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NMR of carotenoids: novel experimental techniques

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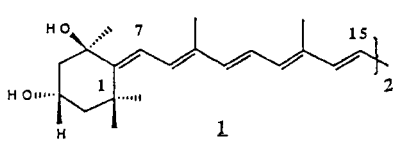
Abstract- A number of novel experimental one- and two-dimensional NMR techniques are presented which have been found to be extremely useful for the elucidation of the structures of carotenoids. These are the rotating-frame experiments TOCSY (**T**otal **C**orrelation **S**pectroscopy, also named HOHAHA) and ROESY (**R**otating Frame **N**uclear **O**verhauser **E**ffect **S**pectroscopy). Moreover, some applications of the very sensitive ^{1}H-detected one-bond and multiple-bond versions of $^{1}H,^{13}C$-correlated 2D COSY will be presented. These techniques will be applied to the derivation of the structures of the novel C_{40} *retro* carotenoid **1** ((3S,5R,3'S,5'R)-4,5-dihydro-4,5'-*retro*-ß,ß-carotene-3,5,3',5'-tetrol) and of the hepta- and octaacetate derivatives **2a** and **2b** of the microalgal glycosidic carotenoid P457 **2** (13'-*cis*-7',8'-dihydroneoxanthin-20'-al 3'-ß-lactoside). Advantages and limitations of these techniques are discussed with respect to structure elucidations in the field of carotenoids.

INTRODUCTION

During the last decade an almost explosive growth in the number of sophisticated multipulse NMR experiments has been encountered (ref.1) and even the expert NMR spectroscopist might sometimes be confused by the myriad of new acronyms appearing in the relevant literature. As my contribution to this conference I have selected a few of these novel one- and two-dimensional (1D and 2D) NMR techniques which I have found particularly useful and advantageous in our laboratory for the structural elucidation of carotenoids. Due to lack of space I shall be unable to explain the physical background of these techniques. In this context I shall rather attempt to use only very simple intuitive pictures and my main goal will be to demonstrate the power and limitations of these methods by applying them to the structural elucidation of some carotenoids (see Scheme 1).

Scheme 1



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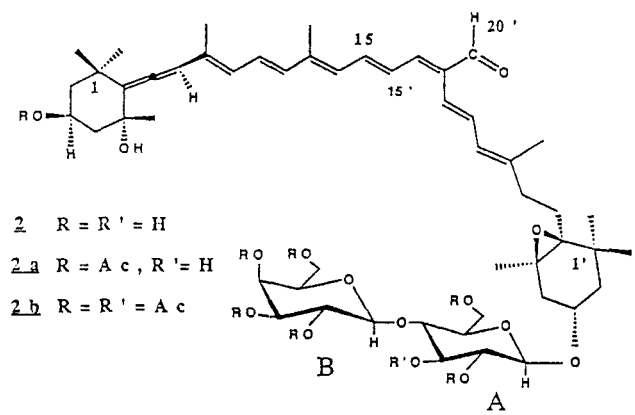

2 R = R' = H

2a R = Ac, R' = H

2b R = R' = Ac

Chemical structures

In what follows I shall present and discuss the following interesting experiments:

1) 1D and 2D TOCSY, sometimes also called HOHAHA (**H**omonuclear **H**artmann-**H**ahn Spectroscopy), which successfully replaces in many cases the well-known COSY experiment;

2) 1D and 2D ROESY (**R**otating Frame **N**uclear **O**verhauser **E**ffect **S**pectroscopy), originally termed CAMELSPIN. This is an important substitute for the normal nuclear Overhauser experiment known as NOESY which fails at medium molecular masses, which are typical for carotenoids;

3) ^{1}H-detected so-called reverse or inverse $^{1}H,^{13}C$-COSY which is a much more sensitive variant of the normal ^{13}C-detected $^{1}H,^{13}C$-COSY.

As will be seen in the following, the use of these new techniques significantly increases the importance and the utility of NMR when applied in the field of carotenoids.

NEW EXPERIMENTAL TECHNIQUES

1D and 2D TOCSY. The TOCSY experiment was proposed and its physical background given by Braunschweiler and Ernst as early as 1983 (ref. 2). However, it took some years for this interesting NMR experiment to find many practical applications.

In the basic 1D TOCSY experiment the magnetization of a selected proton whose signal is sufficiently separated from all other proton signals is first inverted by a selective 180^{0} pulse (ref.3). After a following non-selective 90^{0} pulse, turning the magnetization of all protons into the transverse x,y-plane perpendicular to the magnetic field, a so-called spin-locking pulse for the transverse magnetization is applied, provided by a rotating r.f.-field or a corresponding MLEV-17 multipulse sequence (ref.4). During the time of spin-locking, also called mixing time,which is normally chosen between about 20 and 200 ms, an exchange of magnetization between the inverted spin and its successive neighbours takes place mediated by the proton-proton spin coupling between each pair of subsequent protons. This leads to variations in the intensity of their signals which can be observed in a difference spectrum with the first selective 180^{0} pulse on-resonance minus off-resonance. Thus one observes a subspectrum of only those protons which are directly or indirectly (via relayed processes) spin-coupled to the first inverted proton. The propagation of the magnetization along a chain of coupled protons increases with the duration of the spin-lock and with the magnitude of the couplings. Therefore, the experimental conditions can be chosen such that, in a first experiment, a subspectrum of only one or two neighbours is seen; in further experiments subspectra of up to 6 (in special cases even more) protons are obtained. This means that very complex and overlapping spectra can be separated in a number of pure subspectra. The very interesting practical implications of this technique will be demonstrated now in the following examples.

