Recent Res. Devel. in Phytochem., 2 (1998)

Application of two-dimensional nuclear magnetic resonance methods for structure elucidation of sesquiterpene lactones (guaianolides) from *Anthemis carpatica* and diterpenes (kauranes) from *Achillea clypeolata*

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ABSTRACT

The application of two-dimensional nuclear magnetic resonance (2D NMR) experiments, in combination with other spectrometric methods (*e.g.*, chemical ionization mass spectrometry, electrospray ionization mass spectrometry), for the structure determination of natural products is demonstrated on selected examples of terpenoid compounds such as sesquiterpene lactones (guaianolides) (1) and diterpenes (*ent*-kauranes) (2) isolated from *Anthemis carpatica* and *Achillea clypeolata*, respectively. The examples discussed in this article were chosen to illustrate the potential of the 2D NMR methods for structure determination of natural products, including some new variations of the known techniques (e.g., ^{13}C NMR spectra editing using Heteronuclear Single Quantum Correlation). The spectral analysis of dynamic systems involving either slow conformational or a slow chemical exchange is also discussed.

INTRODUCTION

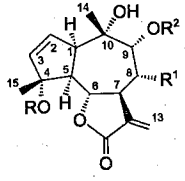
As a part of a comprehensive study regarding Yugoslavian wild-growing plants also aimed at the discovery of new compounds of pharmacologic interest, we examined several genera belonging to a species-rich family, Asteraceae. Since the end of the 1980s, we have focused mainly on sesquiterpene lactones (SLs) of three genera known for the various medicinal properties of its members: *Anthemis*, *Achillea,* and *Centaurea.* SLs constitute one of the biggest groups of natural products -- about 10% of all secondary metabolites isolated so far. More than 5,000 structures of SLs of natural origin and hundreds of the synthetic ones have been reported. Their chemotaxonomic importance and various biologic activities (antitumoral, allergenic, phytotoxic, antimicrobial, insecticidal) are the major reasons for the continuous interest regarding these compounds. The great majority of SLs were isolated from higher plants, mainly those belonging to the large family of Asteraceae, but they also were found in some other plant families as well as in some fungi. Whereas from the species of *Anthemis* (1) and *Achillea* (2-6) we have isolated quite a few new SLs, the investigation of *Centaurea (i.e., C. solstitialis* L., *C. derventana* Vis. et Panč., *C. kosaninii* Hayek and *C. scabiosa* L.) yielded only the known compounds.

In this article, the isolation and structure determination of SLs and kauranoide diterpenes from *Anthemis carpatica* Willd. and *Achillea clypeolata* Sibth. et Sm., respectively, are briefly presented, whereas the application of 2D NMR methods for structure determination of the selected examples (**5**, **8**, **11** and **13**) is discussed in more detail.

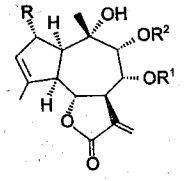
Anthemis carpatica (1)

Genus *Anthemis* L. (family Asteraceae, tribe Anthemidae) comprises about 130 species that commonly occur in the Mediterranean, although some

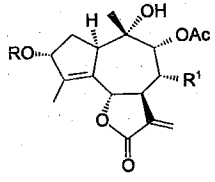
also can be found in southwest Asia and South Africa (7). In Serbia, nine species are known (8). The plant species of this genus have been used as medicinal plants since ancient times. But now the only species that can be found in various pharmacopoeias is *Anthemis nobilis* L. (Roman chamomile) (9). The essential oil of this plant shows the same pharmacologic properties as the *Matricaria chamomilla* oil. The three main classes of compounds of chemosystematic interest, such as polyacetylenes, flavonoids, and sesquiterpene lactones, typical for Anthemidae, have been detected in the genus *Anthemis*



	R	R^1	R^2
1:	H	H	Ac
2:	OH	OAc	H
3:	OH	OH	Ac
4:	OH	OAc	Ac
5:	OH	O-*i*-But	Ac



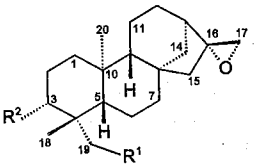
	R	R^1	R^2
6:	H	H	Ac
7:	H	Ac	H
8:	H	Ac	Ac
9:	OOH	*i*-But	Ac



	R	R^1
10:	H	H
11:	H	OAc
12:	OH	OAc

Scheme 1

(10,11). Our examination of this genus involved the aerial parts of two species *(i.e., Anthemis carpatica* Willd. and *Anthemis montana* L.). From *Anthemis carpatica*, the species usually occurring in high, shady, and grassy silicate terrains of the Balkan Peninsula, East Alps, and East Carpathian Mountains (8), a flavonoid quercetin (leaves) (12) and polyacetylenes (roots) (11) were the only secondary metabolites reported before our study. Our plant material (*i.e.*, the aerial parts of *Anthemis carpatica*) was collected at the north part of Sar-planina (Sara mountain, situated between Serbia and Macedonia). Using the usual extraction method for isolation of sesquiterpene lactones (13) in combination with silica gel column chromatography and preparative thin layer chromatography (TLC), we have isolated 12 highly oxygenated sesquiterpene lactones of the same guaiadienolide type, all of them exhibiting an exomethylene 11(13)double bond. According to the position of the remaining double bond, they are divided into three groups: (*i*) Δ^2, (*ii*) Δ^3, and (*iii*) Δ^4, Scheme 1, **1** - **12**. Their ^1H and ^{13}C NMR assignment and structure determination were based on the characteristic chemical shifts and couplings obtained by the first-order analysis combined with 2D NMR measurements, such as Double Quantum Filtered (DQF) COSY, TOCSY, NOESY, (Heteronuclear Single Quantum Correlation), and (Heteronuclear Multiple Band Correlation), performed on compounds **1** and **5** - **11**. Lactones **2** - **5**, **9** and **12** contained a hydroperoxy function that was identified on



