four peaks: 2*R*-stereoisomers constituted the first peak and 2*S*-stereoisomers were separated into three peaks.[13] Use of a chiral stationary phase (CSP) in open-tubular electrochromatography resulted in only two groups of α-tocopherol stereoisomers that were separated.[14] Two chromatography studies have been published in which the eight stereoisomers of (all-*rac*)-α-tocopherol could be separated by combination of a chiral HPLC and

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Received for publication 8 July 2015; Accepted 27 August 2015
DOI: 10.1002/chir.22535
Published online 18 September 2015 in Wiley Online Library (wileyonlinelibrary.com).

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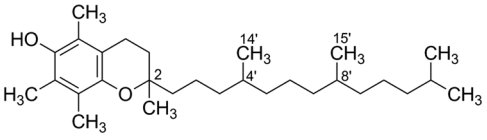

Fig. 1. Structure of α-tocopherol. Chiral carbon atoms are C2, C4' and C8'.

a GC method. In the first study,[15] a derivatization was performed to obtain the ethyl ether derivative of (all-*rac*)-α-tocopherol, followed by two chromatographic separations: first a chiral HPLC separation, which yielded two fractions. One fraction contained the four (2*R*) stereoisomers, the other fraction contained the four (2*S*) stereoisomers. Thus, each fraction contains only diastereomers, and no pairs of enantiomers, and in the next step each fraction could be separated into the four constituent stereoisomers by means of achiral capillary GC. In the second study,[16] derivatization was performed with dimethyl sulfate. The resulting methyl ether derivatives of (all-*rac*)-α-tocopherol were injected on a chiral HPLC column, yielding five peaks corresponding to the 2*S* (*SRS*+*SSR*+*SSS*+*SRR*) and *RSS*, *RRS*, *RRR*, *RSR* stereoisomers. The 2*S* stereoisomer fraction was collected with a fraction collector and analyzed by achiral GC.

It has been shown[17,18] that the four pairs of enantiomers can be discriminated by ^{13}C NMR, albeit after the overnight accumulation of NMR signal in a 10-mm tube.

The aim of the present study was to find conditions to discriminate between all eight stereoisomers of α-tocopherol.

MATERIALS AND METHODS

General NMR Procedures

(all-*rac*)-Isophytol, (all-*rac*)-3-methyl-1,2-cyclopentanediol, and (*S*)-(+)-1-(9-anthryl)-2,2,2-trifluoroethanol (TFAE) were purchased from Sigma-Aldrich (St. Louis, MO).

All-racemic tocols: (all-*rac*)-α-tocopherol is a commercial product of DSM Nutritional Products, Switzerland; (all-*rac*)-β-tocopherol, (all-*rac*)-γ-tocopherol, (all-*rac*)-δ-tocopherol, and (all-*rac*)-tocol were synthesized in-house at F. Hoffmann-La Roche in Basel on lab scale.

Samples were dissolved in $CDCl_3$. Approximately 20 mg of compound of interest and 60 mg of TFAE were dissolved in 0.6 ml of solvent, unless otherwise stated. These concentrations correspond to molar concentrations of 77 mM and 362 mM, respectively.

NMR spectra were recorded on a Bruker (Billerica, MA) Avance III 700 MHz NMR spectrometer, equipped with a cryogenically cooled 5 mm TCI probe. The low-temperature version of this probe was used, which can be operated in a temperature range of 240–340 K.

Standard conditions for NMR data acquisition were as follows: Spectra were recorded with 256 scans and a delay of 5 s between pulses in order to avoid heating of the sample, resulting in an acquisition time of 30 min. A time domain of 64 K data points was used, and FIDs were zero-filled to 256 K data points. The sweep width was reduced to 85 ppm and the aromatic part of the spectrum was filtered out. In this way a high digital resolution was obtained of 0.1 Hz/pt. The low detection limit of 1% for minor stereoisomers was achieved by recording 1 K scans with a delay of 3 s between pulses, which resulted in a total acquisition time of 90 min.

Before Fourier transformation a Gaussian filter was applied with parameters LB = −0.8, GB = 0.25. NMR spectra were also recorded at higher temperatures and at lower concentrations. In these cases the parameter GB was adapted to a maximum value of 0.7 in order to be able to observe the rapidly decreasing peak separation.