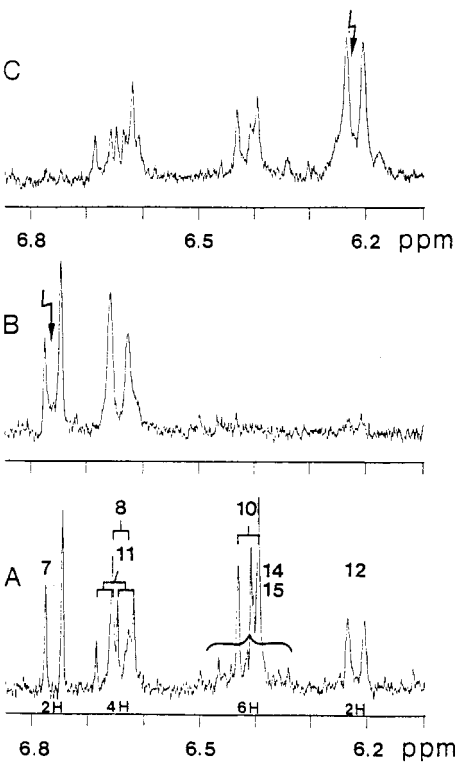

Fig. 1. Low-field section of the 400 MHz ^{1}H NMR spectrum of **1** in $CDCl_3$ (A); 1D-TOCSY with selective 180^{0} inversion by a DANTE pulse train (290 x 2 μs) of the doublets at 6.76 ppm with a spin-locking of 52 ms (B) and at 6.22 ppm with a duration of 20 ms (C).

Figure 1 (A) shows the olefinic part of the 400 MHz ^{1}H NMR spectrum of a new C_{40} *retro* carotenoid **1** isolated by H. Kleinig's group at the University of Freiburg i.Br., F.R.G. (ref. 5). The compound was obtained by an enzymatical reaction from violaxanthin. The proposed symmetric *retro* structure is based on a number of 1D and 2D experiments. In the middle trace (B) of Figure 1 is shown a 1D TOCSY difference spectrum that was obtained upon inversion of the low-field doublet (later to be assigned to proton 7) as indicated by the arrow. Although a medium duration of the spin-lock of about 52 ms was used, only one further proton doublet is seen, showing that only two protons are present in this subsystem and therefore the two doublets must be assigned to protons 7 and 8 (and 7' and 8' of the symmetrical compound).

In the top trace (C) of Figure 1, the high-field doublet at 6.22 ppm is inverted by the selective 180^0 pulse and, despite some minor artifacts in the difference spectrum, a 3-spin subspectrum is clearly seen which must be assigned to protons 10, 11 and 12 (and 10', 11' and 12'). According to the integral (not shown) the only remaining four protons 14, 15, 15' and 14' are identified near 6.4 ppm as expected for *retro* carotenoids (ref. 6).

Figure 2 shows the aliphatic section of the 400 MHz ^{1}H spectrum of the same compound and a corresponding 1D TOCSY spectrum obtained upon inversion of the multiplet signal at 4.25 ppm (2H) assigned to protons 3,3'. Note that the complete subspectrum of the neighbouring methylene groups at positions 2 and 4 (2' and 4') and the doublet of the hydroxyl protons are clearly seen in the difference spectrum. From the observed multiplet structures of this subspectrum, the position of the hydroxyl groups at 3,3' is immediately evident. All the relative assignments in the different subsystems presented in Figs. 1 and 2 and the geometry of the double bonds were derived by 2D ROESY as will be discussed below.

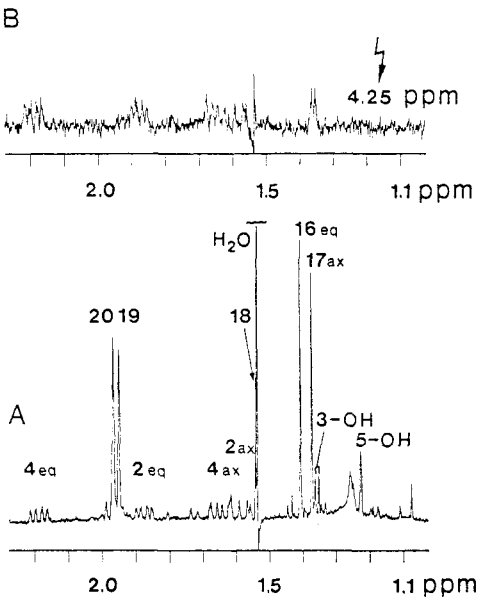

Fig. 2. High-field section of the 400 MHz 1H NMR spectrum of **1** in $CDCl_3$ (A), 1D-TOCSY with selective 180^0 inversion of protons 3,3' at 4.25 ppm by a DANTE pulse train (290 x 2μs) and spin-locking of 70 ms duration.

The detection of a complete subspectrum of the methine and methylene protons of a carotenoid end-group, revealing all chemical shifts and coupling constants despite the presence of strong overlapping methyl signals (and sometimes of strong impurity peaks) is an extremely interesting application of TOCSY.

A further still more impressive example for the power of 1D TOCSY is presented in the next application to the microalgal carotenoid P457 (**2** , see Scheme 1). This highly polar marine carotenoid was first isolated in 1974 by the Trondheim group and, since then, extensive studies have revealed many details of its structure (ref. 7). I intensively investigated this compound, which was

kindly provided by Professor S. Liaaen-Jensen, by several of the new NMR techniques, as will be shown in the following. As a result, the structure of the carbohydrate part of **2** (and of its acetylated derivatives) and its attachment to position 3' were derived.

In order to improve the solubility of P457, it was acetylated and two products became available for spectroscopic investigation in quantities of about 0.2 and 0.4 mg. It became evident that the minor component **2a** had only 7 acetyl groups instead of 8 as in the major component **2b**. Although **2a** was recognized in this sense as an artifact, the interpretation of its spectrum contributed much to the understanding of the spectra of the two other compounds. In addition, the utility of 1D TOCSY is once more nicely demonstrated, since the derivation of the structure of the two sugar moieties and the location of the remaining hydroxyl group in **2a** are readily obtained by this technique.