	R^1	R^2
13:	H	H
14:	OAc	H
15:	H	OAc

Scheme 2

the basis of spectroscopic evidence. All hydroperoxy lactones also exhibited a specific peroxide (red) colored TLC test with N,N-dimethyl-*p*-phenylenediammonium dichloride (14). This sensitive method was useful for the screening of peroxycompounds in our mixtures before the separation. Direct chemical ionization mass spectrometry (DCIMS) (isobutane) afforded additional evidence for the OOH group. Since all the studied lactones contained many polar functionalities, the standard MS electron impact (EI, 70 eV) ionization techniques did not give molecular ions. Among the soft ionization modes applied, DCIMS (isobutane) revealed more structural information compared with electrospray ionization (ESI) applied in some cases. Thus, the standard ESI technique, when using $AcONH_4$ for ionization, yielded only abundant quasimolecular ion in the form of $[M + NH_4]^+$ adduct and practically no fragmentation ions. When the ionization was effected by AcOH, $[M + Na]^+$ and $[M + K]^+$ adducts (the metal ions originating from glass) were detected. At the same

time, DCIMS yielded $[M + H]^+$ ions and also abundant fragmentation ions obtained via elimination of neutral molecules, such as $[M + H - H_2O]^+$, $[M + H - AcOH]^+$ and $[M + H - H_2O_2]^+$, from the lactones containing OH, OAc, and OOH groups, respectively. In addition, the hydroperoxy-lactones exhibited an intense $[M + H - O]^+$ fragment, typical for organic hydroperoxides (15).

Using the same procedure (13), we isolated from the aerial parts of *Anthemis montana* (also collected at the north part of Sar-planina, but not at the same locality as *Anthemis carpatica*) 14 highly oxygenated guaianolides. Six of them (**2**, **4**, **5**, **6**, **7** and **11**) were the same as those isolated from *Anthemis carpatica*, two were new and the remaining six were reported as the constituents *Anthemis aetnensis* (16) and *Anthemis hydruntina* (17).

Achillea clypeolata (2)

Genus *Achillea* L. comprises about 100 species occurring in the temperate areas of the Old World, particularly in the higher hills of the Mediterranean region. In Serbia, 19 species have been detected so far (18). Hitherto, this genus, well known for the medicinal properties of some of its members, has received much attention. The best known among them is yarrow, *Achillea millefolium* L., the species whose extracts and essential oils have been used heavily in folk medicine for different cures since ancient times. Whereas the main constituents of the bitter principles isolated from the aerial parts of *Achillea* species are sesquiterpene lactones (19) (mostly guaianolides) and flavonoids (20), the roots usually contain alkamides (21) (i.e., the amides of polyunsaturated linear carboxylic acids). Our investigations involving the following *Achillea* species: *abrotanoides* Vis. (3), *millefolium* subsp. *pannonica* (4), *crithimifolia* W. et K. (5,6), and *clypeolata* (2) resulted in the isolation of several sesquiterpene lactones. The only exception was *clypeolata*, originating from Sicevacka klisura, southeast Serbia. An attempt to isolate SLs from the aerial parts of this species using the standard procedure failed (2). At the same time, by means of the usual procedure for isolation of the alkamides (22), we have isolated from its roots (instead of the alkamides) diterpenes **13** - **15** belonging to the *ent*-kaurane group, Scheme 2 (2). The assignment of diterpene **13** as 16α,17-epoxy-*ent*-kaurane was based on the analogy of its ^{1}H NMR spectral data (assigned by means of DQF COSY, HSQC, and NOESY) and optical rotation to those reported for the same compound originating from the aerial parts of *Baccharia minutiflora* (23). The ^{13}C

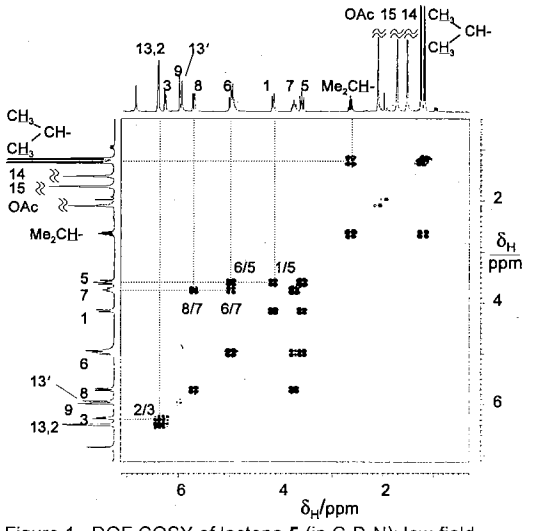


Figure 1. DQF COSY of lactone **5** (in C_5D_5N); low-field OOH (δ 13.30) not shown; weak cross-peaks (e.g., 1/2, 1/3, 7/13, 7/13' and 8/9) observed in contour plot obtained with lower threshold and also in TOCSY (see Fig. 2).

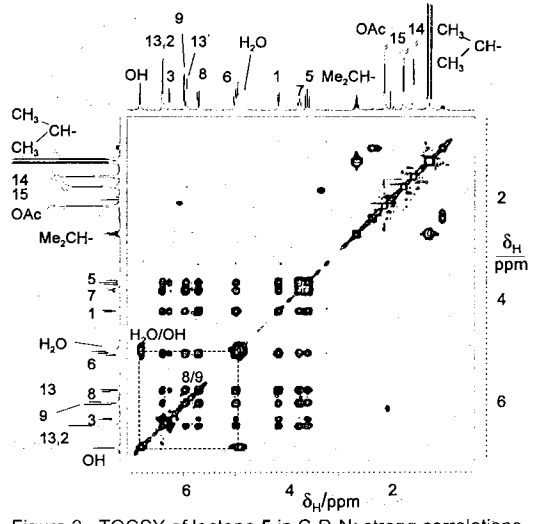


Figure 2. TOCSY of lactone **5** in C_5D_5N; strong correlations due to a chemical exchange OOH/OH not shown.

