PROTON AND CARBON-13 NMR OF GINKGOLIDES

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Abstract : Complete proton and 13C NMR data sets are presented for the
main ginkgolides isolated from Ginkgo biloba. Due to their unique cage
structure, assignment of the complete spectra requires sophisticated
approaches since the spectral properties can be strongly affected by
local strains. A combination of one and two-dimensional analysis was
used, homo- and heteronuclear correlation experiments providing a very
efficient means of achieving a complete description of the structural
and conformational properties in this series. The resulting database
can be used for direct correlation of biological activities with
structural or conformational properties in solution.

Ginkgolides, terpenoids isolated from Ginkgo biloba, are known as specific antagonists of
platelet-activating factor (PAF), an autacoid mediator implicated in a diverse range of
pathological conditions.[1] Ginkgolides represent a challenge for the organic chemist due to
their unique cage structure and their potent biological activity. The high variability of
this activity across the complete ginkgolide series constitutes a unique system for esta-
blishment of structure-activity relation ships since the basic molecular backbone is iden-
tical in all ginkgolides. Thus the observed variations of activity should only be due to
subtle structural or conformational changes. In many cases, NMR has been found to be
powerful tool for structural and conformational analysis of bioactive molecules.[2] Our
purpose was thus to perform an extensive and complete NMR analysis of this series in order
to elucidate the basic molecular origins of their activity. Owing to the large number of
unprotonated carbon atoms in ginkgolides, proton-NMR alone is not sufficient to fully
characterize these compounds, therefore we also employed carbon-13 (^{13}C) NMR. The complete
assignment of all protons and carbons requires the use of sophisticated approaches such as
those described here.

RESULTS AND DISCUSSION

The four ginkgolides under study are presented in Figure 1 and henceforth will be
referred to as ginkgolides A,B,C and J. They were obtained from the Institut Henri Beaufour
(France) and used without further purification.

Proton NMR experiments

Since these molecules contain a limited number of protons, they yield very simple
proton NMR spectra, especially at a high magnetic field. In some cases, however, the
complete assignment is not unequivocal and specific techniques are required. This
limitation has precluded a complete analysis in previous works performed at much lower
fields.[3][4] Since the various ginkgolides differ only in the number and position of
substituents, it is expected that proton NMR could yield a very specific fingerprint
for these compounds. The complete proton NMR spectra of the four ginkgolides are shown
in Figure 2.

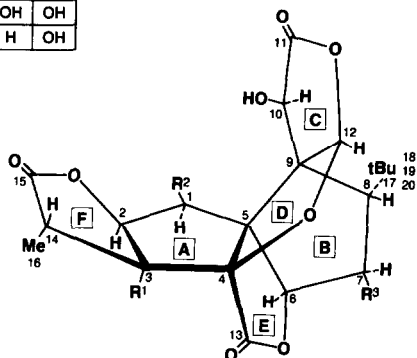| Ginkgolide	IHB nomenclature	R_1	R_2	R_3
A	BN 52020	OH	H	H
B	BN 52021	OH	OH	H
C	BN 52022	OH	OH	OH
J	BN 52024	OH	H	OH

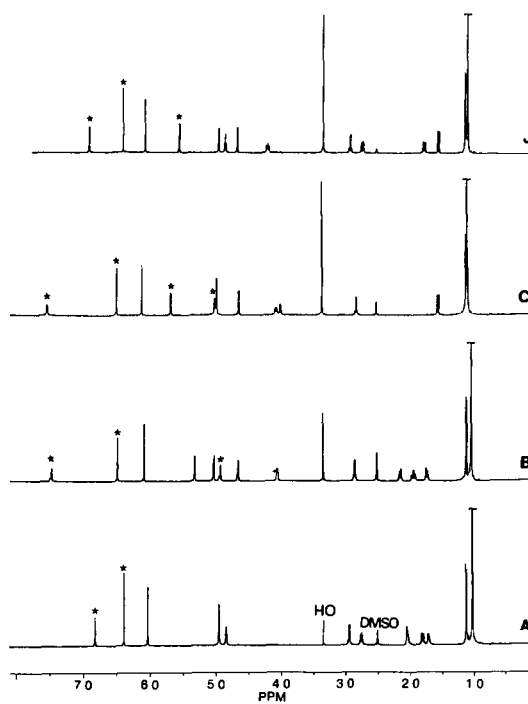Figure 1 : Structure and atom numbering of the ginkgolides.

Figure 2 : 500 MHz proton NMR spectra of the ginkgolides in DMSO-d6 at 300° K. Peaks labelled by stars indicate labile protons from the hydroxyl groups wh disappear upon addition of D_2O.

