Monatshefte für Chemie 133, 1249–1261 (2002)

Monatshefte für Chemie
Chemical Monthly

Homonuclear Selective One-Dimensional Gradient NMR Experiments in the Structure Determination of Natural Diterpene Derivatives

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Summary. The solution structure of two natural diterpene derivatives, the secondary metabolites esulatin-A and esulatin-B of *Euphorbia esula*, was investigated by homonuclear NMR experiments. Since the spectral dispersion of the ^{1}H NMR spectra at 500 MHz was sufficient to separate several skeletal protons of the title compounds, they were selectively excited with a double pulsed field gradient spin-echo (DPFGSE) sequence using 180° *Gaussian* pulses sandwiched between sine shaped gradients. With the use of selective excitation, scalar as well as dipolar interactions of the selected spins were monitored through one-dimensional (1D) COSY, TOCSY, and NOESY experiments. The chemical shifts of the coupling partners could be accurately extracted from the 1D COSY and TOCSY spectra recorded with high digital resolution. The selective TOCSY experiment provided an excellent opportunity to identify spins belonging to the same scalarly coupled spin system. The solution state conformation was investigated by selective gradient enhanced NOESY experiments. Proton–proton distances were evaluated from the cross-relaxation rates obtained from a quantitative analysis of the NOESY spectra recorded with different mixing times. The NMR derived distances were compared to the results of solid state X-ray diffraction measurements.

Keywords. ^{1}H NMR; Selective NMR experiments; DPFGSE; Diterpene; Esulatin.

Introduction

In recent years, considerable attention has been paid to macrocyclic diterpenoids based on the jatrophane, lathyrane, casbane, and cembrane skeleton because of their structural complexity, biogenic relevance, and remarkable biological activity such as cytotoxic, antibacterial, and vascular activities [1–12]. These compounds are formed *via* the cembrene cation in the plant biosynthesis and are found in nature in various oxygenated forms. Phytochemical investigations of Euphorbiaceae (spurge) species have resulted in structurally unique therapeutically relevant diterpenes.

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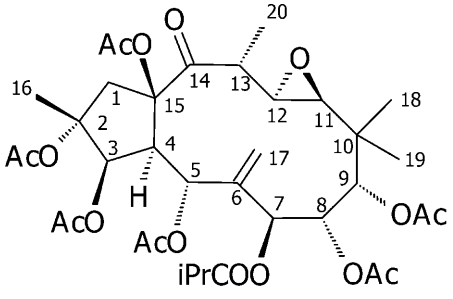
Since natural diterpenoids belong to the class of moderate sized compounds, their NMR spectra have numerous well resolved multiplets that are ideal targets for selective excitation. Thus, selective experiments can be attractive alternatives of the corresponding two-dimensional methods, mainly because of the reduced experimental time required and the high resolution of the resulting spectra. These experiments yield scalar or dipolar coupling information only for the selected spin. Several protocols are available to achieve selective excitation using hard-pulse trains [13, 14], chemical shift selective filters [15], shaped pulses [16], or gradient enhanced spin-echo sequences combined with selective inversion pulses [17, 18].

The present work represents an application of selective homonuclear experiments based on the double pulsed field gradient spin-echo sequence (DPFGSE). Our objective was to demonstrate the advantages of these experiments in the structure elucidation of natural diterpene compounds. The solution structures of the two jatrophane diterpene derivatives esulatin-A (**1**) and esulatin-B (**2**), isolated from *Euphorbia esula* L. [8], were established employing the DPFGSE-COSY [19], DPFGSE-TOCSY [20], and DPFGSE-NOESY [21] sequences.



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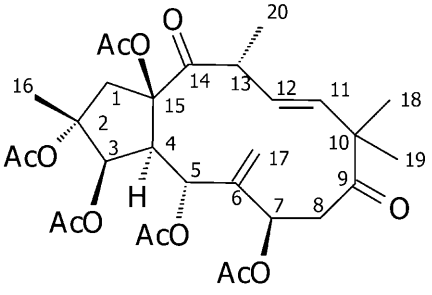

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Results and Discussion

Natural jatrophane diterpene derivatives incorporate a 20 carbon containing skeleton based on a fused ring system, one with five and the other one with twelve members, and substituted with methyl groups at C-2, C-6, C-10, and C-13. The numerous derivatives of these compounds are due to the different substituent patterns present on the skeleton (e.g. various acyl, hydroxy, epoxy, and keto functions).

Figure 1 displays the ^{1}H NMR spectra of **1** and **2**. Selective gradient enhanced one-dimensional COSY (1D DPFGSE-COSY) experiments were performed with selective excitation of H-12, H-9, and H-3 in **1** (Fig. 2) as well as H-1b, H-4, H-8a, and H-11 in **2** (Fig. 3). The coupling partners of H-12 (H-13 and H-11) in **1** were identified from the 1D COSY spectrum (Fig. 2, trace b). The selective excitation of H-9 (Fig. 2, trace c) in **1** provided a COSY response on H-8. The selective COSY experiment on H-3 (Fig. 2, trace d) afforded a COSY signal to H-4, which is a multiplet having a small coupling constant to H-3.