Synthetic Procedures for Enriched Stereoisomers

General. All reactions were performed under an argon atmosphere. Room temperature was 20–24 °C. All reagents and chemicals not mentioned separately were obtained from commercial suppliers and were used without further purification, if not stated individually. Tetrahydrofuran (THF) was freshly distilled from Na/ benzophenone under argon. For transfer of solvents, reagents (if liquid), or their solutions, syringes, cannulas, and rubber septa were used. The reactions were monitored by thin-layer chromatography (TLC) using glass plates coated with silica gel 60 F254, thickness 0.25 mm (Merck, Germany); spots were visualized by UV-light and by spraying with a solution of ammonium molybdate / cerium sulfate in water / sulfuric acid, and subsequent heating. Flash chromatography was performed using silica gel 60, 0.040–0.063 mm (Merck) with max. 0.2 bar gauge pressure argon. The identity and purity of intermediates were checked by using standard spectroscopic and physicochemical methods and compared to literature data. A schematic representation of the procedure for the synthesis of enriched α-tocopherol stereoisomers is given in Figure 2 and experimental procedures are detailed below.

Trolox. (*R*)-Trolox, content 99.9% (GC), enantiomeric excess (*ee*) > 99.8% (GC); (S)-Trolox, content 99.9% (GC), *ee* > 99.9% (GC); both compounds were prepared by optical resolution in the kilo-lab of F. Hoffmann-La Roche, Basel, according to a published procedure.[19]

Hexahydrofarnesol. Two samples of the following stereoisomeric composition have been prepared according to published procedures.[20,21] Sample 1: 3*R7R* 93.45%, 3*R7S* 5.81%, 3*S7R* 0.75%, 3*S7S* 0%; sample 2: 3*R7R* 0.12%, 3*R7S* 6.22%, 3*S7R* 1.36%, 3*S7S* 92.30%. The stereoisomeric

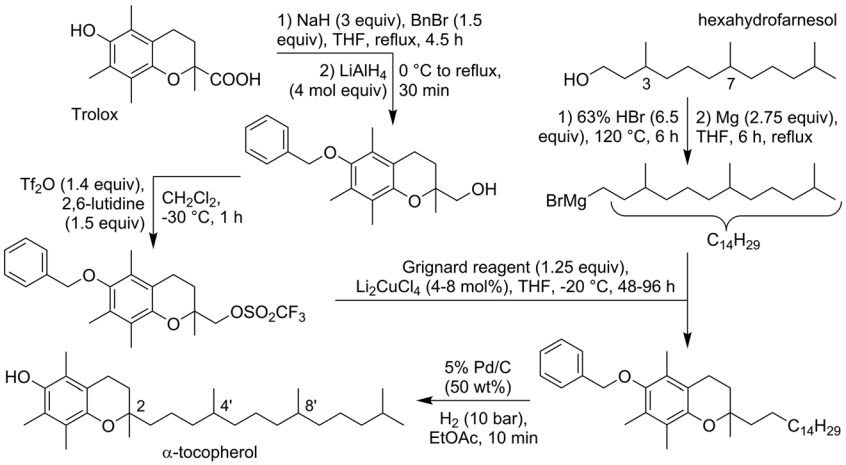

Fig. 2. Schematic procedure for the synthesis of enriched α-tocopherol stereoisomers.

composition was determined by using the acetal method (after oxidation of the primary alcohol to the corresponding aldehyde) as described in the literature.[22,23]