Figure 3 (A) shows the complex sugar section of the 400 MHz ^{1}H NMR of the heptaacetate **2a** between 5.6 and 3.4 ppm. In this strongly overlapping region with about 17 protons, the doublets of the two axial anomeric protons A1 and B1 of the two rings A and B are easily identified. In contrast to P457 and its octaacetate they have sufficiently different chemical shifts and can serve therefore as a good starting point for a series of 1D TOCSY experiments.

In the other spectra shown in Figure 3 the magnetization of proton A1 is inverted and, with increasing duration of the mixing time (10 ms in spectrum B, 30 ms in C, 100 ms in D and 140 ms in E) the magnetization of A1 is propagating through the whole sequence of A protons until the 6-methylene protons are reached and hence the complete subspectrum of all coupled protons of ring A is seen. Whereas the first three experiments, B, C and D, were each performed with 15 min acquisition time, spectrum E was run in 45 min in order partly to compensate for the decaying signal-to-noise ratio due to the distribution of the primary magnetization along the chain of coupled protons. It is worth mentioning that the attachment of the hydroxyl group at position A3 is immediately evident from the high-field shift of proton A3 compared to that in compound **2b** and from the small coupling between both protons.

In Figure 4 is presented a corresponding 1D TOCSY experiment starting from the anomeric proton B1. In contrast to ring A the transfer of magnetization is interrupted at the equatorial proton B4 since the coupling $J_{4,5}$ is obviously too small (<1Hz) for a further relay of the magnetization. The remaining three protons of ring B, therefore, had to be located by other techniques, e.g. by ROESY, since a strong through-space interaction is present between proton B1 and the two 1,3-diaxial partners

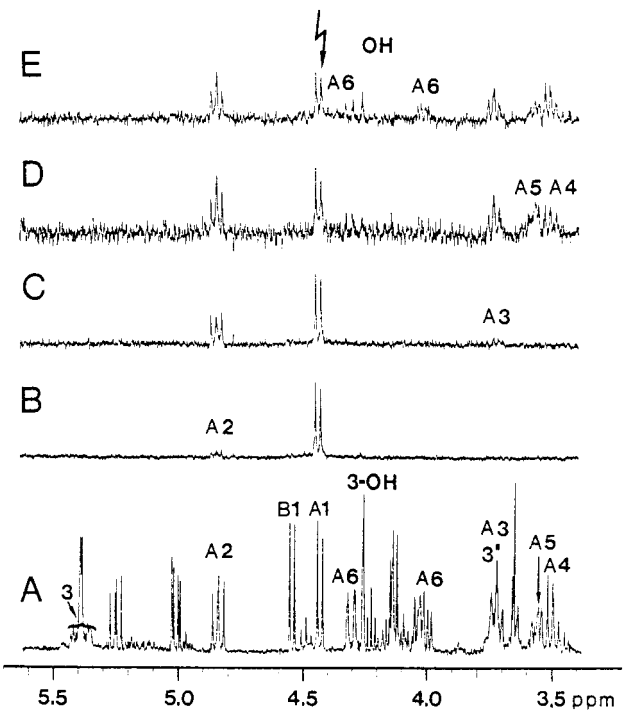

Fig. 3. Section of the 400 MHz ^{1}H NMR spectrum of ca. 0.2 mg of **2a** in $CDCl_3$ (A); 1D-TOCSY spectra with selective inversion of proton A1 of ring A at 4.427 ppm by a DANTE pulse train (1020 x 2μs). Spin-locking 10 ms (B), 30 ms (C), 100 ms (D), and 140 ms (E).

B3 and B5. In the case of the octaacetate **2b**, the signal for proton B5 at 3.87 ppm was sufficiently separated from all other neighbours for a further 1D TOCSY experiment to be started from there yielding the multiplets of protons B6. The ROESY experiments performed with both compounds also gave information on the relative positions of rings A and B since an inter-ring ROE between protons B1 and A4 was detected. The attachment of the ß-lactoside at position 3' was also supported by ROESY and, moreover, by a $^{1}H,^{13}C$-COSY experiment, as will be shown below.

The examples discussed so far clearly indicate the great potential and also the limitations of the 1D TOCSY experiment. Compared to the corresponding 2D experiment with typical acquisition times of about 15 h, the 1D variant has the advantage of a much shorter acquisition time and a better digital resolution. This means that subspectra with optimum resolution are obtained, enabling one to determine accurate values of coupling constants and chemical shifts, as in normal 1D spectra. The 1D version is therefore generally to be preferred in cases of low and medium complexity. We have successfully applied this technique in numerous cases in order to generate subspectra of olefinic protons and of end groups. As examples, the subspectra of the methylene groups at positions 2,4 and 2',4' of P457 and its hepta- and octaacetates were measured by selective inversion of the signals of protons 3 and 3'. This greatly helped in the identification and confirmation of the two end groups (ref. 7).

1D and 2D ROESY. In a regular nuclear Overhauser effect (NOE) experiment one measures the change of the intensity of proton signals upon irradiation of the signal of another spatially close proton. The effect is proportional to $1/r^6$, r being the distance between the two protons or group of protons. The effect is normally observable for distances of up to 4 - 5 Å. It is caused by an exchange of longitudinal or z magnetization (parallel to the magnetic field axis) arising by fluctuating dipolar magnetic fields due to the molecular motion (refs. 8,9). The NOE has been used in countless applications for the elucidation of chemical structures and some cases have been reported in the field of carotenoids (refs. 10-12).