Coupling partners in **2** were identified using a similar approach. The chemical shifts of the isolated CH_2 group (C-1) could be identified with the COSY

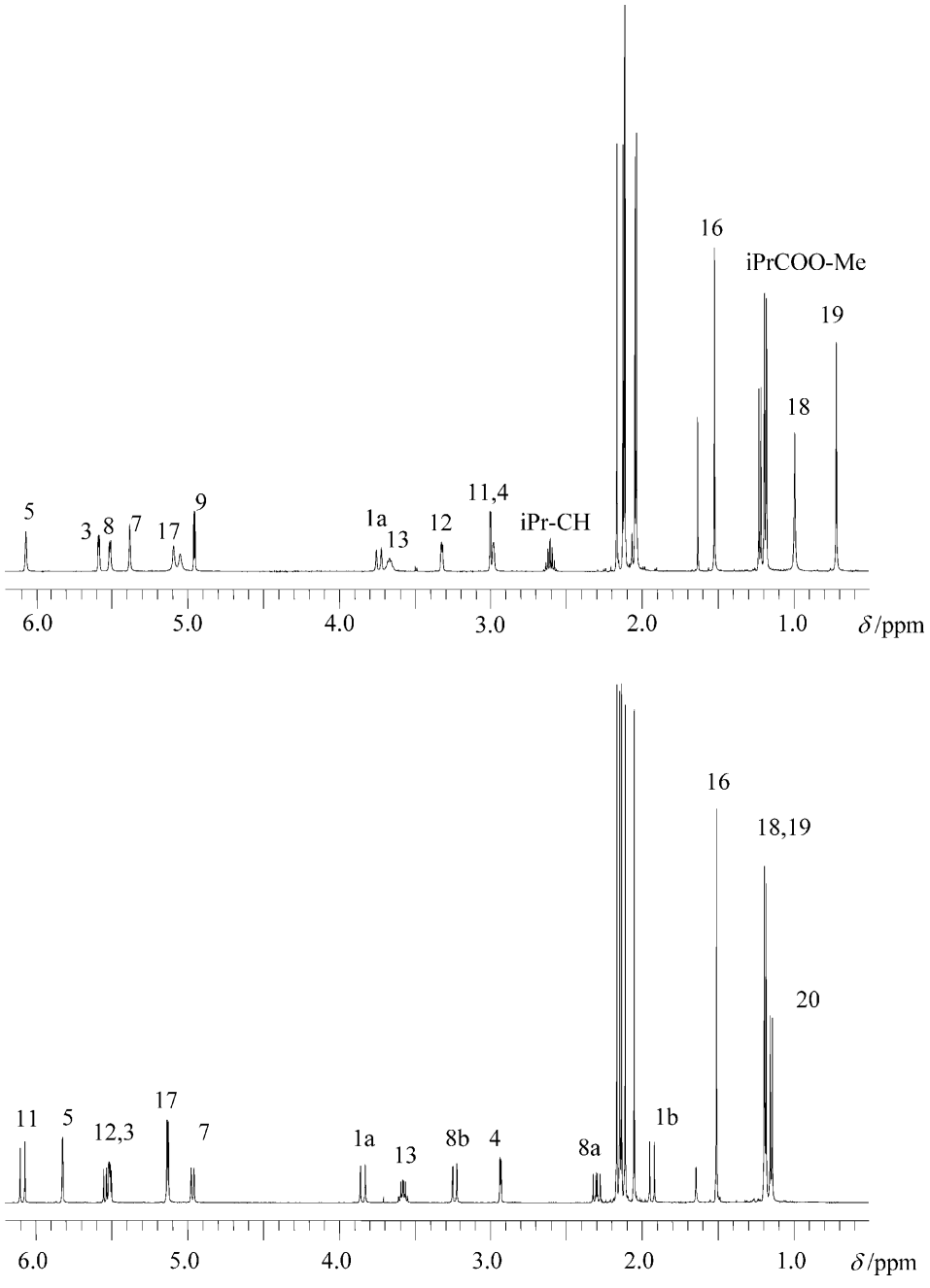

Fig. 1. ^{1}H NMR spectra of esulatin-A (**1**, top) and esulatin-B (**2**, bottom)

experiment (Fig. 3, trace b). The selective excitation of H-4 (Fig. 3, trace c) provided the COSY signal of H-5 and H-3, the latter being highly overlapping with H-12. The selective COSY experiment of H-8a (Fig. 3, trace d) yielded correlations to H-8b and to H-7, whereas the excitation of H-11 provided a strong COSY response on H-12.

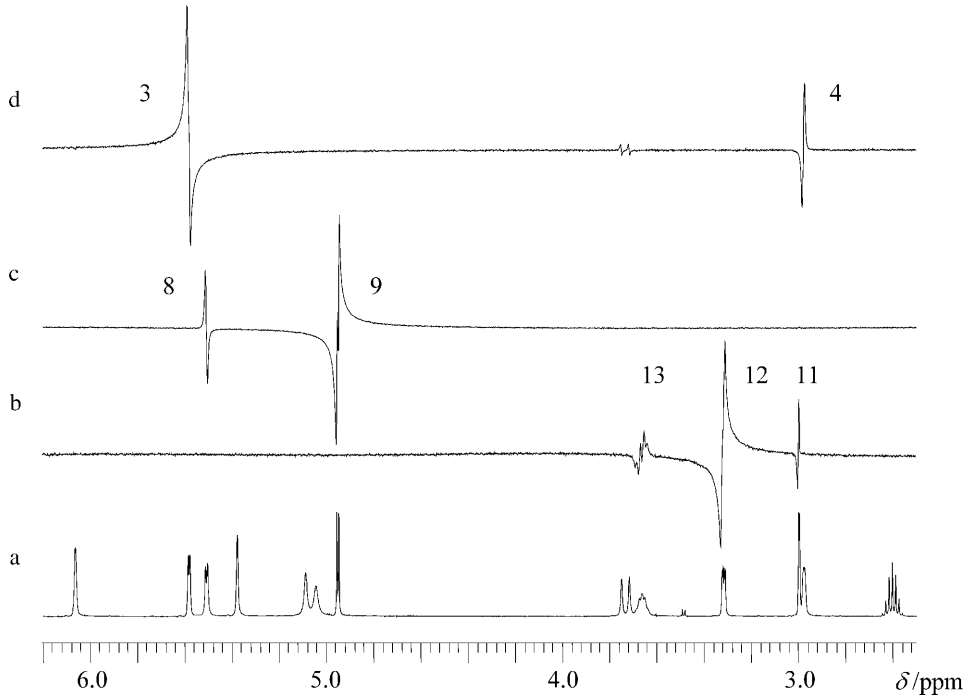

Fig. 2. 1D COSY spectra of **1** with selective excitation of H-12 (b), H-9 (c), and H-3 (d)