3,4-Dihydro-6-benzyloxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl]methanol. To a suspension of NaH (7.42 g, 97%, dry, Aldrich, Milwaukee, WI, 300.0 mmol, 3.0 equiv.) in THF (200 mL) the solution of Trolox (25.06 g, 99.9%, 100.0 mmol) was added dropwise at 26 °C under stirring. Additional THF (150 mL) was added to facilitate stirring. The beige suspension was heated to reflux (oil bath 90 °C) under stirring for 2 h. Benzyl bromide (26.18 g, 98%, 300.0 mmol, 3.0 equiv.) was added, and the mixture further stirred at reflux temperature for 2.5 h. The mixture was cooled to 0 °C, and $LiAlH_4$ (15.65 g, 97%, 400.0 mmol, 4 mol equiv.) was added carefully in portions. After heating for 30 min at reflux temperature (oil bath 95 °C), the mixture was stirred at 23 °C for 16 h. At 0 °C small portions of ice were added slowly and carefully until foaming ceased, and 2 N HCl (750 mL) was added to the gray suspension. The organic phase was separated, and the aqueous phase extracted with diethyl ether (250 mL, twice). The organic phases were washed separately with 1 N HCl and sat. NaCl solution (100 mL each), combined, dried over sodium sulfate, filtered, evaporated (20 mbar, 40 °C), and dried (30 min, 0.022 mbar, 24 °C). The 36.34 g red-yellow oil was dissolved in n-pentane/diethyl ether (85 mL/ 15 mL). After crystallization (−20 °C, 16 h) and washing with cold n-pentane/ diethyl ether (9:1, 75 ml) the crystals were dried (0.022 mbar, 24 °C): 28.76 g (88%) off-white crystals. From the mother liquor, additional 4.79 g yellow oil was obtained. An analytically pure sample (colorless crystals, 98.1%) was obtained from the crystals by recrystallization from the same solvent mixture.

3,4-Dihydro-6-methoxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl] methyl trifluoromethane-sulfonate. Triflic anhydride (19.75 g, 11.5 mL, 70.0 mmol, 1.4 equiv.) was added dropwise during 20 min to the stirred solution of the alcohol (16.93 g, 96.4%, 50.0 mmol) and 2,6-lutidine (8.20 g, 8.90 mL, 98%, 75.0 mmol, 1.5 equiv.) in dichloromethane (240 mL) at −30 °C.[24] After stirring for 1 h at this temperature, the mixture was poured onto 2 N H_2SO_4 of 0 °C (100 mL). The organic phase was separated and washed with sat. $NaHCO_3$ and NaCl solutions (100 mL each). The water phases were extracted with dichloromethane (100 mL), and the combined organic extracts dried over sodium sulfate, filtered, evaporated (20 mbar, 40 °C), and dried (1 h, 0.022 mbar, 24 °C). The 24.73 g slightly beige crystals were dissolved in diethyl ether (200 mL), treated with active carbon (1.0 g), filtered through Dicalite, and the solution evaporated under reduced pressure to ~40 g. n-Pentane (30 mL) was added, and crystallization (room temperature, then −20 °C, 16 h) gave, after filtration, washing (cold n-pentane / diethyl ether 1:1, 100 mL) and drying (2 h, 0.021 mbar, 24 °C) 17.33 g (97.7% by NMR, yield 73.8%). The colorless crystals should be stored at −20 °C in order to avoid decomposition. From the mother liquor, additional 4.84 g yellowish crystals (yield 21%) were obtained.

Hexahydrofarnesylmagnesium bromide. The mixture of hexahydrofarnesol (11.67 g, 97.9%, 50.0 mmol) and HBr (63% in water, 41.7 g, 325 mmol, 6.5 equiv.) was heated under stirring to 120 °C (oil bath 130 °C) for 5 h. Completion of the conversion was checked by TLC control (SiO_2, n-hexane/EtOAc 9:1): alcohol Rf 0.07, bromide Rf 0.65. After cooling to room temperature the mixture was poured onto ice-water (50 mL) and extracted with n-hexane (75 mL, twice). The combined organic phases were washed with sat. $NaHCO_3$ and NaCl solutions (50 mL each), dried over sodium sulfate, filtered, evaporated (20 mbar, 40 °C), and dried (1 h, 0.022 mbar, 24 °C): 13.93 g yellowish oil which was filtered through SiO_2 (100 g). The fractions containing the bromide were evaporated (20 mbar, 40 °C) and dried (0.021 mbar, 24 °C). The colorless oil (13.24 g) was distilled (0.27 mbar, 140 °C, Kugelrohr oven): 12.96 g colorless liquid (98.9% by NMR, yield 88%).