The longitudinal NOE has, however, the substantial disadvantage that its magnitude decreases with increasing molecular mass of the compounds under study and also with increasing spectrometer frequency. At typical frequencies of 400 MHz or above and molecular masses of 600 to 800 Da the

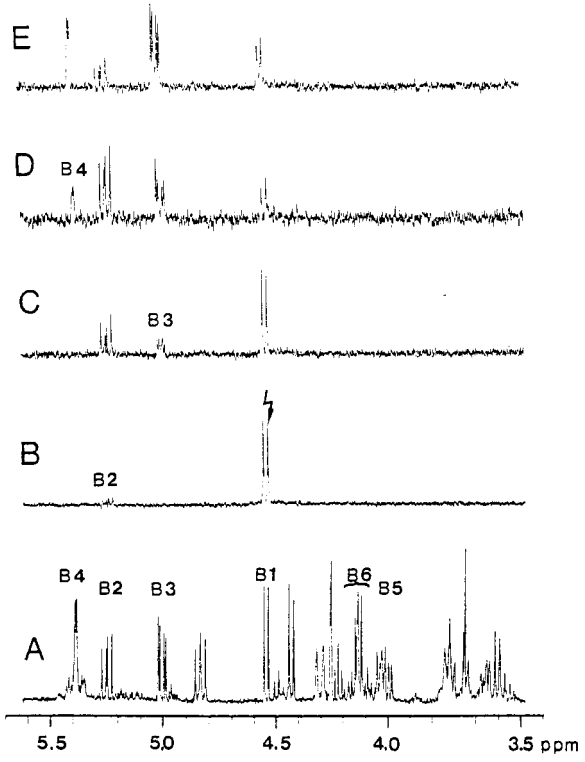

Fig. 4. Section of the 400 MHZ ^{1}H NMR spectrum of **2a** in $CDCl_3$ (A). 1D-TOCSY with selective inversion of proton B1 at 4.54 ppm by a train of DANTE pulses (1020 x 2 µs). Spin-locking of 10 ms (B), 30 ms (C), 100 ms (D), and 140 ms (E).

NOE can actually vanish. At still higher molecular masses it becomes negative and is again detectable (ref. 8, 9). In a recent investigation of the structure of 19'-butanoyloxyfucoxanthin and of its 9'-*cis* isomer, we have shown this unfavourable null condition to be present (ref. 12).

For molecules of intermediate size the only known remedy is the application of the rotating-frame or transverse NOE spectroscopy, also called ROESY. It is known always to give a positive effect and,in this range of medium molecular size, its intensity is stronger than the longitudinal NOE (refs. 9,13,14). Therefore, the ROESY variant is now generally preferred in our laboratory for applications to carotenoids.

In this experiment the longitudinal z-magnetization is rotated by a 90^0 pulse into the transverse x,y-plane and then locked there by a rotating spin-locking field as in the TOCSY experiment. This can again be achieved by a continuous r.f. field or a suitable "chopped" spin-lock provided by a train of short pulses (ref. 15). This process averages to zero the longitudinal z-magnetization of all the spins, i.e. the chemical shift differences of the spin-locked transverse magnetizations are effectively quenched. In this state, flip-flop processes due to through-space dipolar interactions can lead to an exchange of transverse magnetization, depending in the same $1/r^6$ manner on the distance as in NOESY. Since the exchange of magnetization takes place in the spin-locked mode, i.e. in the rotating frame, this experiment is therefore called transverse NOE or rotating-frame nuclear Overhauser effect spectroscopy ROESY (refs. 13,14). In the same way as TOCSY it can be performed in 1D or 2D versions.

In 1D ROESY, the experiment starts again with a selective 180^0 pulse inverting a selected proton signal. In the next step the non-selective 90^0 pulse and then the spin-locking process follow before the FID is acquired. This leads to a change of the intensity of the signals of spatially close neighbouring

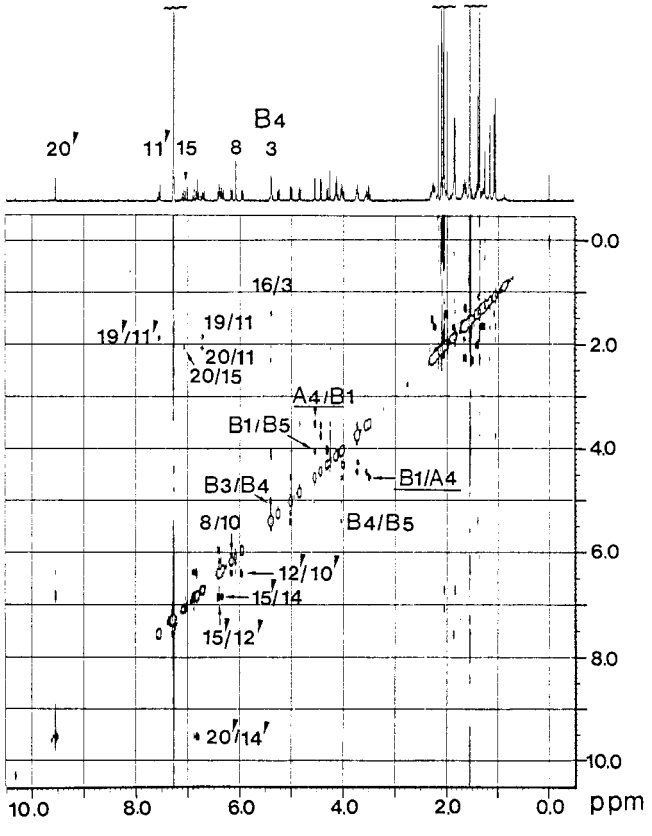

Fig. 5. Phase-sensitive 400 MHz 2D ROESY of < 0.1 mg of 2a (purified by HPLC) in $CDCl_3$. Experimental data: acquisition 4K x 400 experiments, zero-filling to 2K x 1K data points, spectral width 8064 Hz in both directions, cosine-square filters, 3.9 and 7.8 Hz digital resolution, 1.5 s relaxation delay, coaddition of 3 experiments with 0.4, 0.6 and 0.8 s spin-locking durations, 0.08 duty cycle, total acquisition time 14 h.

protons. In the 2D case, the pulse sequence starts with the 90^0 pulse and the incremented evolution period before the spin-locking is applied. Spatially close protons are here revealed by cross-peaks in the 2D spectrum.