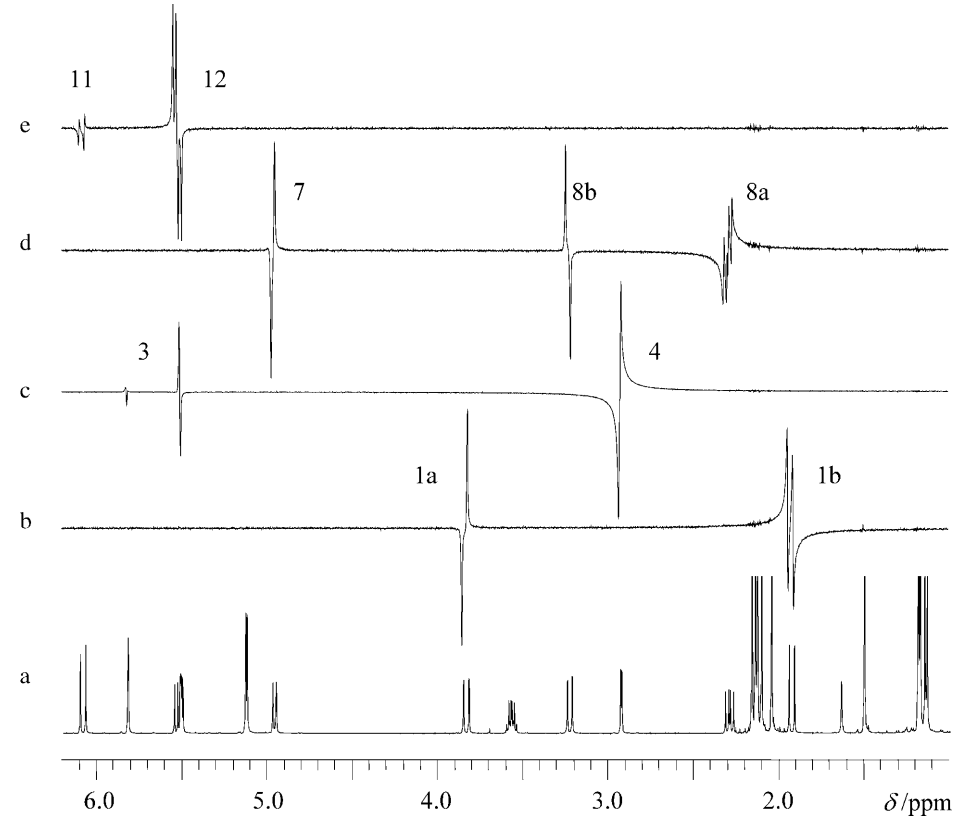

Fig. 3. 1D COSY spectra of **2** with selective excitation of H-1b (b), H-4 (c), H-8a (d), and H-11 (e)

The 1D COSY experiment provides only direct correlations between coupling partners which are less than four bonds apart. Moreover, COSY yields antiphase multiplets, which render the experiment less sensitive due to possible signal cancellation; in addition, the phasing procedure may cause difficulties.

The 1D TOCSY experiment also provides scalar coupling information, but the magnetization of the selectively excited proton can be transferred through the entire coupling network, irrespective of the number of bonds involved. The efficiency of the magnetization transfer strongly depends on the magnitude of the coupling constants. By arraying the duration of the isotropic mixing time, magnetization can be transferred to protons remote from the selectively excited proton, resulting in different subspectra of the same spin system. The signals in the TOCSY spectrum are pure absorptive thus eliminating problems related to phasing and signal cancellations. In general, the experiment provides clean spectra with high sensitivity. Several isolated spin systems could be identified in compounds **1** and **2**.

When H-3 in **1** (Fig. 4, trace b) was selectively excited, a relatively strong TOCSY signal appeared on H-4. However, the TOCSY signal on H-5 is very weak,