Grignard magnesium turnings (469 mg, Alfa Aesar 99.8%, 19.25 mmol, 2.75 equiv.) were placed in an oven-dried 25 mL two-necked round-bottomed flask and heated in an oil bath to 90 °C. THF (4 mL) was added, and the solution of bromide (2.06 g, 98.9%, 7.0 mmol) in THF (8 mL) was added dropwise under magnetic stirring for 30 min. After 6 h heating under reflux (TLC control: no bromide detectable) the mixture was cooled to room temperature and the supernatant filtered through a syringe filter (0.45 μm) into an oven-dried two-necked round-bottomed flask. The remaining Mg turnings were washed with THF (5 mL) and the supernatant was filtered through a syringe filter (0.45 μm) and added to the solution obtained above. The combined solutions of the Grignard reagent (14.51 g yellowish liquid) were stored at room temperature in a flask sealed by rubber septa. Titration of the solution (sec. butanol, o-phenanthroline method[25]) gave a 73% yield (0.35 M solution).

6-Benzyloxy-α-tocopherol[26–28]. The triflate (3.52 g, 97.7%, 7.5 mmol) and a magnetic stirrer bar were placed in an oven-dried 100 mL two-necked round-bottomed flask. After threefold evacuation followed by purging with argon, THF (4 mL) was added, and the flask placed in a cooling bath of −20 °C. The Grignard solution in THF (0.35 M, 32.1 mL, 11.25 mmol, 1.5 equiv.) was dropped in, followed by the solution of Li_2CuCl_4 in THF[29] (3 mL, 0.1 M, 0.3 mmol, 4 mol%). After stirring for 48 h at −20 to 24 °C additional Li_2CuCl_4 in THF (3 mL, 0.1 M, 0.3 mmol, 4 mol%) was added. After overall 95 h (TLC control; SiO_2, n-hexane/toluene 7:3, benzyl-tocopherol Rf 0.38, triflate Rf 0.14) the reaction mixture was poured onto ice (25 g). The organic phase was separated and washed subsequently with 2 N H_2SO_4, sat. $NaHCO_3$ and NaCl solutions (50 mL each). The water phases were extracted with diethyl ether (100 mL, three times), and the combined organic extracts dried over sodium sulfate, filtered, evaporated (20 mbar, 40 °C), and dried (2 h, high vacuum, room temperature): 6.39 g black solid. Column chromatography (SiO_2, n-hexane/toluene 7:3) delivered, after evaporation and drying (20 mbar, 50 °C/0.021 mbar, 23 °C), 2.42 g (purity 97.3% by NMR, yield 60%) benzyl-tocopherol as a yellowish oil, and in a second fraction 0.53 g (yield 15%) triflate as a purple-colored solid.

α-Tocopherol. α-Tocopheryl benzyl ether (2.36 g, 97.3%, 4.4 mmol), EtOAc (30 mL) and 5% Pd/C (1.17 g, 50 wt%) were placed in an autoclave (50 mL, glass, gassing stirrer). After threefold evacuation followed by purging with argon, and 3-fold purging with hydrogen gas (5 bar) and subsequent pressure release, the autoclave was pressurized with hydrogen gas (10 bar), and the stirrer (1000 rpm) was started. After 9 min, hydrogen uptake was completed. After pressure release the reaction mixture was filtered, and the catalyst residue washed with additional EtOAc (~4 g). Evaporation of the filtrate (20 mbar, 40 °C) and drying (0.1 mbar, 23 °C, 2 h) gave 1.89 g slightly yellowish oil, which was purified by column chromatography (SiO_2, n-hexane/EtOAc 9:1), evaporated (20 mbar, 40 °C) and dried (0.021 mbar, 23 °C, 2 h): 1.80 g almost colorless oil, purity 97.6% by quant. GC, yield 92.6%.

Three samples of the following stereoisomeric composition were prepared. Reference sample 1 ("*RRR*"): 2*R*,4'*R*,8'*R* 93.40%, 2*R*,4'*R*,8'*S* 5.81%, 2*R*,4'*S*,8'*R* 0.75%, 2*R*,4'*S*,8'*S* 0%; reference sample 2 ("*SRR*"): 2*S*,4'*R*,8'*R* 93.40%, 2*S*,4'*R*,8'*S* 5.81%, 2*S*,4'*S*,8'*R* 0.75%, 2S,4'S,8'S 0%; reference sample 3 ("SSS"): 2S,4'R,8'R 0.12%, 2S,4'R,8'S 6.21%, 2*S*,4'*S*,8'*R* 1.36%, 2*S*,4'*S*,8'*S* 92.21%.