As an example, Figure 5 shows the 400 MHz 2D ROESY spectrum of the heptaacetate **2a** with the 1D ^1H NMR plotted along the horizontal ω_2-axis. The intensity of the cross-peaks is represented by contour lines with stronger peaks having several contour levels, weaker peaks only one or two . The strongest peaks with positive intensity (sign not shown in Figure 5) occur along the diagonal axis $\omega_1=\omega_2$ which represents essentially the 1D spectrum. Numerous weaker and negative off-diagonal ROESY cross-peaks are also shown, each connecting two diagonal peaks, thus indicating that the two protons are spatially close to each other. It is immediately obvious that this overview spectrum is not suitable for a detailed interpretation and, to avoid overcrowding, only some relevant cross-peaks were designated here. The full wealth of information contained in such a 2D spectum can only be extracted from a number of expansion plots comprising many more cross-peaks of smaller intensity.

A quantitative interpretation of the Overhauser effect in terms of distances is, in general, rather tedious work. However, a simple qualitative picture is easily obtained if structurally relevant cross-peaks only are taken into account. This is demonstrated in Figure 6 for the heptaacetate **2a**. Each arrow represents the two cross-peaks found in the 2D ROESY spectra and hence indicates that the two protons or group of protons are spatially close to each other.

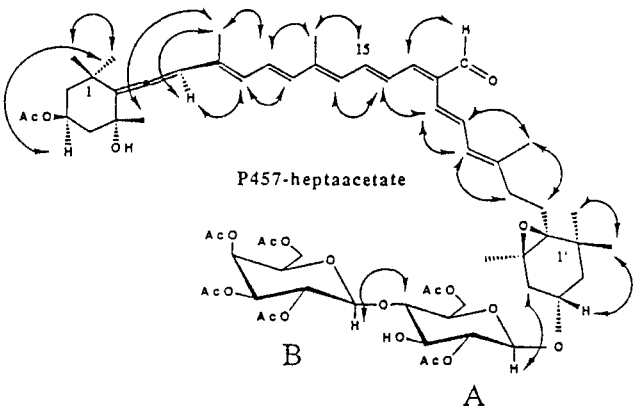

Fig. 6. Part of the results extracted from the 2D ROESY of **2a** of Fig. 5. Each arrow indicates relevant through-space ROESY contacts between the indicated protons.

Thus, in the two-end groups, we have through-space contacts between the two geminal methyl protons. The axial methyl groups of both end-groups give strong 1,3-diaxial interactions with the axial protons 3 and 3'. Along the olefinic chain we observe all expected interactions of protons on the same side of the chain and these fully define the geometry of all the double bonds. As an exception, the cross-peaks between protons 14 and 12 could not be detected because the two have approximately the same chemical shift and are therefore too close to the strong diagonal. Important are the cross-peaks between protons 20' and 14' as well as those between 15' and 12' which define the geometry of the Δ-13' double bond and the orientation of the aldehyde proton as depicted in the formula. Moreover, the relative position of rings A and B is indicated by cross-peaks between protons B1 and A4 and the attachment of the lactoside at the right-hand end-group by cross-peaks between A1 and the equatorially oriented proton 4'.

Further through-space contacts were identified between protons 10' and 8', and between 19' and one of the 7'-methylene protons. The complete subspectrum of the two neighbouring 7'- and 8'-methylenes was defined by a 1D TOCSY in the same way as discussed above. These results clearly support the structure proposed for these parts of the molecule.

In the left-hand end-group a cross-peak of medium intensity between protons 19 and 18 was seen; this supports the assumed relative stereochemistry at position 6. Moreover, all the chemical shifts of this end-group are in agreement with those of suitable reference compounds (ref. 7).

Further cross-peaks between geminal protons of both end groups as well as between methyl protons and neighbouring protons on the same side of each end-group are not designated in Figure 6, although they also contributed to the reliability of all the assignments. The same is true for all other through-space contacts such as the 1,3-, 1,5- and 3,5-diaxial contacts in rings A and B as well as between protons 2 and 4, and 4 and 5 in ring B which were all in agreement with the proposed structure. These experiments, together with those for P457 and its derivative **2b**, clearly prove the shown 3'-ß-lactoside partial structure and the geometry of all the double bonds.

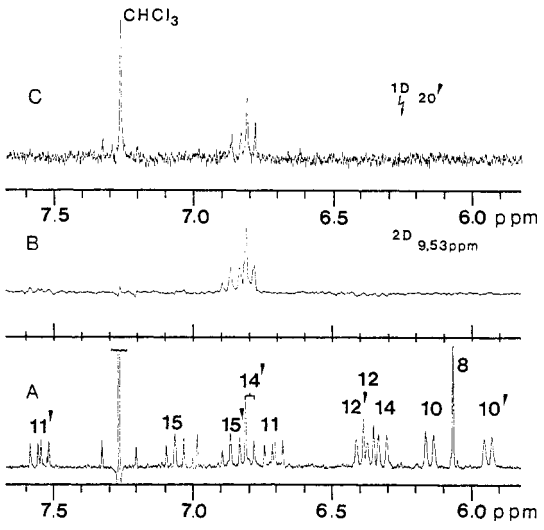

Fig. 7. A: Olefinic section of the 400 MHz ^{1}H NMR of ca. 0.4 mg of the octaacetylated P457 **2b**. B: Selective row at 9.53 ppm (proton 20') of the 2D ROESY obtained with similar experimental conditions as in Fig. 5. C: 1D ROESY experiment obtained upon selective inversion of the signal of proton 20' by a DANTE pulse train (1000 x 2 μs). Mixing times 0.4, 0.6 and 0.8 s, duty cycle 0.08, total measuring time 50 min.