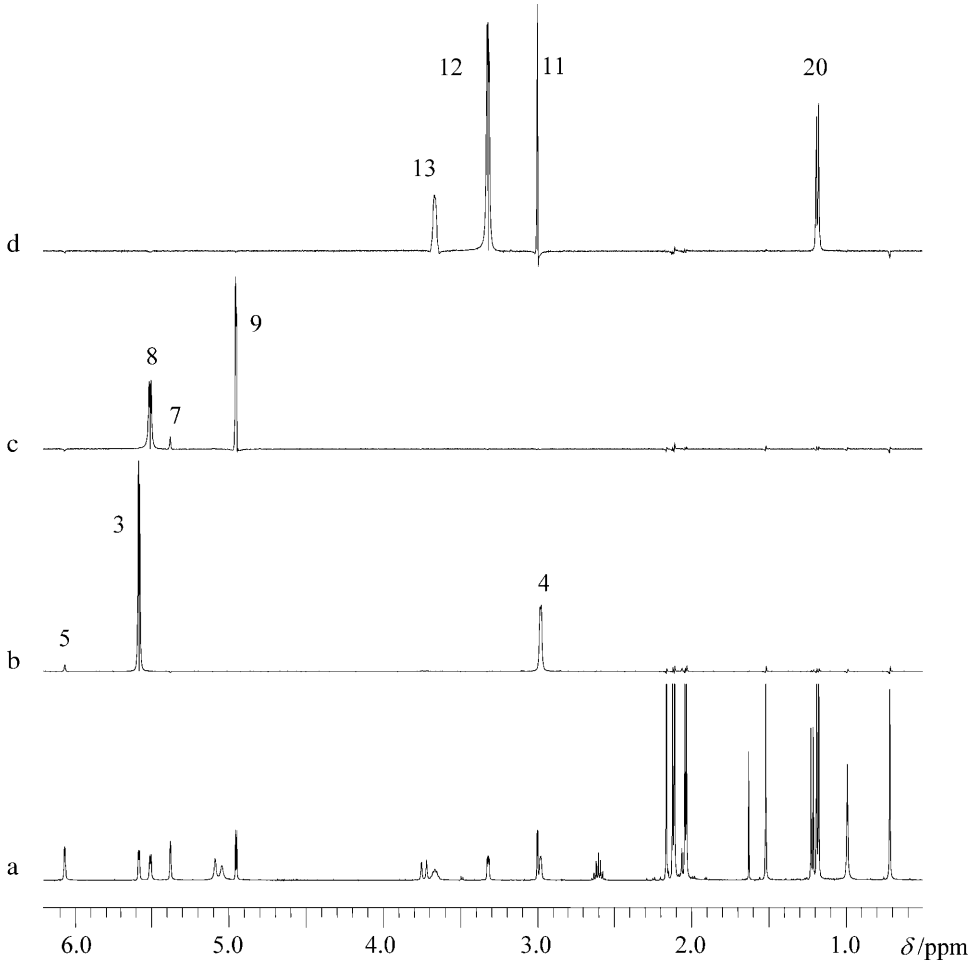

Fig. 4. 1D TOCSY spectra of **1** with selective excitation of H-3 (b), H-8 (c), and H-12 (d)

since the scalar coupling between H-4 and H-5 is very small. The magnetization of H-8 is transferred to H-9 (Fig. 4, trace c). Because of the small scalar coupling between H-8 and H-7, the TOCSY transfer is strongly attenuated and provides a relatively weak TOCSY signal on H-7. The selective excitation of H-12 (Fig. 4, trace d) provided more responses because this spin belongs to an extended spin system; TOCSY peaks were observed on H-13, H-11, and H-20.

The same procedure was applied to investigate the scalar coupling patterns in **2** as shown in Fig. 5. Three protons belonging to different spin systems (H-11, H-8b, and H-1a; (Fig. 5, traces b–d) were excited in separate 1D TOCSY experiments.

The solution conformations of compounds **1** and **2** were established with 1D DPFGSE-NOESY experiments. Whereas the DPFGSE-NOESY sequence works well for small molecules, it fails for medium sized compounds because the intensity of the NOE signal passes through zero as the molecule size increases.

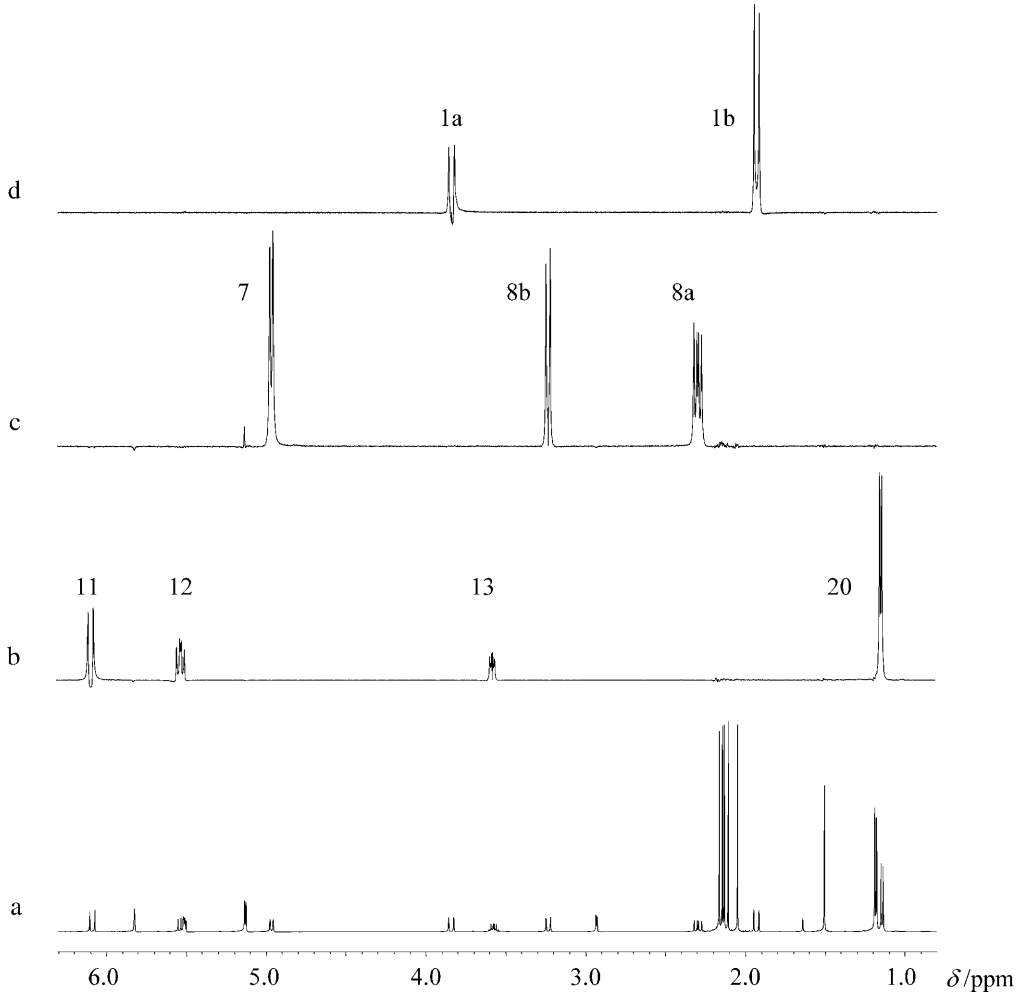

Fig. 5. 1D TOCSY spectra of **2** with selective excitation of H-11 (b), H-8b (c), and H-1a (d)