NMR spectrum of **13** was assigned completely by a heteronuclear one-bond C,H-correlation (HSQC) and comparison of the ^{13}C chemical shifts with those of *ent*-

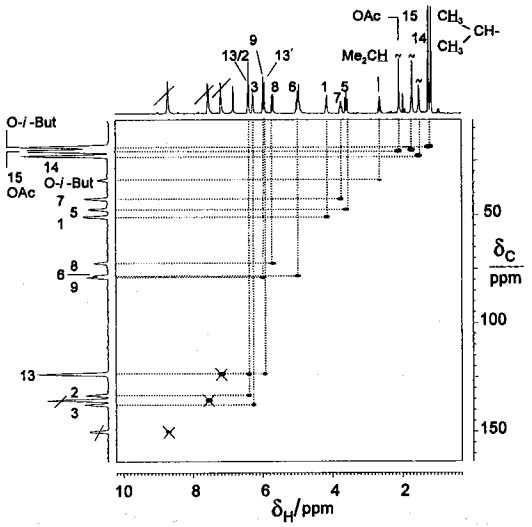


Figure 3. One-bond heteronuclear C,H-correlation (HSQC) spectrum of lactone **5** in C_5D_5N.

kaurene (24). In this case, an HSQC technique for spectrum editing providing information which could be obtained by a combination of the HETCOR and the standard DEPT (Fig. 13) for spectral assignment has been applied. Isomers **14** and **15** (new compounds at that time), both exhibiting molecular ion *m/z* 346 ($C_{22}H_{34}O_3$) in the EIMS and an acetate carbonyl IR band (about 1730 cm^{-1}), were identified as 19- and 3α-acetoxy-16α,17-epoxy-*ent*-kauranes, respectively. Whereas, for the identification of **15**, the same 2D techniques as in case of **13** have been applied, diterpene **14** was mostly identified by the analogy of its ^{13}C and ^{1}H NMR data to those of the structural analogs with 19-OAc (25,26) and 19-OH (23) functions.

2D NMR SPECTRA OF SELECTED COMPOUNDS

Sesquiterpene lactones

Δ²-Group: 8-O-isobutyryl-9-O-acetylanthemolide B (**5**)

Among the Δ²-lactones isolated from *Anthemis carpatica* and *Anthemis montana*, compound **5** ($C_{21}H_{28}O_9$) was one of the most extensively studied by means of 2D NMR methods (DQF COSY, TOCSY, NOESY, HSQC, and HMBC), and its ^{1}H and ^{13}C NMR data are presented elsewhere (1). Chemical shifts and multiplicity of the ^{1}H signals together with the COSY correlations (Fig. 1) led to an 8α,9α-diacyloxyguaia-2,11(13)-dienolide gross structure (acyl = acetyl and isobytyril) also containing tertiary OH and OOH groups. These data allowed neither acylation pattern nor the positions of tertiary (C-4 and C-10) oxygen functions to be established unambiguously. The TOCSY spectrum (Fig. 2) demonstrating only two independent spin systems, such as the isobutyrate moiety and all the protons attached to the guaianolide ring system was compatible with the proposed gross structure. Compared with COSY, in which small scalar couplings (e.g., $J_{8,9}$) gave weak cross peaks, in TOCSY they exhibited much stronger correlations. In addition, the TOCSY spectrum contains cross-peaks connecting signals of protons exhibiting mutual (slow) chemical exchange (*e.g.*, OH, OOH in Fig. 2), but such correlations are not distinguishable from the remaining ones, as is the case in NOESY (Fig. 5), in which the cross-peak originating from dipolar couplings and those from the chemical exchange are of different sign. The

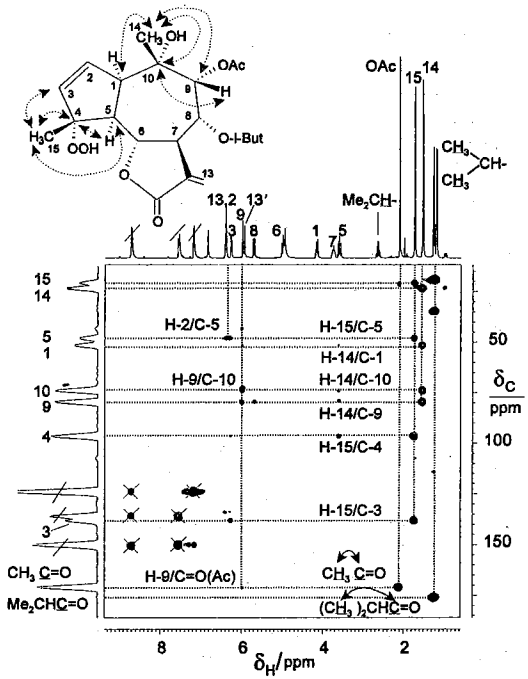


Figure 4. Long-range heteronuclear C,H-correlation (HMBC) spectrum of lactone **5** in C_5D_5N.

one-bond heteronuclear correlation spectra (HSQC) (Fig. 3) enabled detection and assignment of signals of protonated carbons. The signals of the majority of non-protonated carbons were visible and assigned in a long-range HETCOR (HMBC) (Fig. 4). The advantage of the HMBC technique over COSY for the assignment of a connectivity pattern involving carbons bearing no protons, such as C-4 and C-10, was fully demonstrated in this case. Thus, the observed (two-bond) correlation of a carbon, assigned according to the characteristic chemical shift (δ 96.4) as the hydroperoxidation site, to H-15 fully accorded with 4-OOH substitution. The remaining long-range H,C-correlation of this portion of the molecule, together with those of H-14 to C-1 (δ 51.18), C-9 (δ 79.22) and C-10 (δ 73.62) corroborated the 4-OOH, 10-OH assignment. A weak cross-peak connecting H-9 and the acetate carbonyl carbon, δ 170.75, (the latter also exhibiting a strong two-bond correlation to the protons of the acetate methyl, δ 2.11) indicated an 8α-*i*-butyryloxy-9α-acetoxy arrangement. This esterification pattern also was confirmed by the NOEs between H-13' and the protons of isobutyrate

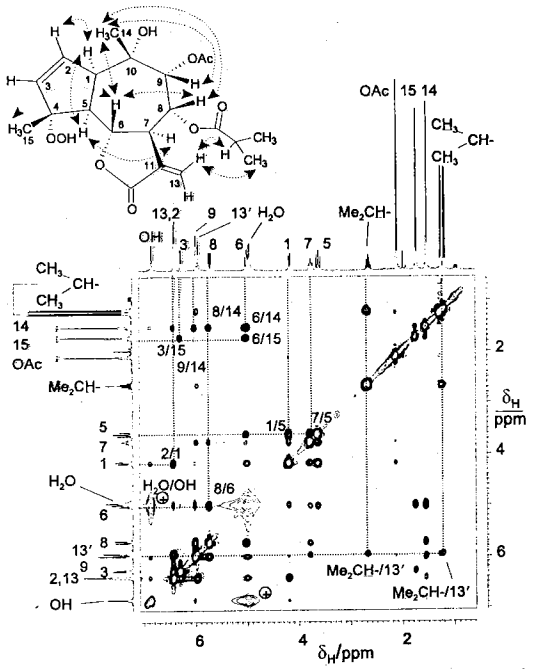


Figure 5. NOESY spectrum of lactone **5** in C_5D_5N; chemical exchange cross-peaks (e.g. H_2O/OH) are positive.