These applications convincingly demonstrate the power and utility of 2D ROESY. The only drawback is the fact that long acquisition times, typically 15 h, have to be endured with sample quantities below 1 mg. Therefore, if instrument time is scarce, it is often sufficient to acquire only a few relevant 1D ROESY spectra which can be obtained in a much shorter time. A comparison of the two cases is given in Fig 7. Here the olefinic section of the 400 MHz ^{1}H NMR of the octaacetate **2b** is presented in trace A. The middle trace (B) shows the olefinic part of a horizontal row of the 2D spectrum taken at the chemical shift of the aldehyde proton 20'. The upper trace (C) gives the corresponding 1D ROESY obtained on selective inversion of the aldehyde proton. This latter experiment was performed in less than one hour compared to the 14 hours which were needed for the 2D. Both spectra indicate in a fully analogous way that proton 20' is very close to 14'. No effect on proton 11' could be detected in either experiment. Note that the enhancement for proton 15' in these spectra is an artifact caused by the small chemical shift difference between protons 15' and 14' and the mixing of their eigenfunctions.

Figure 8 shows the results of a 2D ROESY experiment performed with the *retro* compound **1**. Here, the geometry of all double bonds and, in addition, all the local stereochemical assignments are clearly evident. In this experiment, it was not possible to use the through-space interactions between protons 20 and 15 since the latter had an almost identical chemical shift to proton 14.

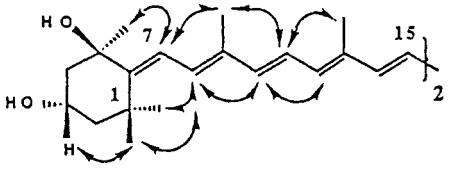

Fig. 8. Part of the observed 2D ROESY through-space interactions of **1**. Each arrow represents a medium to strong cross-peak in the 2D spectrum indicating that the corresponding protons are spatially close to each other.

1H-DETECTED ^{1}H,^{13}C-COSY. In a proton-carbon-13 correlated 2D spectrum, abbreviated as ^{1}H,^{13}C-COSY, one observes connectivities between ^{1}H and ^{13}C chemical shifts. The modulation of the ^{13}C magnetization by ^{1}H chemical shifts during the evolution period of the pulse sequence is mediated either by one-bond $^1J_{CH}$ couplings or by corresponding long-range couplings $^{2,3}J_{CH}$ over two or three bonds. Thus, if the assignment of one nuclear species is known, the assignment of the other is immediately obvious by cross-peaks in the 2D spectrum. Whereas the one-bond version mainly serves for the assignment of the protonated carbons, the long-range variant gives important structural

information on neighbouring carbons and protons separated by two or three bonds and, moreover, allows the safe assignment of the quaternary carbons. I have previously discussed the great potential of this technique when applied to carotenoids (ref. 16).

A significant disadvantage of the regular $^{1}H,^{13}C$-COSY, however, is the fact that the signal of the low-sensitivity nucleus ^{13}C is acquired and hence minimum sample quantities of several mg and relatively long acquisition times are needed; and this often precludes its application.

In 1975 Luciano Müller (ref. 17) proposed that, the high-sensitivity nucleus, the proton should be used as the reporter for the chemical shift of the spin-coupled, less sensitive carbon-13. This ^{1}H-detected, so-called reverse or inverse $^{1}H,^{13}C$-COSY experiment yields a considerable gain in sensitivity and hence sample quantities of less than 0.1 mg can be measured, although long acquisition times must be accepted under these extreme conditions. Note that in this experiment only the 1% of protons which are coupled to ^{13}C are actually detected so the much stronger signal of the 99% of protons that are attached to ^{12}C must be suppressed. As will be seen below, this severe problem has been solved by modern pulse techniques.

In Figure 9 is shown the 400 MHz ^{1}H-detected one-bond $^{1}H,^{13}C$-COSY spectrum of 75 µg of **1** (0.12 µmol; determined by UV / VIS assuming $A_1^1 = 2000$) in 0.3 ml $CDCl_3$ (0.4 mM) recorded in 36 h. Along the horizontal ω_2-axis, the 1D ^{1}H NMR spectrum is plotted. Each cross-peak in the 2D spectrum links a previously assigned proton to its attached carbon-13 and the assignment of the latter is therefore straightforward. The doublet splitting by ^{13}C along the ω_2-axis was removed by application of GARP decoupling during acquisition (ref. 18). Along the perpendicular ω_1-axis the projection of the cross-peaks onto this axis is plotted. Each signal reveals the chemical shift of a protonated carbon-13. Note that it would be extremely time consuming to record a normal 1D ^{13}C spectrum of such a small sample quantity (see example below).

Particularly informative are the ^{13}C shifts of the "in-chain" olefinic carbons C-8 to C-15 at 129.1, 137.7, 124.9, 132.4, 137.7 and 129.1 ppm, respectively, which are in excellent agreement with the shifts of other *retro* compounds such as rhodoxanthin (ref. 6). Moreover, the existence of two methylene groups at positions 2 and 4 (2' and 4') is indicated by two cross-peaks to each of these