RESULTS AND DISCUSSION

Method Development

^{1}H NMR spectra are shown in the Supporting Information (S1). It is clear that spectra are too crowded to extract information on the stereochemical composition. Therefore, the discrimination of all eight stereoisomers was attempted with a ^{13}C NMR measurement, which is outlined below. The full ^{13}C NMR spectrum is shown and its assignment according to previous results[17,18] is shown in Supporting Information S2.

In the abovementioned previous NMR work[17,18] separate ^{13}C NMR signals for each of the four pairs of enantiomers for C3' and C2' were detected. NMR spectra were recorded in acetone as solvent, but, unfortunately, acetone is not suitable for separation of signals of enantiomers with the aid of the chiral solvating agent TFAE, because acetone is too polar. Enantiodiscrimination requires the use of an apolar solvent such as chloroform or benzene. In deuterated chloroform

($CDCl_3$) the abovementioned signals show overlap with other peaks, but in this solvent the signal of C14', the methyl group on the chiral carbon C4' appears in a relatively uncrowded part of the spectrum. Figure 3 shows that (*R*,*R*,*R*)-α-tocopherol gives, as expected only one signal for C14', the all-racemic tocopherol gives rise to four different NMR signals, and in the presence of TFAE each of these four signals is split into two peaks, yielding eight peaks in total, which represent the eight stereoisomers of α-tocopherol.

It can be seen from Figure 3 that all peaks have shifted to high-field in the presence of TFAE, which is due to the magnetic anisotropy of the anthryl moiety of TFAE. It is due to the different magnitudes of these effects (different association constant between TFAE and each stereoisomer, and different spatial arrangement of tocopherol carbons relative to the anthryl ring) that the carbon signals of each stereoisomer shift to a different extent, which enables the separation of peaks.

The spectra shown in Figure 3 were obtained at 250 K and the concentration of α-tocopherol was 77 mM and TFAE was added in 4–5-fold molar excess. These two conditions are extremely important for the successful application of the CSA method. In Figure 4 the temperature dependence of the separation between the signals of one pair of enantiomers is shown (peak no. 5 and peak no. 6 in Fig. 3). The distance between the peaks of other enantiomeric pairs behaves in a similar way. Only the magnitude of the separation is slightly different. It is clear that full resolution is obtained at 260 K or 250 K. In fact, the separation of peaks of enantiomers is even better at lower temperatures (240 K), but some extra line-broadening was observed, and moreover at temperatures below 250 K separation between the signals of two enantiomers is even too large, and the peaks numbers 2 and 3 partly overlap. The same is happening with peaks 6 and 7. Thus, 250 K emerged as the optimal temperature to achieve the separation of signals. At this temperature the line width of the signals at 10% of the peak-height is 1.3 Hz. It is shown in Figure 4 that the separation of signals is small but more than sufficient to obtain baseline resolution ($\Delta\delta$ = 2.1 Hz).

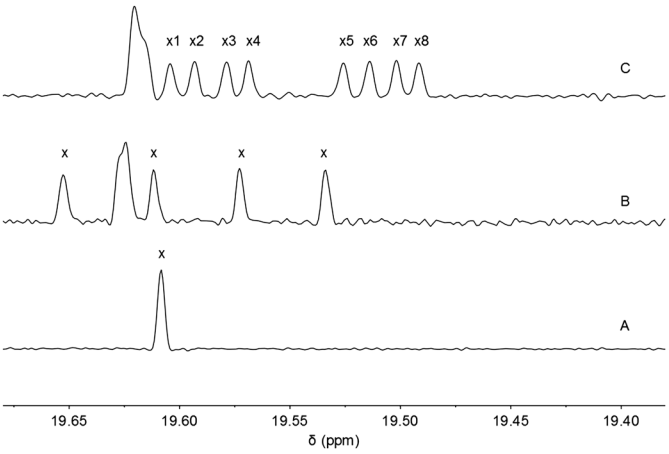

Fig. 3. Part (0.3 ppm) of the carbon spectrum of α-tocopherol. A: (*R*,*R*,*R*)-α-tocopherol, B: all-racemic α- tocopherol, C: all-racemic α-tocopherol + (*S*)-TFAE. Signals marked with "x" are due to the methyl group C14'.