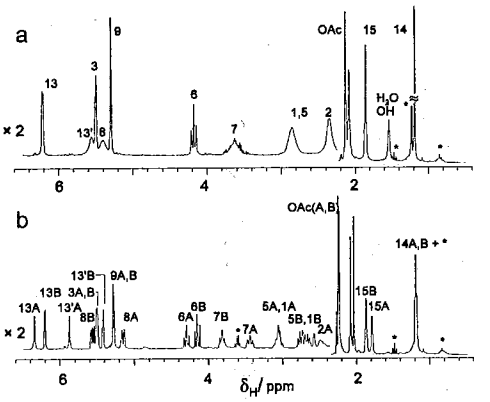


Figure 6. (a) ^{1}H NMR spectrum of lactone **8** in $CDCl_3$ at (a) 297 K and (b) 216 K; * - impurities.

moiety observed in NOESY (Fig. 5), giving a complete map of dipolar coupling between the spatially close groups of protons ($r \leq 4$ Å) and thus the relative stereochemistry in this lactone. The ^{1}H NMR signals of such dipolarly coupled groups are connected by negative cross-peaks in the NOESY. At the same time, the resonances of protons undergoing mutual slow (stereo)chemical exchange exhibit positive cross-peaks (*e.g.*, OH and H_2O in Fig. 5). The detection of slow conformational exchange by means of positive NOESY cross-peaks is illustrated in the example of compound **8** *(see below)*.

Δ^3-Group: 9α-acetoxycumambrin A (**8**)

As far as the ^{1}H NMR spectra are concerned, lactones **6** ($C_{17}H_{22}O_6$) and **8** ($C_{19}H_{24}O_7$) behaved differently compared with the remaining members of Δ^3-guaianolide series by exhibiting rather broad resonances at room temperature, both in $CDCl_3$ (Fig. 6a) and C_6D_6 (not shown), which was due to a conformational exchange at intermediate rate (on the NMR time scale). Such a behavior, not typical for guaianolides, is observed more frequently among the conformationally mobile germacranolides. The existence of conformational exchange was confirmed by a low-temperature NMR ($CDCl_3$) study. At 216 K, most of the broad multiplets (observed at the room temperature) split into pairs of sharp, well-resolved signals (with the intensity ratio of about 1:1.16 within each pair) (Fig. 6b and 7). These pairs of signals were connected by positive cross-peaks in NOESY (Fig. 8b), thus

indicating the existence of two conformers (**8A** and **8B**), with the latter slightly predominating. In systems mutually exchanging at a slow rate, such as **8A** ⇌ **8B** at a low temperature, each form exhibits a separate correlation network in DQF COSY (Fig. 9) and also in HSQC (Fig. 10). The first-order analysis of the low-temperature ^{1}H NMR spectrum (Fig. 6 and 7), in combination with the scalar H,H-coupling networks in **8A** and **8B**, unambiguously assigned in DQF COSY (Fig. 9) as well as ^{13}C data measured in HSQC (Fig. 10) enabled ^{1}H and ^{13}C NMR spectral assignment and identification of compound **8** as 9α-acetoxycumambrin A. These NMR data were in accordance with different (distorted chair) conformations of the 7-membered ring in **8A** and **8B**, with (pseudo)axial and (pseudo) equatorial 10β-methyl, respectively. The similar situation also was encountered in the closely related lactone **6** eluted as the major component of the inseparable (8.5:1.5) mixture with the isomeric lactone **7**. Lactone **6** gave broad ^{1}H NMR lines, also indicating the conformational equilibrium similar to that in **8**. At the same time, the coeluting isomer **7** exhibited ^{1}H (well-resolved) NMR resonances and ^{13}C NMR data similar to those of **8A**, which could be explained by the existence of a single conformation (like **8A**). A more detailed conformational analysis of these lactones, including evaluation of the geometry of the conformers together with the corresponding kinetic parameters is in progress.

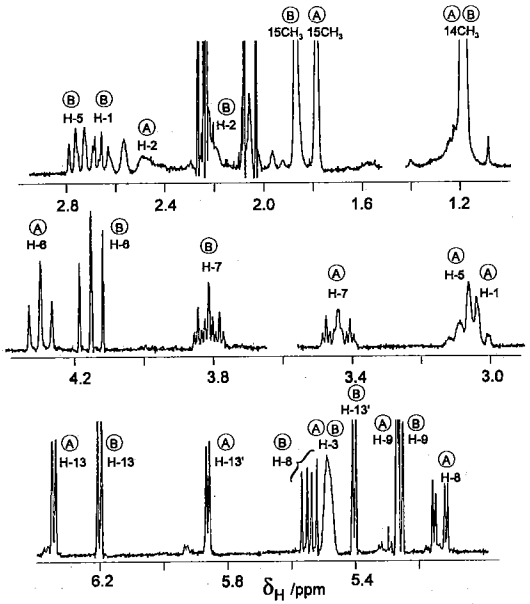


Figure 7. Expansions of the low-temperature (216 K) ^{1}H NMR spectrum of lactone **8** in $CDCl_3$.