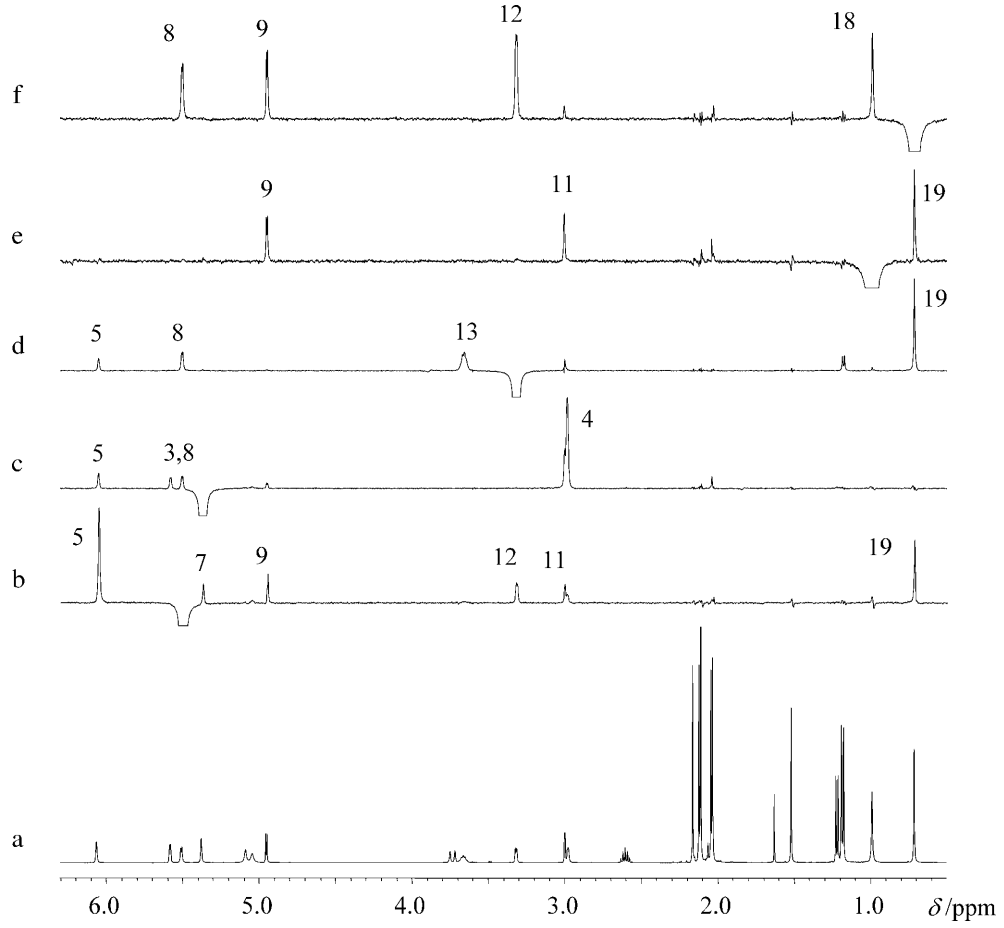

Fig. 6. 1D NOESY spectra of **1** with selective excitation of H-8 (b), H-7 (c), H-12 (d), H-18 (e), and H-19 (f)

In that case, the 1D-DPFGSE-ROESY experiment [22] proves to be superior. The following protons were selected in different 1D-DPFGSE-NOESY experiments: H-8, H-7, H-12, H-18, and H-19 in **1** (Fig. 6), H-5, H-13, H-8b, H-4, and H-20 in **2** (Fig. 7). The selective excitation of H-8 in β-position in **1** (b in Fig. 6) resulted in strong NOE signals on H-5, H-9, and H-19, which are located in β-position as well; however, a weak NOE was detected on H-7 as a result of its α-position. Moreover, the bridge protons H-11 and H-12 of the epoxide moiety responded with relatively weak NOE signals. The selective excitation of H-7 (Fig. 6, trace c) yielded weak NOE signals on H-5, H-3, and H-8 as well as an intense NOE response on H-4. The selective NOE experiment gave enhancements on distant protons (H-5 and H-8) as well as the expected NOE correlation to H-13 when H-12 was excited (Fig. 6, trace d). The excitation of H-18 (Fig. 6, trace e) gave an intense signal on the neighbouring H-19, responses on one of the epoxide bridging protons of H-11, and on H-9. When H-19 was selected (Fig. 6, trace f), H-12, H-9, and H-8 showed intense responses in addition to H-18. On the basis of