The spectral assignment can be easily initiated from the identification of labile hydroxyl protons which can be exchanged to deuterons by addition of a very small amount of D_2O and are indicated by stars on the spectra. This exchange also simplifies some of the multiplets in the spectrum due to the suppression of scalar couplings.

Since most of the signals appear as multiplets with the exception of H-12, OH-3 and the t-butyl group, a complete neighbour analysis could be performed using two dimensional (2D) experiments which give complete separation of chemical shifts and homonuclear coupling by connectivity determination.

COSY experiments[5] yield an assignment of most protons, but an uncertainty still remains concerning the H-6 - H-7α - H-7β - H-8 systems since the coupling constant between H6 and one of the H7 protons is very close to zero. Using the multiplet analysis only, several assignments are possible and all yield different sets of chemical shifts and coupling constants. To overcome this difficulty we used a pulse sequence version (COSY 45)[6] which is potentially capable of indicating the relative signs of the coupling constants connecting different protons. Since it is well known that the vicinal coupling constants are positive and that the geminal ones are negative, the assignment of the protons H-7α, H-7β and H-8 becomes unequivocal. We will show later that heteronuclear correlation experiments confirm this assignment.
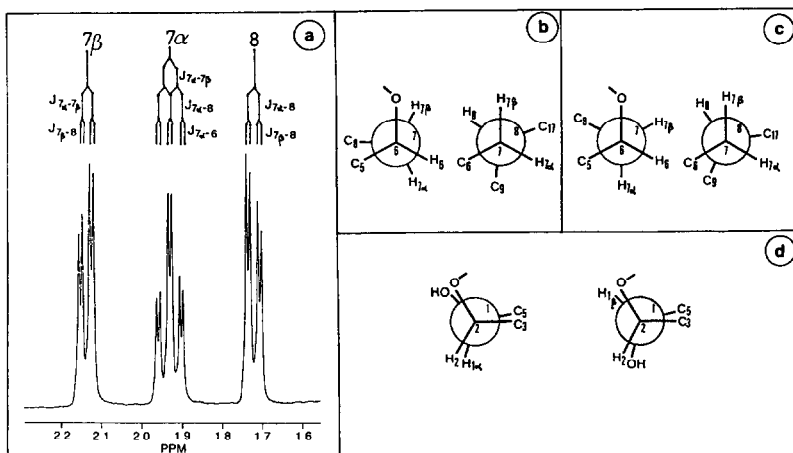

Figure 3 : a) Partial spectrum of ginkgolide B showing the complete multiplet analysis.
b) Ring B conformation in ginkgolides B,C and J.
c) Ring B conformation for ginkgolide A.
d) Two possible conformations for the C2-C1 bond in ginkgolides B and C corresponding to OH-1 in H-1α or H-1β position. In both cases, the torsion angles (0° or 120°) yield identical J1-2.

- Conformation of ring A and orientation of the OH-1 group.

A more complex situation is encountered concerning the orientation of the OH-1 group of ring A in ginkgolides B and C and is still a matter of controversy. Spin coupling analysis around the C-2 - C-1 bond should be expected to solve this problem. However, as shown in Figure 3d, the two possible solutions yield 0° or 120° torsion angles between H-2 and H-1 and both situations correspond to approximately the same coupling constant if a classical Karplus law is considered.

Attempting to overcome this problem, saturation transfer experiments were carried out. Thus, relations between the exchangeable protons can be rationalized. If a given labile proton is saturated by irradiation it will disappear from the spectrum. Moreover, if this

proton further exchanges with other labile protons and if the spin-lattice relaxation time
is larger than the exchange rate, saturated spins can be transferred from one site to the
other. This is visualized as a decrease in the intensity of the other sites as one of them
is saturated. It should be stressed that this phenomenon can be attributed to the nOe
effect due to through-space dipolar transfer of magnetization. In the present case this
by-effect is negligibly small since, at room temperature and 500 MHz, nOe effects are very
close to zero for this type of molecule.

Using this technique of two dimensional correlation all protons can be safely assigned
for all ginkgolides. The relevant coupling constants can be derived from multiplet analysis
as shown in Figure 3a for the H-6 - H-7α - H-7β - H-8 system of ginkgolide B. The complete
data for proton NMR are presented in Table 1.