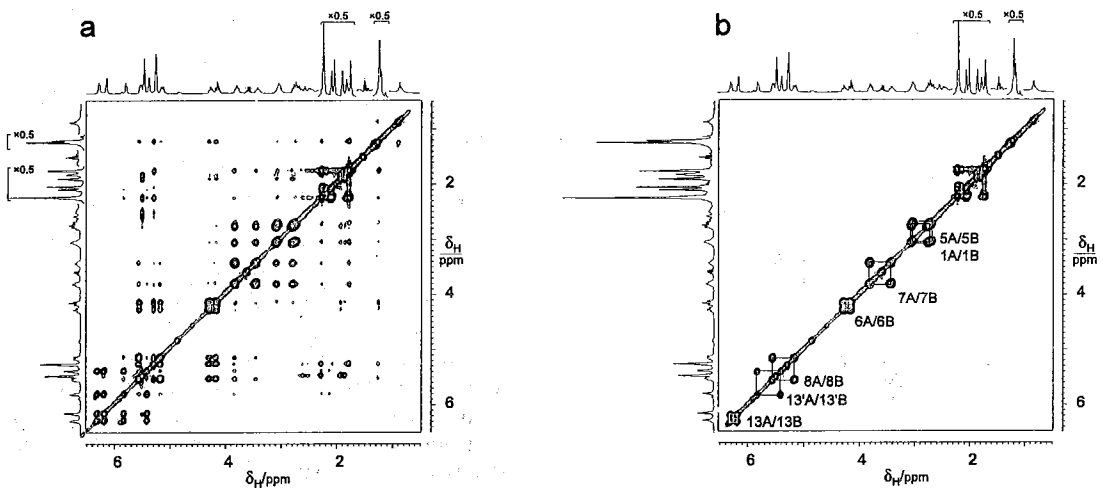


Figure 8. NOESY of lactone **8** in $CDCl_3$ at 243 K: (a) all correlations and (b) only positive correlations due to a slow conformational exchange.

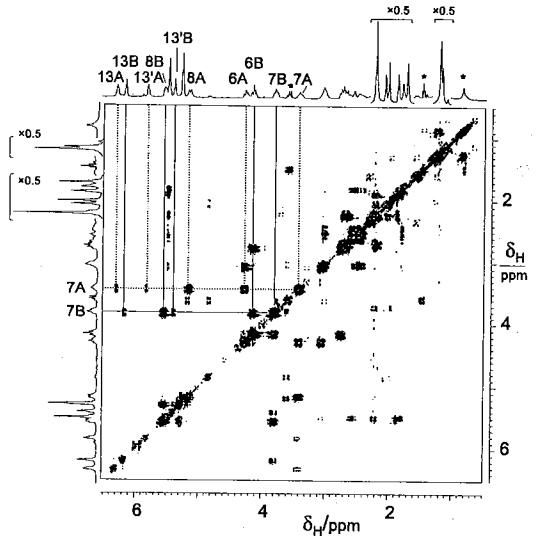


Figure 9. DQF COSY of lactone **8** in $CDCl_3$ at 243 K; * - impurities.

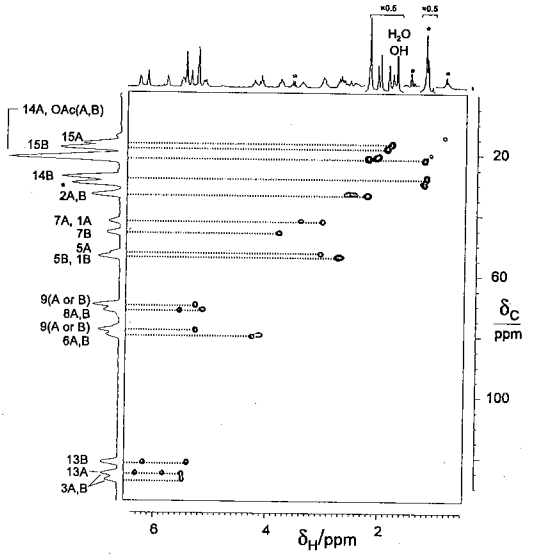


Figure 10. HSQC of lactone **8**, in $CDCl_3$ at 243 K; * - impurities.

Δ^4-Group: anthemolide D (**11**)

The assignment of Δ^4-structure to the lactones of this group was based on the following ^1H and ^{13}C NMR

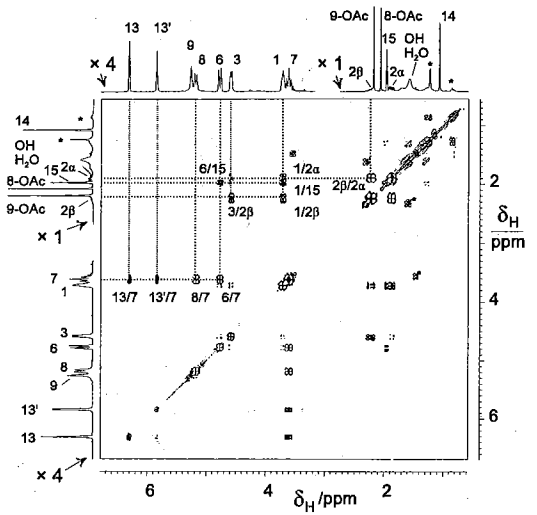


Figure. 11. DQF COSY of lactone **11** in $CDCl_3$.

evidence: (a) the occurrence of a doublet for H-6 (δ about 4.7 - 4.8, J about 11 Hz) with a fine structure caused by a homoallylic coupling to H-15 (3H, δ about 2, t, J = 1.7 Hz) in the NMR ($CDCl_3$), both shifted downfield in (C_5D_5N) (1), and (b) the detection of signals of two nonprotonated olefinic carbons in HMBC of **10** and **11** at δ about 145 and 132 (in $CDCl_3$), assigned, according to characteristic chemical shifts (27) to C-4 and C-5, respectively (1). In addition to the molecular formula ($C_{19}H_{24}O_8$) based on the $[M + H]^+$ ion, the DCIMS revealed functional groups in lactone **11**. The consecutive eliminations of two water molecules and also two acetic acids, giving the following abundant fragments: $[M + H - 18]^+$, $[M + H - 2\times18]^+$, $[M + H - 60]^+$, $[M + H - 18 - 60]^+$, $[M + H - 2\times18 - 60]^+$ and $[M + H - 2\times60]^+$, accorded with two OH and two OAc groups. The presence of two acetoxy groups was also obvious from the occurrence of two three-proton acetoxy singlets (δ ≥ 2.0). The characteristic chemical shifts and couplings, obtained by the first-order analysis in the spectra measured in $CDCl_3$ and also in C_5D_5N, together with the interproton scalar coupling connectivity pattern (Fig. 11) were in accordance with 3,10-dihydroxy-8α,9α-diacetoxy substitution in this lactone. An additional proof for this structure was obtained from the long-range (HMBC) heteronuclear C,H-correlations of C-3, C-5 and C-4 to H-15 and C-1, C-10, and C-9 to H-14. The NOESY data (Fig. 12) were in agreement with the above structural and stereochemical proposal,