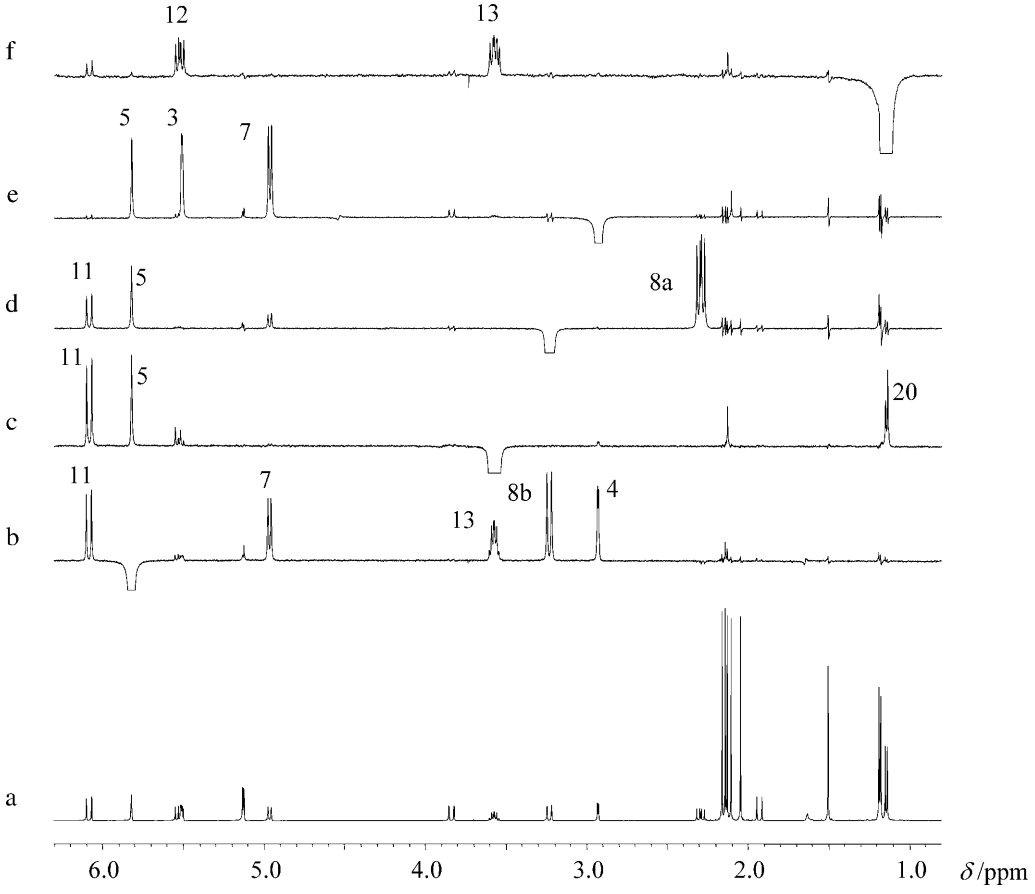

Fig. 7. 1D NOESY spectra of **2** with selective excitation of H-5 (b), H-13 (c), H-8b (d), H-4 (e), and H-20 (f)

the NOE experiments the epoxide moiety was found to be in *trans* configuration because the selective excitation of H-18 and H-19 provided only enhanced signals either on H-11 or H-12.

The selective excitation of H-5 in **2** (Fig. 7, trace b) resulted in a strong NOE signal at H-11 and H-13 which can only be explained by a twisted shape of the twelve-membered ring. A relatively strong NOE was measured on H-7, which is surprising because these protons are in *trans* orientation to each other. One of the H-8 protons at 3.23 ppm also showed a strong NOE correlation with H-5. Since H-4 is orthogonal to H-5, a strong enhancement was observed when H-5 was excited. The selective excitation of H-13 (Fig. 7, trace c) resulted in a strong NOE at H-11 and H-20 as expected and at H-5, which also corroborates the twisted shape of the ring. When H-8b (Fig. 7, trace d) was excited, its geminal proton H-8a gave a strong response, and a NOE was observed on H-5 and H-11. The selective excitation of H-4 (Fig. 7, trace e) provided strong NOEs on H-7, H-3, and H-5. When H-20 was the target of the excitation (Fig. 7, trace f), only the expected enhancements appeared at H-13 and H-12.

Quantitative analyses of the NOESY experiments

Analyses of NOE enhancements by determination of cross-relaxation rates (σ) provide distances between the relevant nuclei. The cross-relaxation rate for a proton pair in an isotropically tumbling molecule is given by Eq. (1) [23].

$$\sigma_{H,H} = \left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_H^4 \hbar^2}{r^6} \left(\frac{3}{5}\left(\frac{\tau_c}{1+4\omega_H^2\tau_c^2}\right) - \frac{1}{10}\tau_c\right) \quad (1)$$

In this equation, μ_0 is the vacuum permeability constant, γ_H is the magnetogyric ratio of the proton, r is the distance between the interacting nuclei, ω_H is the *Larmor* frequency, and τ_c is the rotational correlation time of the molecule. The cross-relaxation rate can be obtained from NOE experiments performed with different mixing times (Fig. 8).

The integral intensities of the NOE signals are measured as a function of the applied mixing time. The NOE buildup follows a linear function at short mixing times, and the cross-relaxation rate can be extracted as the slope of the line (initial rate approximation, Fig. 9) [23].

According to Eq. (1), the distance calculation requires the knowledge of the value of the molecular rotational correlation time. The τ_c values were evaluated