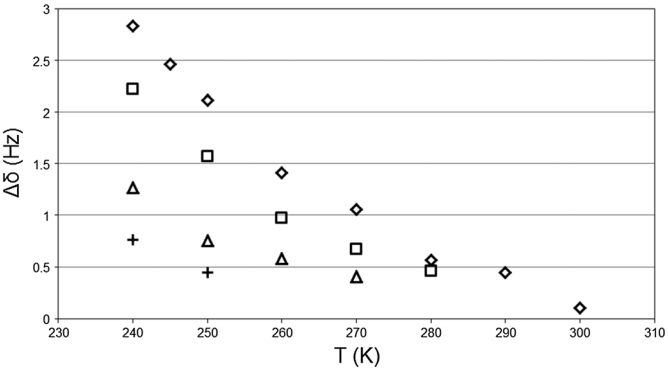

Fig. 4. Chemical shift difference between peak 5 and 6 as a function of temperature and of concentration of the sample. ◊ undiluted, □ 2× diluted, Δ 4× diluted, + 8× diluted.

For other compounds the optimal temperature can be different (see Supporting Information S3–S9).

The importance of the second-mentioned condition, the concentration of both compounds, is also demonstrated in Figure 4.

The solution of α-tocopherol with TFAE was diluted 2×, 4×, and 8×. In this way the ratio between the two compounds remained the same, i.e., 4–5-fold molar excess of TFAE. From Figure 4 it can be seen that at lower concentrations the separation between signals of enantiomers rapidly decreases, and it is concluded that the highest concentration achievable must be employed. It should be noted that higher concentrations of TFAE are not compatible with the low temperature of 250 K, because crystallization starts to occur, which should be avoided, because it negatively affects the resolution of the NMR spectrum, and moreover, the desired condition of 4-fold molar excess of CSA is no longer fulfilled.

Assignment of Individual Stereoisomers

Samples of the individual stereoisomers were synthesized from stereochemically pure or enriched building blocks. Each reference compound contained one major and one minor stereoisomer and a third stereoisomer in even lower quantity. The composition of the reference compounds was derived from the known composition of the reactants used in the synthesis of the α-tocopherol stereoisomers and is shown in Table 1.

All spectra shown in the previous paragraphs were obtained in a total acquisition time of 0.5 h. In order to detect small quantities of minor stereoisomers, a sufficient signal-to-noise ratio is needed. Significant signal-to-noise improvement was obtained by increasing the total acquisition time. We show in Figure 5 the spectrum of reference sample 1 (main component *R*,*R*,*R* stereoisomer) containing 5.8% of *R*,*R*,*S* and 0.75% of *R*,*S*,*R*.

It can be seen from Figure 5 that with a total acquisition time of 1.5 h (1024 scans, 3 s interpulse delay) 1% of a minor compound can be detected in a sample of a single stereoisomer of α-tocopherol. The amount of *R*,*R*,*S* was determined by setting the integral of the *R*,*R*,*R* signal to 93.4% (expected value). A value of 5.8% was found for *R*,*R*,*S*, which is in

TABLE 1. Stereochemical composition of the enriched stereoisomers (%)

Reference sample	Major	2nd	3rd
1	*RRR* 93.4	*RRS* 5.8	*RSR* 0.75
2	*SRR* 93.4	*SRS* 5.8	*SSR* 0.75
3	*SSS* 92.2	*SRS* 6.2	*SSR* 1.4