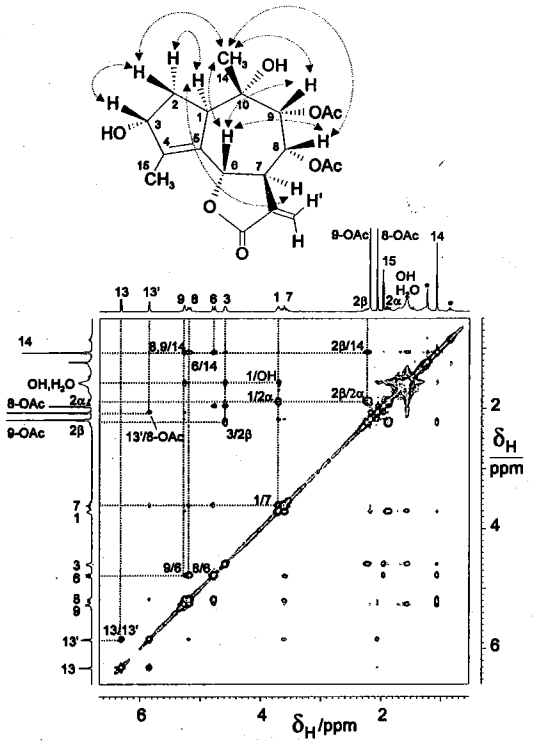


Figure 12. NOESY spectrum of lactone **11**; double-headed arrows denote the NOEs significant for determination of structure and stereochemistry; * - impurities.

also indicating 3α,10α-dihydroxy configuration in this lactone. Structure determination of the related 3α-hydroperoxy lactone **12** (anthemolide E, $C_{19}H_{24}O_9$) by analogy with the spectral data to those of **11** was reported elsewhere (1).

Diterpenes - 16α,17-epoxy-*ent*-kaurane (13)

Although diterpene **13** was assigned by the analogy of its spectral data and optical rotation to those reported for the same compound originating from other sources (24), to check some previous spectral assignments, we have run DQF COSY, NOESY, and HSQC spectra of this compound. A modification of the standard HSQC sequence *(see below)* enabled spectrum editing and separation of the C,H-correlations with respect to the number of the attached protons. This could be extremely useful for the assignment of signals

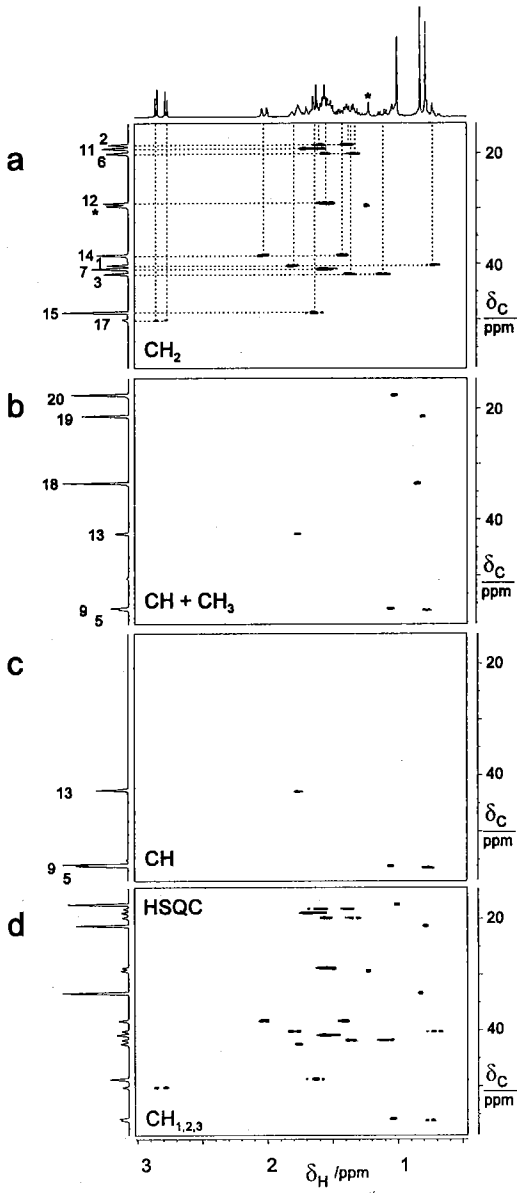


Figure 13. Edited HSQC spectra of diterpene **13** in $CDCl_3$: (a) correlations of CH_2-groups, (b) correlations of CH_3 + CH groups (c) correlations of CH-groups and (d) unedited HSQC spectrum; * - impurities.

of protonated carbons and (the attached protons) in the crowded NMR spectra, as shown by the example of the set of the edited HSQC spectra of diterpene **13** (Fig. 13).

NMR METHODS

NMR spectroscopy is a versatile instrumental method capable of providing structural and dynamic data on an atomic scale (28,29,30,31). Application of NMR in the study of natural products was particularly invigorated by development of 2D spectroscopy (32). Perhaps the most important application of NMR spectroscopy is in elucidation of solution structure of proteins and nucleic acids (33,34). Thus, most of the modern methods were developed in relation to macromolecular structure determination. Many of the new methods are useful in the study of smaller molecules, which invigorated their application in the study of natural products. In this section, we briefly describe the ones we have used most successfully.

Homonuclear experiments

DQF COSY (35) is the basic 2D experiment that reveals connectivities through the network of scalar couplings. Appearance of cross-peaks indicates that respective diagonal lines originate from the spins that are coupled directly. Because scalar coupling diminishes with the number of chemical bonds, this experiment reveals contacts among directly coupled protons that are 2 to 4 bonds away.