Ginkgolide	A	B	C	J
H-1 alpha	1.80	——	——	1 77
H-1 beta	2 75	4 04	3 98	2.72
H-2	4 83	4 61	4 62	4 83
H-6	4 92	5 30	4 96	4 64
H-7 alpha	2 05	1.93	4 04	4.16
H-7 beta	2.05	2 14	——	——
H-8	1.72	1.72	1.54	1 54
H-10 alpha	4.92	5.02	4.99	4.94
H-12	6.02	6.07	6.10	6.03
H-14	2.93	2.84	2 81	2 85
t-Bu	1.02	1.03	1 09	1 09
H-16	1.18	1.11	1 10	1 05
OH-3	6.38	6.47	6.48	6.40
OH-10	6 81	7.47	7 54	6 90
OH-1	——	4.92	4.97	——
OH-7	—	——	5 66	5 53
Coupling constants (Hz)				
J 1-1	—15.2	——	——	—15 2
J 2-1	8 3	——	——	7 8
J 2-1	7 2	7.4	7 3	8 2
J 6-7	2.5	4 1	4 2	4 1
J 6-7	2.5	0	——	——
J 7-7	—13.0	—14 0	——	——
J 7-8	11 0	14 6	12 4	12 3
J 7-8	8 0	4 7	——	——
J 14-16	7 2	7 0	7.1	7.1

Table 1 : Chemical shifts and coupling constants of protons

- Conformation of the ginkgolides.

The coupling constant derived from proton NMR analysis can be used to determine the ring
conformations and eventually to derive the orientation of the OH groups relative to these
rings.

- Conformation of ring B.

A complete set of coupling constants is available for bonds C-6 - C-7 and C-7 - C-8 for
all ginkgolides and allows a full determination of the torsion angles of these sites. As
shown in Figure 3b and 3c, the same conformation was found for ring B in ginkgolides B, C
and J and a slightly distorted form was encountered in ginkgolide A. The beta orientation
of the OH group at C-7 in ginkgolides C and J is unequivocally determined by this analysis.

For ginkgolides A and J, no saturation transfer could be observed between hydroxyl
protons whatever the location or duration of the saturation pulse. Conversely, for
ginkgolides B and C, strong transfer could be observed between OH-1 and OH-10 as shown

in Figure 4. This strongly supports the β orientation of OH-1 creating a very specific interaction between these two groups.
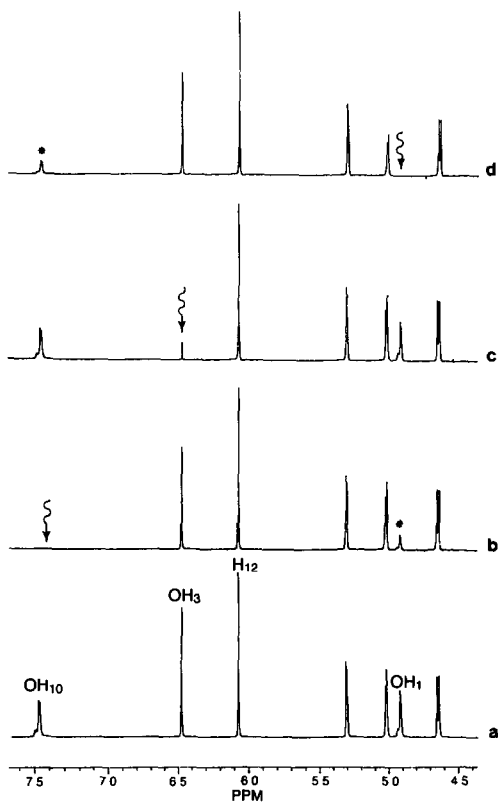

Figure 4 : Saturation transfer experiments for ginkgolide B in DMSO.
      a) Reference spectrum with assignments.
      b) Saturation of OH-10 and transfer to OH-1.
      c) Saturation of OH-3 showing no transfer.
      d) Saturation of OH-1 and transfer to OH-10.
      All experiments were performed by 0.5 sec. low power saturation at the frequency indicated by an arrow. The * symbol indicates signal saturated by transfer.

This is further supported by the chromatographic behaviour of ginkgolide B which appears as the least polar compound, while being the most hydroxylated ; this strange behaviour can be explained if OH-1 is buried in the cage of the molecule.

Carbon-13 NMR experiments

The ginkgolide molecules contain a very large number of non-protonated carbons and their cage structures induce very important strains which may severely affect the NMR parameters.