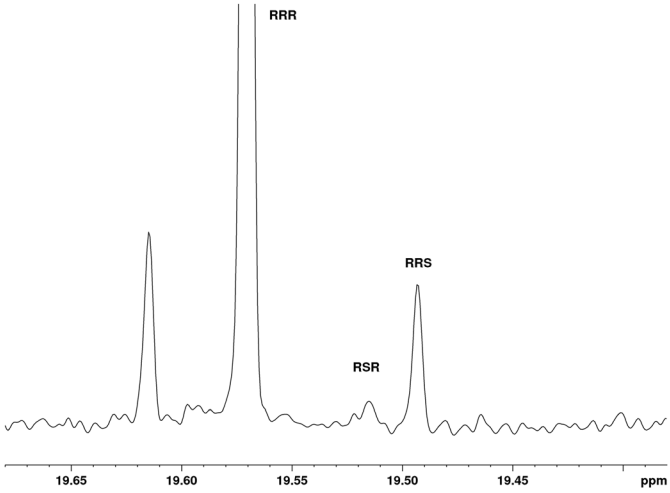

Fig. 5. NMR spectrum of reference sample 1. *RRR*-α-tocopherol with minor amounts of *RRS*- and *RSR*-α-tocopherol. Amounts detected by NMR 5.8% and 1.2%, respectively. Signal at 19.615 ppm is due to C15'. Expected concentrations see Table 1.

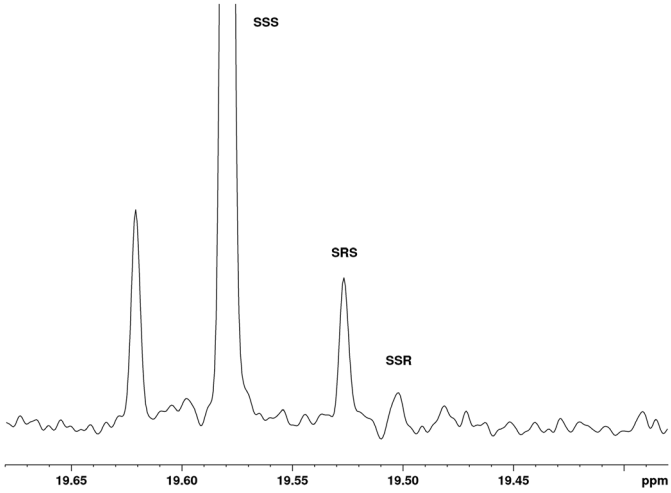

Fig. 7. NMR spectrum of reference sample 3. *SSS*-α-tocopherol with minor amounts of *SRS*- and *SSR*-α-tocopherol. Amounts detected by NMR 6.6% and 1.7%, respectively. Signal at 19.615 ppm is due to C15'. Expected concentrations see Table 1.

excellent agreement with the expectation. For the *R*,*S*,*R* stereosiomer we find 1.2%, which is slightly different from the expectation due to the fact that the peak of *R*,*S*,*R* is just at the detection limit of the present method. Therefore, quantification at this level is less precise, but the impurity is clearly detected.

The NMR spectra of reference samples 2 and 3 are shown in Figures 6 and 7, respectively. Also in these two cases there is excellent agreement between the expected concentrations of impurities and the experimental concentrations.

With the samples enriched in individual stereoisomers, assignment of the eight peaks is an easy task. In fact, only two enriched stereoisomer samples are sufficient to assign all eight peaks. At this point some explanation is needed. Because the temperature-dependent behavior of the racemate in the presence of TFAE was studied, there is no doubt as to which pair of peaks is due to a pair of enantiomers. It follows that if one peak is assigned, automatically the peak of its enantiomer is assigned as well. Furthermore, each reference sample contains two well-detectable stereoisomers with

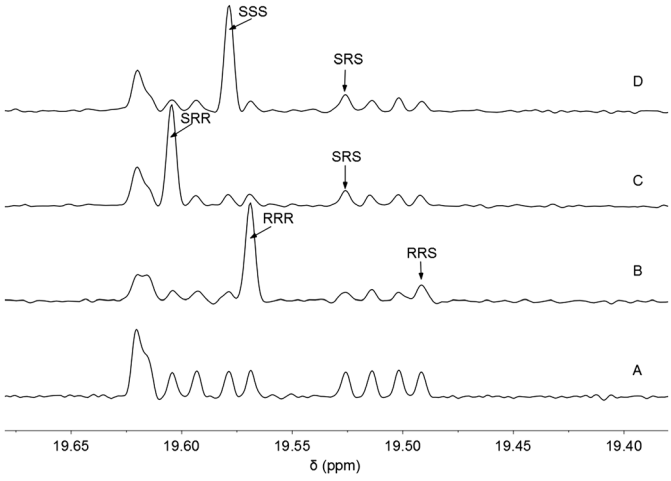

Fig. 8. A: all-racemic α-tocopherol. B: same with spike of *RRR*- (and *RRS*-) α-tocopherol. C: with spike of *SRR*- (and *SRS*-) α-tocopherol. D: with spike of *SSS*- (and *SRS*-) α-tocopherol.