TOCSY (36) (TOtal Correlated SpectroscopY) or **HOHAHA** (37) (HOmonuclear HArtman HAhn spectroscopy) reveals connectivities among all the spins from the coupling network. Both experiments are based on the Hartman-Hahn magnetization transfer and differ only in the pulse sequence during the mixing period. For short mixing times cross-peaks arise only among directly coupled spins. For longer mixing times cross-peaks appear also among the spins that are not coupled directly but share a common coupling partner. Thus, the experiment is useful for identification of whole spin networks.

To enable Hartman-Hahn magnetization transfer in the TOCSY experiment the pulse sequence during the mixing time locks magnetization along the effective magnetic field. Under this condition, chemical exchange that may take place irrespective of the NMR experiment gives rise to the chemical exchange cross-peaks, in the same manner as in the NOESY (*see below*) or ROESY (38) (Rotating frame nuclear Overhauser Effect SpectroscopY) experiment. The difficulty is that chemical exchange peaks have the same sign (positive) as TOCSY cross-peaks and, thus, are hard to identify. They can be recognized in a series of TOCSY experiments recorded at different mixing times by the difference in the mixing time evolution.

The TOCSY cross-peaks have oscillatory transfer function (oscillate as a function of mixing period), whereas chemical exchange cross-peaks first build and then decay steadily. This may be ineffective and in practice chemical exchange cross-peaks are identified by comparison TOCSY to other experiments (e.g. by identifying chemical exchange network in the NOESY /EXSY experiment (*see below*) or by independent knowledge of potential sources of chemical exchange). For example, it is well known that the protons in labile groups like -OH and =NH tend to exchange mutually or with traces of water. Then, their lines are usually exchange broadened, which is a good indication of the potential problem. For example, in the TOCSY spectrum of lactone **5** (Fig. 2), chemical exchange takes place between the -OH group and residual water. The nature of the process can be deduced from exchange broadened water line in the 1D spectrum and the singlet structure of the -OH signal. They both indicate the absence of measurable coupling of these spin sites and thus eliminate the possibility that

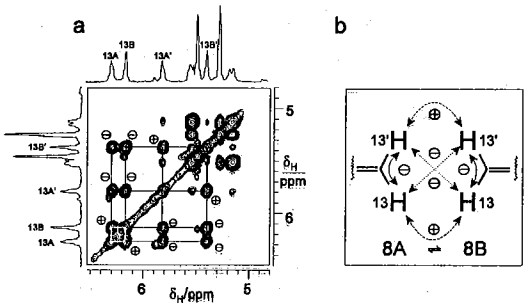


Figure 14. Combined effects of cross-relaxation (NOE) and chemical exchange: a) Enlargement of the section from the NOESY spectrum of lactone **8** (c.f. Fig. 8) that shows positive and negative cross-peaks. b) Scheme of magnetization exchange pathways: full lines indicate NOE, dashed, chemical exchange and dotted, relayed magnetization transfer by both, NOE and chemical exchange.

these are the TOCSY cross-peaks.

NOESY (39,40) (Nuclear Overhauser Effect SpectroscopY) or EXSY (39,41) is an experiment for monitoring incoherent magnetization transfer (i.e., chemical exchange and cross-relaxation). Chemical exchange in the NMR sense is any process by which the spin site changes its environment. It can be intermolecular or intramolecular rearrangement including conformational changes. In chemical exchange, magnetization between the two spin sites is transferred by direct mass transfer. In cross-relaxation, magnetization is transferred by dipole-dipole interaction. Nuclear Overhauser effect (NOE) is just an experimental manifestation of cross-relaxation. Dipole-dipole interaction takes place through space and produces cross-peaks between proximal spins irrespective of the existence of chemical bonds. Because the dipole-dipole interaction depends on the inverse sixth power of interproton distance, cross-relaxation vanishes quickly with the distance and rarely can be detected for spins more than 4 Å apart.

In small molecules, NOE is positive and so is the cross-relaxation rate constant. Then, the cross-peaks are of opposite phase with respect to the (negative) diagonal peaks. Cross-peaks are apparently negative because their sign is determined relative to the diagonal peaks. Similarly, in large molecules, NOE and cross-relaxation rate constants are negative but cross-peaks in the NOESY spectra are positive (in phase with the diagonal peaks). Chemical exchange, expressed by the (positive) chemical exchange rate constant, produces always positive cross-peaks.

In small molecules (MW up to 1,000), it is easy to distinguish cross-relaxation from chemical exchange merely by the sign of cross-peaks. As is shown in Figure 5, all cross-relaxation cross-peaks are negative except the peak pair between water and the -OH group which is positive. Thus, the OH/H_2O exchange is due to chemical exchange rather than to cross-relaxation between the respective spin groups. If chemical exchange is much faster than cross-relaxation, then, at the mixing times necessary to observe cross-relaxation, chemical exchange can contribute also to the relayed magnetization transfer. Magnetization transferred from one site to another by cross-relaxation can further be transferred to another site by fast chemical exchange. This gives rise to an additional set of cross-peaks that can cause a false impression of spatial proximity among distant spins (Fig. 8 and 14). Positive peaks between the protons 13A/13B and 13'A/13'B indicate chemical exchange between conformations A and B. Negative cross-peaks, 13A/13'A and 13B/13'B indicate close spatial proximity of the protons within given conformations. However, negative cross-peaks between protons 13A/13'B and 13'A/13B are not genuine cross-relaxation peaks that indicated spatial proximity, but they are created by two-step magnetization transfer:

$$13A \rightleftharpoons_{NOE} 13'A \rightleftharpoons_{Chem.\ Exc.} 13'B$$

and

$$13A \rightleftharpoons_{Chem.\ Exc.} 13B \rightleftharpoons_{NOE} 13'B.$$

In the presented case almost all the lines are doubled, and it is relatively easy to recognize the two sets that belong to conformers A and B. However, if undetected, the indirect magnetization transfer might be interpreted as a genuine contact in space that produces false information about the structure. The safest way to eliminate undesired effects of chemical exchange is to record the NOESY spectrum at a lower temperature. This reduces the chemical exchange rates and increases the cross-relaxation rates, which can be seen easily as a redistribution of cross-peaks' intensities (42,43).