The proton-decoupled ^{13}C spectra of the four ginkgolides in DMSO are shown in Figure 5. The multiplicity information can be derived from a number of techniques, among which the DEPT approach[7] is commonly used as its pulse sequence is short and it is relatively insensitive to variations in the value of ^{13}C-^{1}H coupling constants.
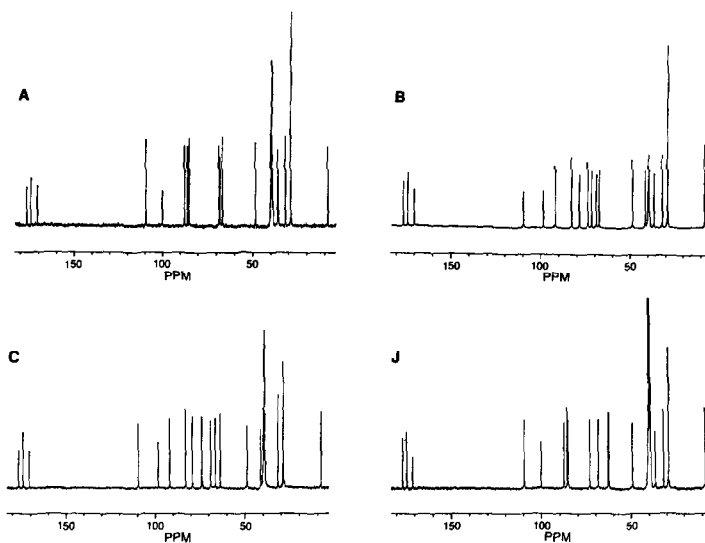
Figure 5 : Complete proton decoupled C-13 spectra of the four ginkgolides A,B,C and J in
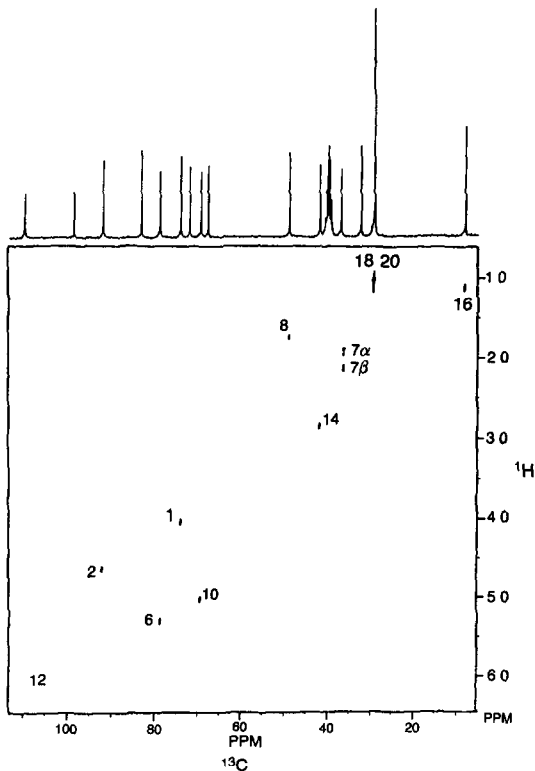DMSO-d6 at 75.47 MHz. The septuplet signal at 39.6 ppm arises from DMSO-d6.

Figure 6 : Heteronuclear proton-carbon shift correlation using direct H-C couplings. The horizontal (F2) dimension contains the ^{13}C chemical shifts only (quaternary carbons suppressed) and the vertical dimension (F1) the chemical shifts of connected protons.

The connection of the protonated carbons to their respective protons was achieved by a heteronuclear correlation experiment. The experiment relies on specific magnetization transfer between a proton and the bond carbon via the coupling constant.[8] Figure 6 shows a typical heteronuclear correlation experiment and allows an accurate determination of proton-carbon pairs.