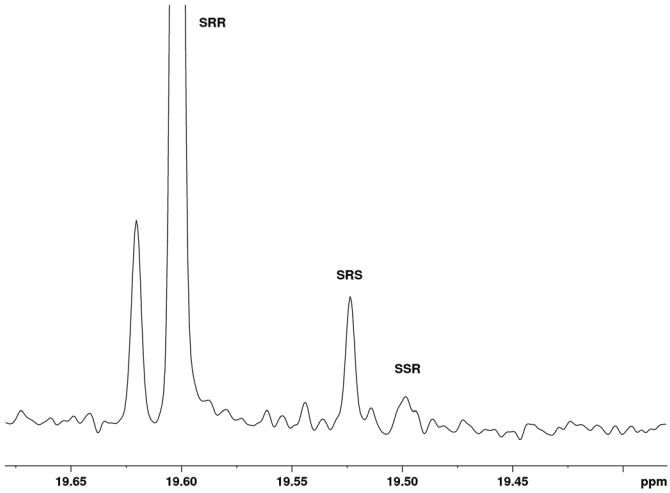

Fig. 6. NMR spectrum of reference sample 2. *SRR*-α-tocopherol with minor amounts of *SRS*- and *SSR*-α-tocopherol. Amounts detected by NMR 6.2% and 2.2%, respectively. Signal at 19.615 ppm is due to C15'. Expected concentrations see Table 1.

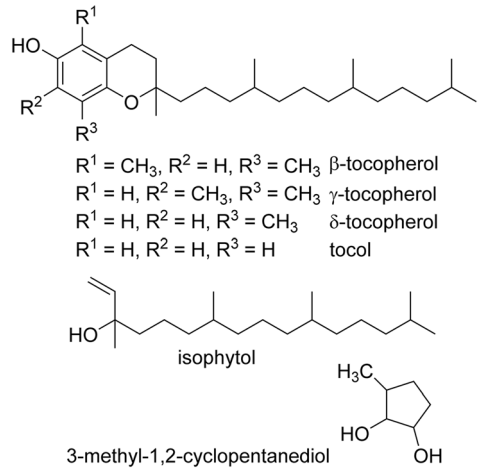

Fig. 9. Structures of additional compounds possessing three chiral centers and successful observation of eight stereoisomers.

a characteristic ratio; the third stereoisomer is not useful for this purpose. As a consequence of these two conditions, with each spike eventually four peaks could be assigned, and thus two spikes assigned all eight stereoisomers. The third spike merely served for confirmation of the results obtained through the first two spikes.

The results of the spiking experiments are shown in Figure 8.

With similar conditions it is also possible to observe eight different stereoisomers of other compounds containing three chiral carbon atoms (structures shown in Fig. 9). The spectra of (all-*rac*)-β-tocopherol, (all-*rac*)-γ-tocopherol, (all-*rac*)-δ-tocopherol, (all-*rac*)-tocol, (all-*rac*)-isophytol, and (all-*rac*)-3-methyl-1,2-cyclopentanediol are shown in the Supporting Information S3–S9. The interaction of the compound of interest with TFAE requires the presence of an aliphatic or aromatic hydroxy (OH) group. If such a group is not present, the resolution of enantiomers seems to be insufficient.

CONCLUSION

In summary, we have shown that the discrimination of all eight stereoisomers of α-tocopherol, as well as six other representative examples possessing three chiral centers, by means of ^{13}C NMR is possible, provided that the right experimental conditions are applied. The important conditions are: low temperature (250 K) and the highest concentration achievable at the low temperature (80 mM of α-tocopherol and 4–5-fold molar excess of TFAE). In the case of α-tocopherol, all individual stereoisomers were identified. It is expected that this ^{13}C NMR method using the abovementioned experimental conditions is applicable to most chloroform soluble organic compounds bearing a hydroxyl group.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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