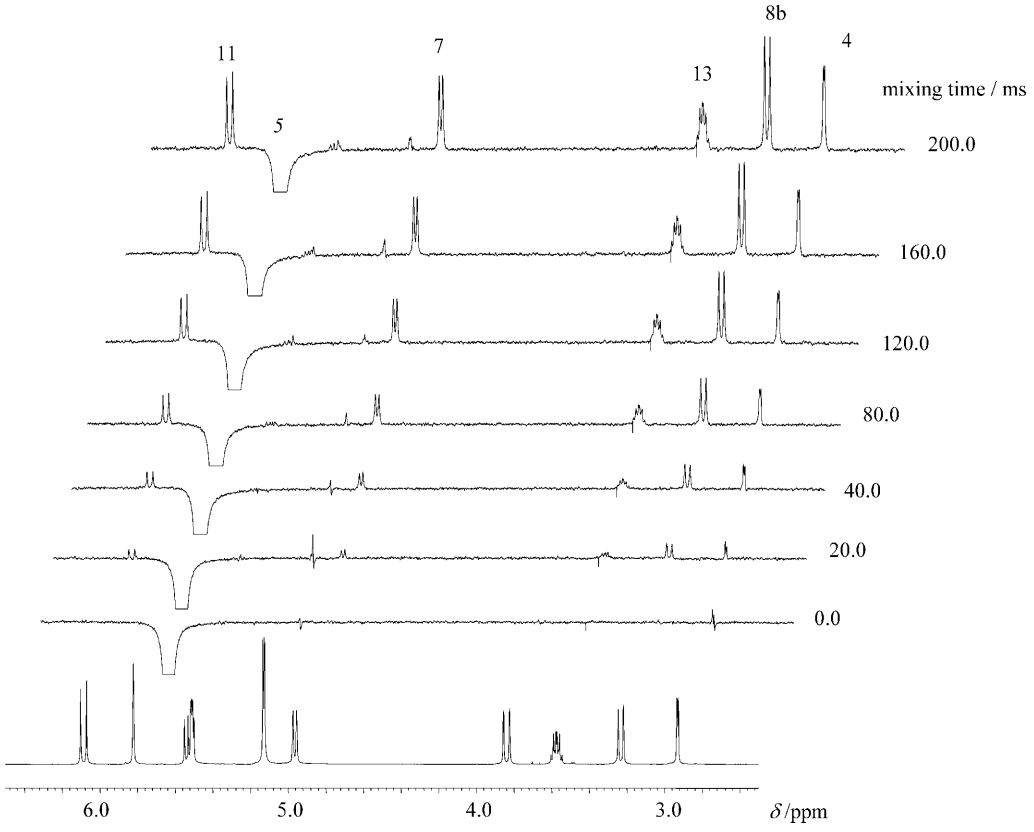

Fig. 8. 1D NOESY spectra recorded with different mixing times; the selective excitation was carried out on H-5 in **2**

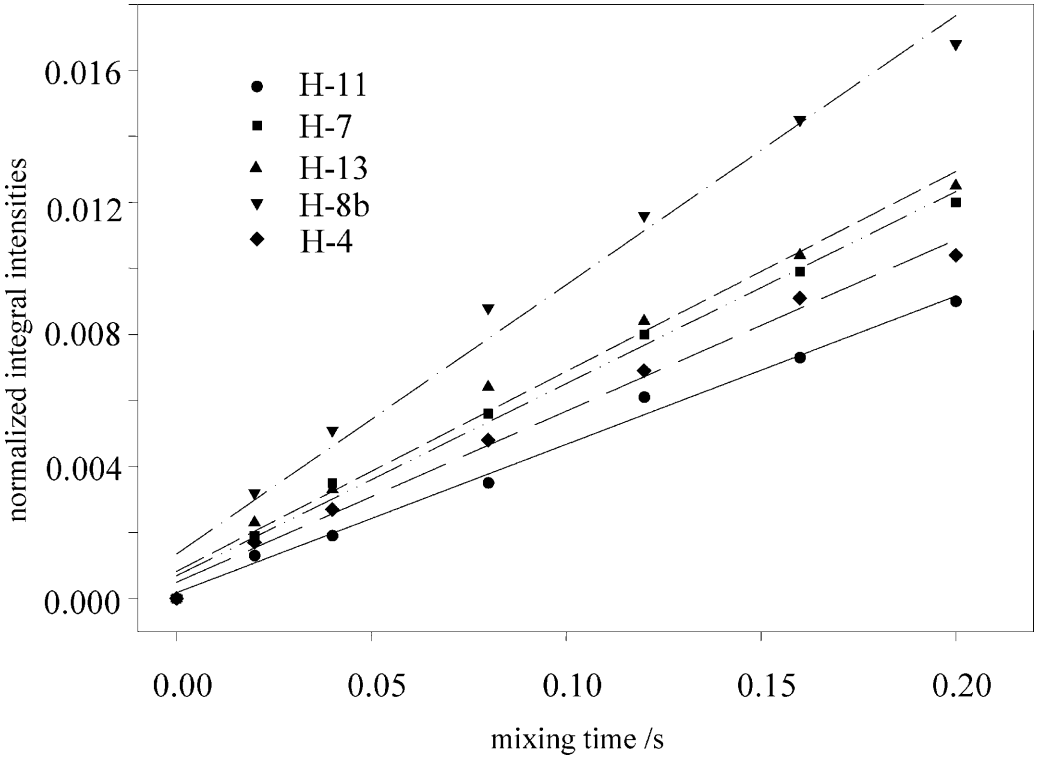

Fig. 9. 1D NOESY buildup rates measured for five different multiplets (H-11, H-7, H-13, H-8b, H-4) when H-5 was selectively excited in **2**

from ^{13}C spin-lattice relaxation data assuming fast isotropic rotational motion of the molecules in solution. The ^{13}C spin-lattice relaxation times of the skeletal methine resonances varied between 0.69 and 0.71 s, and an average value of 0.7 s was used for the calculation in both compounds. The rotational correlation time was found to be 6.65×10^{-11} s. Quantitative evaluations of the NOESY experiments were carried out on H-3, H-5, H-7, and H-12 in **1** (Table 1) and on H-4, H-5, H-7, H-8b, H-11, and H-13 in **2** (Table 2).