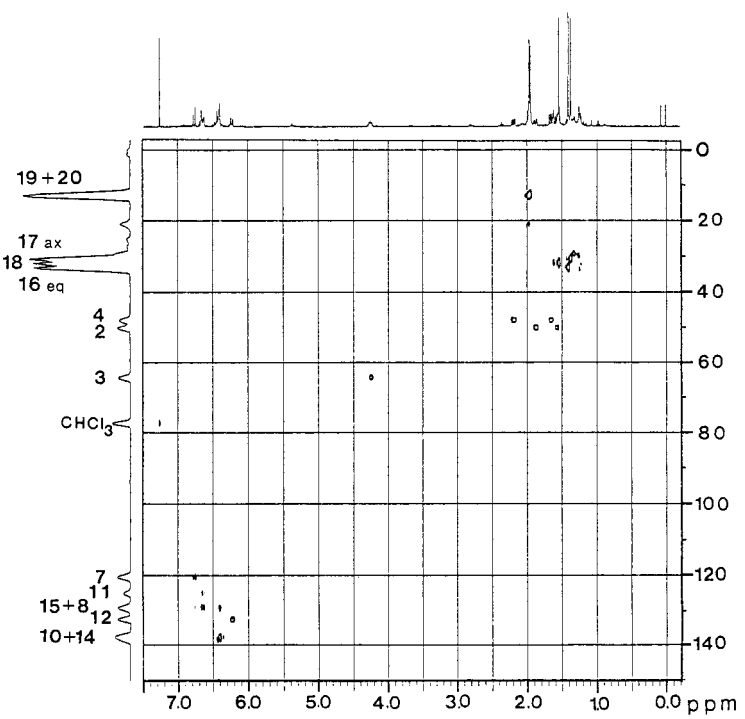

Fig. 9. Phase-sensitive proton-detected 400 MHz one-bond 2D $^{1}H,^{13}C$ COSY spectrum of ca. 75 µg (0.4 mM) of **1** in $CDCl_3$. Experimental conditions: 1500 x 300 experiments, zero-filling to 4K x 1K, 4000 and 20000 Hz spectral width in F_2 and F_1 , 3.9 and 39 Hz digital resolution, cosine-square filters, 1.2 s relaxation delay, 224 scans and 4 dummy scans per experiment, ^{13}C GARP decoupling during acquisition, ca. 36 h total acquisition time.

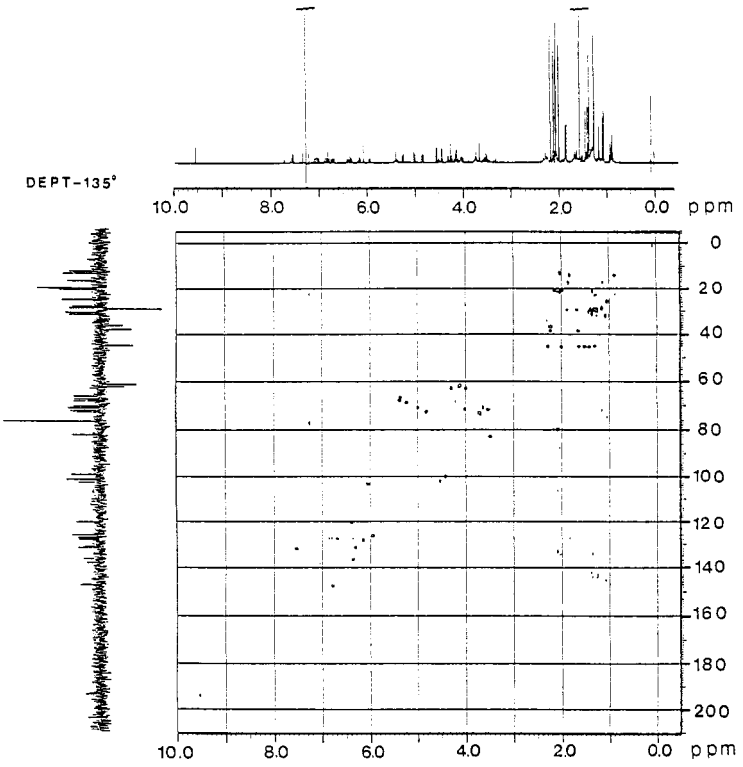

Fig. 10. Phase-sensitive proton-detected 400 MHz one-bond 2D $^{1}H,^{13}C$ COSY spectrum of ca. 0.2 mg (0.5 mM) of **2a** in $CDCl_3$. Top: ^{1}H NMR; Left: DEPT-135 ^{13}C spectrum (see text). Experimental conditions: 1500 x 500 experiments, zero-filling to 4K and 2K, 5556 and 22726 Hz spectral width, 2.7 and 22.2 Hz digital resolution, cosine-square filters, 1.2 s relaxation delay, 384 scans and 4 dummy scans per experiment, ^{13}C GARP decoupling during acquisition. The delay was tuned to 140 Hz. Total acquisition time 85 h.

carbons. It should be mentioned that the cross-peak intensities are proportional to the number of contributing protons. This means that a methyl cross-peak has at least three times the intensity of a methine cross-peak which is often additionally reduced in intensity by proton-proton splittings.

As a further example, Figure 10 shows the ^{1}H-detected one-bond $^{1}H,^{13}C$-COSY of ca. 0.2 mg of the heptaacetate **2a** (0.4 mM). Instead of the normal 1D ^{13}C spectrum, a DEPT-135 ^{13}C spectrum is plotted at the left margin (ref. 19). I have previously shown that this technique provides useful information for the elucidation of the structures of carotenoids (ref.16). In this experiment only protonated carbon signals are observed and the spectrum can be phased such that methylene carbons give negative, methine and methyl carbons positive signals. From an expanded plot, it can be deduced that **2a** possesses a total of 8 methylene groups, in agreement with the proposed structure. From the known assignments of the proton signals we obtain the complete assignment of the carbon signals, as will be shown elsewhere (ref. 7).

It is worth mentioning here that, owing to the very small sample quantity available, extremely long acquisition times of ca. 75 and 85 h had to be endured for the ^{13}C DEPT and the 2D spectra compared to a few minutes for the ^{1}H spectrum. Normally this can not be tolerated and this example, therefore, demonstrates the present limits of the technique. However, by using instruments operating at 500 or 600 MHz, a reduction of the measuring time by a factor of roughly 3 or 6, respectively, is feasible.

In Figure 11, a corresponding multiple-bond ^{1}H-detected $^{1}H,^{13}C$-COSY of compound **2b** is presented, which shows connectivities between protons and carbon-13 over two and three bonds. Less than 50% of the cross-peaks were designated here in order to avoid overcrowding. In the following, a few general remarks will be made on this method and only a few relevant cross-peaks can be specifically discussed .

First, it is obvious that, in this experiment, some artifactual ridges along the vertical ω_1-axis are unavoidable. They are, in general, much stronger than in the one-bond version and are positioned at the proton chemical shifts of strong and sharp methyl signals.