Heteronuclear experiments with proton (inverse) detection

One of the most important steps in the application of multinuclear NMR to chemical and biochemical problems is development of proton-detected (also called inverse-detected) heteronuclear experiments; for a review, see the compilation by Croasmun and Carlson (44). Briefly, instead of detecting insensitive heteronuclei directly, proton polarization is transferred to heteronuclei for frequency labeling and then transferred back to protons for detection. This brings immense savings in measurement time. For example, with proton detection the same signal-to-noise in ^{13}C spectrum can be achieved 64 times faster than in the direct detection method. For ^{15}N nuclei this factor is 1,000! For the analysis of natural products, an obvious consequence of this improvement is that heteronuclear NMR spectra can be recorded with the minute quantities of the material. With a conventional 5-mm inversion detection probe, at 500 MHz, heteronuclear spectra can be recorded from 100 to 200 μg of moderate size molecular weight sample. Further improvement can be achieved using microprobes, in which the amount of material can be reduced to 10 to 20 μg (45).

HSQC (46) is the proton-detected equivalent of the HETCOR (HETeronuclear CORrelated

spectroscopy) experiment. It establishes correlations between protons and directly bonded heteronuclei; thus, it discerns resonances of protonated heteronuclei (Fig. 3 and 10). Nonprotonated heteronuclei can be detected by an HMBC experiment.

HMBC (47) is the proton-detected equivalent of long-range HETCOR. It establishes correlation through smaller heteronuclear coupling constants, thus, through several chemical bonds. This enables detection of nonprotonated heteronuclei, as is demonstrated in Figure 4. The HMBC spectrum of lactone **5**, besides new correlations with already observed carbons (H-15/C-3,4,5 and H-14/C-1,9,10) shows correlations to nonprotonated carbons (e.g., carbonyl carbon in -OAc and -*i*-But groups).

Double INEPT (48,49) (Insensitive Nucleus Enhancement by Polarization Transfer) or **INEPT edited HSQC** is described in several different implementations (50) with different aims. We have used our own implementation (Fig. 15), which we derived from the NOESY editing sequence (51). The principal aim of the double INEPT experiment in our implementation is to sort out heteronuclear correlation in the HSQC experiment according to the number of attached protons. The sequence can be derived from the standard HSQC sequence by inserting an editing delay in front of the second polarization transfer block. In the HSQC experiment, polarization from protons is transferred by an INEPT-type sequence to directly attached heteronuclei (here carbon) via $^1J_{CH}$ coupling. The ^{13}C magnetization evolves during the evolution period t_1. A proton 180° pulse in the middle of the evolution period eliminates splitting in the carbon lines by $^1J_{CH}$ coupling. At the end of the evolution period, magnetization labeled with carbon frequencies is transferred back to protons by a reverse INEPT sequence and subsequently detected during detection period t_2. Antiphase proton magnetization after reverse INEPT is refocused after $1/2^1J_{CH}$ and then carbons can be decoupled during acquisition. This yields a phase sensitive HSQC spectrum with all the lines positive. There is no difference between differently protonated carbons.

Insertion of additional delay Δ' ($\Delta/2 + \Delta/2$) at the end of the evolution period and two refocusing pulses allows carbon to evolve further under only the influence of coupling. (Refocusing pulse on carbon eliminates ^{13}C chemical shift evolution and the pulse at proton restores $^1J_{CH}$ coupling.) Because of the influence of the number of attached protons, magnetization

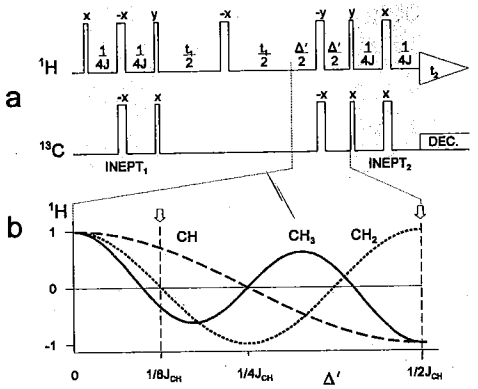


Figure 15. INEPT edited HSQC: (a) Pulse sequence. (b) Evolution of magnetization during the editing delay.

evolution of CH, CH_2, and CH_3 carbons will be distinctly different (Fig, 15 b). Obviously, for $\Delta' = 0$, as in HSQC, all lines are n phase. For $\Delta' = 1/2^1J_{CH}$ signals of CH and CH_3 become inverted with respect to signals from CH_2. At $\Delta' = 1/4^1J_{CH}$, only signals from CH_2 are detected, and finally, for $\Delta' = 1/8^1J_{CH}$, only CH signals are observed. If chemical shifts of CH and CH_3 do not overlap, then it is sufficient to record the experiment with $\Delta' = 1/2^1J_{CH}$ only. To eliminate any doubts we also record the experiment with $\Delta' = 1/8^1J_{CH}$. Figure 13 shows INEPT edited HSQC of diterpene **13**. Panels a and b show positive and negative contours from the experiment with $\Delta' = 1/2^1J_{CH}$, and panel c represents the contour plot of the experiment with $\Delta' = 1/8^1J_{CH}$. For comparison, HSQC contours are shown in panel d. It is good practice always to record HSQC because accidental overlap of CH_2 and CH_3 resonances may lead to cancellation of cross-peaks in the edited experiment.

Note that the same editing effect can be achieved by suitable modification of other proton-detected experiments (e.g., DEPT) edited HMQC, DEPT-HMQC (52).

EXPERIMENTAL

NMR experiments were performed on a Bruker AMX 300 spectrometer with 5-mm broad band inverse probe. Two-dimensional spectra were recorded overnight: homonuclear 8 to 10 h and heteronuclear 12 to14 h. Amount of isolated and analyzed material

ranged from 2.5 mg (lactone **8**) to 5 mg (lactones **5**, **11** and diterpene **13**).

CONCLUSION

We have reviewed the application of modern NMR methods to elucidate the structure of the new small molecular weight natural products such as sesquiterpene lactones (guaianolides from *Anthemis carpatica*) and diterpenes (kauranes from *Achillea clypeolata*). The application of the techniques described above enabled complete structural assignment and determination of relative stereochemistry of these compounds as well as the detection of conformational equilibrium in one case (compound **8**).

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