Table 1. Proton–proton distances (Å) obtained from NOE buildup curves for **1**; data obtained from X-ray studies are given in parentheses

Exc. \ Obs.	H3	H4	H5	H7	H8	H13
H3		2.45 (2.34)		3.11 (2.31)		
H5				2.91 (3.22)	2.21 (2.24)	2.47 (2.39)
H7	3.10 (2.31)		2.94 (3.22)			
H12			3.10 (3.39)		2.73 (2.49)	

Table 2. Proton–proton distances (Å) obtained from NOE buildup curves for **2**; data obtained from X-ray studies are given in parentheses

Exc. \ Obs.	H3	H4	H5	H7	H8b	H11	H13
H4	2.39 (2.26)		2.59 (2.39)	2.27 (2.48)			
H5		2.61 (2.38)		2.54 (2.39)	2.40 (2.33)	2.67 (2.50)	2.54 (2.71)
H7		2.28 (2.48)	2.54 (2.39)				
H8b			2.40 (2.33)			2.50 (2.33)	
H11			2.64 (2.50)		2.49 (2.33)		2.46 (2.33)
H13			2.54 (2.71)				

Nine distances were obtained in **1** (Table 1), whereas the analysis resulted in sixteen distances in **2** (Table 2). Distances for proton pairs could be estimated from two separate measurements with selective excitation of each member of the interacting proton pair. Duplicate distance information was extracted for proton pairs H-3/H-7 and H-5/H-7 in **1** and for H-4/H-5, H-4/H-7, H-5/H-7, H-5/H-8b, H-5/H-11, H-5/H-13, and H-8b/H-11 in **2**. These data were used to examine the quality of the distance measurement. It was found that the difference between the corresponding data was less than 0.1 Å. The distances in **1** varied between 2.21 Å (H-5/H-8) and 3.11 Å (H-3/H-7), those in **2** between 2.27 Å (H-4/H-7) and 2.67 Å (H-5/H-11). The obtained solution state proton–proton distances were compared to those of the solid state obtained by X-ray crystallography [24, 25]. The difference between the relevant distances was found to be 0.1–0.2 Å. The only notable difference observed in the distances measured by the two different methods occurred for H-3/H-7 of **1**. The solution state distance was around 3.10 Å, whereas the X-ray analysis gave 2.31 Å. This contradiction can be explained by the different conformational mobility around the exocyclic double bond between C-6 and C-17, as was predicted by molecular calculation methods [26].

Conclusions

Gradient enhanced selective one-dimensional homonuclear NMR experiments were used to study the solution structure of the two diterpene derivatives esulatin-A (**1**) and esulatin-B (**2**). The selective excitation was performed by the double pulsed field gradient spin-echo (DPFGSE) sequence including selective *Gaussian* inversion pulses. The scalar and dipolar interactions of the excited spins were monitored in COSY, TOCSY, and NOESY experiments. The selective COSY experiment provided the scalar coupling connectivity of the selectively excited spin, whereas the entire spin system could be identified with the help of the selective

TOCSY experiment allowing spectral editing. The solution state conformation was studied with the selective NOESY experiment. Quantitative analysis of the NOE enhancements provided proton–proton distances which appeared to be close to those measured in the solid state with X-ray crystallography.

Experimental

All experiments were carried out using a Bruker Avance DRX-500 instrument equipped with a 5 mm inverse probe with *z*-pulsed field gradient capability. Compounds **1** and **2** were isolated as described in Ref. [8]. Samples were prepared using 10–15 mg of material dissolved in 0.6 cm^3 $CDCl_3$. The samples were thoroughly degassed by three freeze-pump cycles and then sealed under vacuum. NMR experiments were carried out at room temperature. The duration of the hard 90° proton pulse was 9 μs. A *Gaussian* inversion pulse of 80 ms duration was used in the middle of the gradient spin-echo sequence. Sine-shaped gradients of 1 ms duration followed by 100 μs recovery delays were applied in the DPFGSE sequence. The gradient strengths (as percentage of the maximum gradient, *ca.* 50 G/cm) were set as 40:40:7:7. The evolution period in the COSY sequence varied between 30 and 50 ms. A 90° hard pulse separating two gradients with different polarity was applied at the end of the COSY sequence to select detectable antiphase coherences with a 20:–20 gradient strength ratio. In the TOCSY experiment, an isotropic mixing time of 60 ms was employed ($\gamma B_1 = 7140$ Hz). The mixing sequence was flanked by two gradients (10:10) applied simultaneously with the trim pulses. To identify the dipolar coupled partners in spatial proximity, a 1D DPFGSE-NOESY sequence was used. The mixing time was 200 ms, and a hard 180° pulse sandwiched between two gradients (with gradient strength ratio 20:–20) was applied in the middle of the mixing period. For the evaluation of proton–proton distances, 1D NOESY experiments were set up with different mixing times (0, 20, 40, 80, 160, and 200 ms). Time domain data were acquired into 16 k complex data points and weighted with a 1 Hz *Lorentz* window function prior to *Fourier* transformation.

Acknowledgments

This work was supported by grants OTKA-T035200 (*P. Forgo*, *J. Hohmann*), OTKA-T022587 (*J. Hohmann*), OTKA-T029089 (*K. E. Köver*), OTKA-T034515 (*K. E. Köver*), FKFP-0598/1999 (*J. Hohmann*), FKFP-0024/2001 (*P. Forgo*), and ETT T-01222/99 (*J. Hohmann*).

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Received November 21, 2001. Accepted (revised) January 9, 2002

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Österreichische Akademie der Wissenschaften, Dr.-Ignaz-Seipel-Platz 2, A-1010 Wien, und Gesellschaft Österreichischer Chemiker, Eschenbachgasse 9, A-1010 Wien. – Redaktion: Währinger Straße 38, A-1090 Wien. – Satz und Umbruch: Thomson Press Ltd., Chennai, India. – Offsetdruck: Manz Crossmedia, A-1051 Wien. – Verlagsort: Wien. – Herstellungsort: Wien. – Printed in Austria.