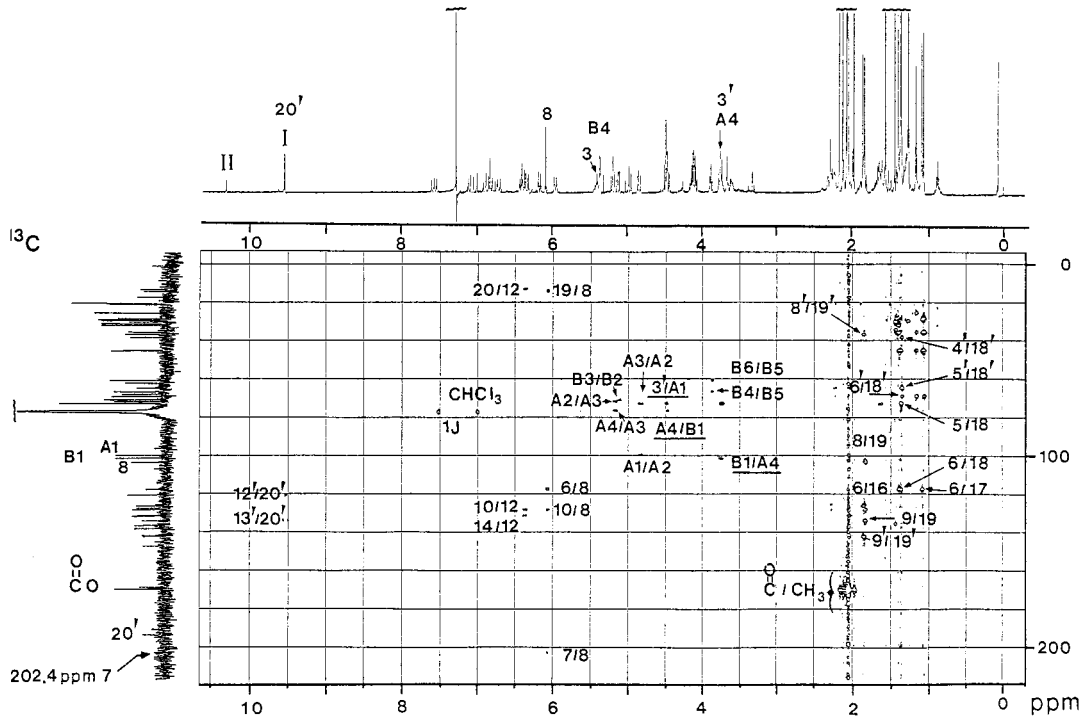

Fig. 11. Multiple-bond, proton-detected, 2D $^{1}H,^{13}C$ COSY (absolute value) of ca. 0.4 mg (0.9 mM) of **2b** in $CDCl_3$ .Top: 400 MHz ^{1}H NMR. Left: 100 MHz ^{13}C NMR, 38 h acquisition time. Experimental conditions: 3K x 388 experiments, zero-filling to 4K x 1K, 4504 and 22726 Hz spectral width, 2.2 and 22.2 Hz digital resolution, cosine-square filters in both directions, 352 scans and 4 dummy scans per experiment, 0.3 s acquisition time, 1.2 s relaxation delay. The delays were tuned to 139 Hz (3.6 ms) and 8 Hz (60 ms). Total measuring time 65 h.

Secondly, in the olefinic part of the spectrum two- and three-bond cross-peaks are observed, having about the same medium intensities. Typical connectivities are between C-13'/H-20', C-12'/H-20'; these confirm the position of the aldehyde group. A cross-peak of medium intensity between C-7 and proton 8 helps to determine the chemical shift of C-7 at 202.4 ppm, since this signal is not observable in the ^{13}C spectrum even though an acquisition time of 38 h was used. Relatively strong cross-peaks are also observed between protons of each methyl group and the carbons two and three bonds away. Examples are cross-peaks between C-4', C-5' and C-6' and H-18', between C-1, C-2 and C-6 and protons H-16, H-17, between C-8, C-9 and C-10 and protons H-19, etc. Not all of these cross-peaks are designated in Figure 11.

Thirdly, within the two carbohydrate moieties only two-bond cross-peaks have medium or strong intensities. This helps the assignment of a few, structurally relevant, cross-peaks in this spectral region which were underlined in Figure 11. These are inter-ring cross-peaks between carbons and protons A4 / B1 and B1 / A4 , thus confirming once more the linkage of the two rings A and B. The attachment of the lactoside to carbon 3' is supported by a cross-peak between carbon 3' and proton A1. Moreover, a cross-peak between carbon 8' and protons 19' is also in agreement with the proposed structure. All these results discussed so far strongly support the proposed structure of P457. We are convinced that only the question of the absolute stereochemistry of the epoxy end-group remains open for further discussion.

CONCLUSION

In my contribution to this conference, I have reported the application of a few novel pulse techniques for the elucidation of the structure of the *retro* carotenoid **1** and the glycosidic carotenoid P457 **2** and its acetylated derivatives **2a** and **2b**. Further experimental details will be presented elsewhere (ref. 7). It was shown that the rotating-frame experiments TOCSY and ROESY are of great use for the solution of structural problems. Moreover, the possibility to acquire ^{1}H-detected $^{1}H,^{13}C$-correlated 2D spectra with sample quantities of the order of a tenth of a milligram has greatly extended the applicability of NMR for the solution of structural problems in the field of carotenoids.

EXPERIMENTAL

All of the spectra were run on a 400 MHz AM-400 Bruker-Spectrospin FT spectrometer equipped with Aspect-3000 computer, 160 Mbyte disk and process controller. A dual and a reverse 400 MHz probe head were available. The selective pulses were generated by a DANTE pulse train (ref. 20). The spin-locking and reverse experiments were performed in the reverse mode, with the decoupler as a source for ^1H excitation. The sample quantities were determined by UV/VIS spectroscopy. Further experimental details, the description of the applied pulse sequences and all references to the pertinent literature are given in ref. 12.

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