The present version of the pulse sequence[9] is designed to cancel vicinal proton-proton couplings and the ^{1}H projection spectrum contains only chemical shift for simplification and sensitivity enhancement. However, this is not true for geminal couplings and explains why the protons bound to the single CH_2 (C-7) appear as a quadruplet due to inequivalence and non-suppression of their mutual coupling. This further confirms the previous assignment of H-7α and H-7β protons from COSY 45.
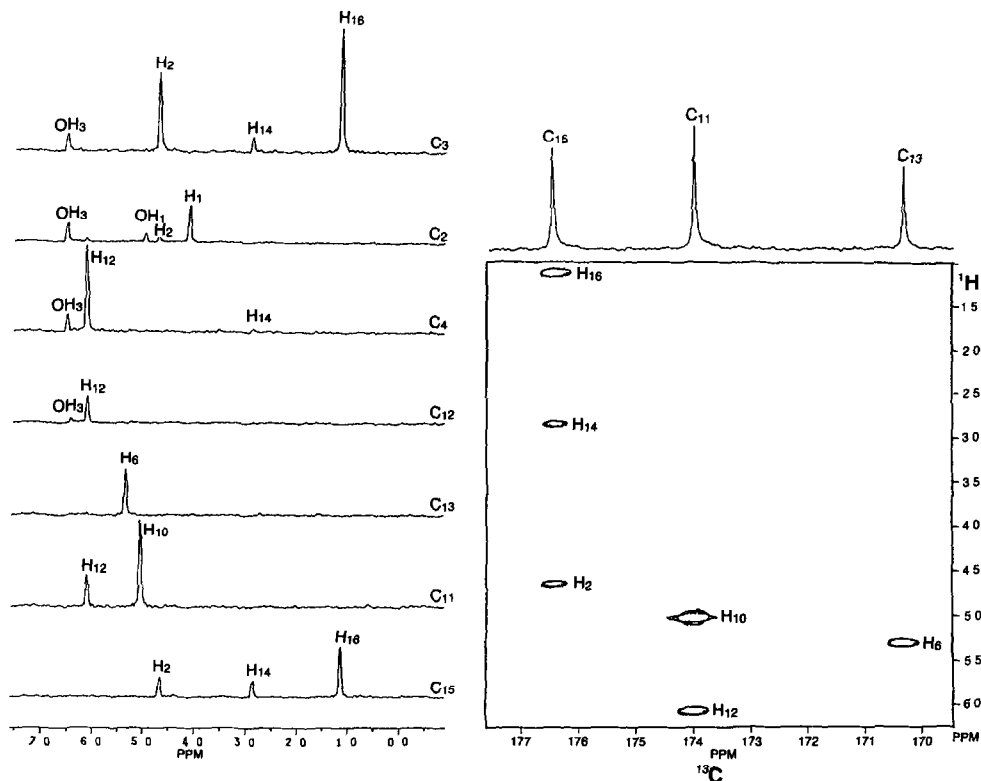

Figure 7 :   Heteronuclear H-C correlation via long-range couplings (COLOC).

The neighbour determination for quaternary carbons was achieved using the COLOC experiment[10] using polarization transfer through the small long range coupling between any carbons and remote protons from neighbouring carbons. This is illustrated in Figure 7 (left panel) by a contour plot for ginkgolide B and the several relevant sections taken through this plot to show how each carbon atom is coupled with remote protons and thus can be assigned (Fig. 7, right panel). The assignment of the three carbonyl carbons is very representative. For all other carbons, the assignments are not always completely

unequivocal since different carbons can experience long-range couplings with the same protons.
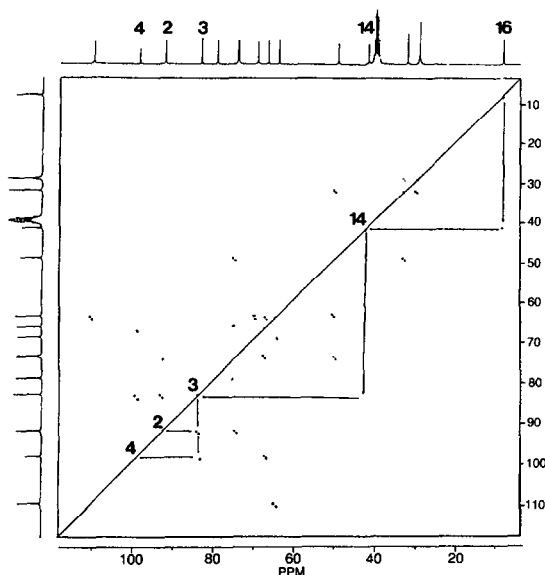

Figure 8 : Carbon correlation via double quantum coherences (INADEQUATE) showing a complete analysis of the carbon skeleton starting from t-butyl group.

The most comprehensive method used to assign all carbons in one step and to confirm our previous assignment was a bidimensional $^{13}C-^{13}C$ homonuclear correlation experiment (^{13}C-INADEQUATE) at natural abundance. This two quanta experiment[11] is highly insensitive, requires large sample concentration (in the 0.2 - 0.4 M range) and long experimental periods (24-48 h). A complete INADEQUATE contour plot for ginkgolide B is shown in Fig. 8 and illustrates the usefulness of this experiment. The interpretation is very

Ginkgolide	A	B	C	J
C-1	36 01	73 87	73 65	36 56
C-2	85 20	91 68	91 93	87 72
C-3	68.14	83 01	82 92	86 05
C-4	100 33	98 55	98 25	100 06
C-5	86 14	71.79	66 42	63 16
C-6	87.77	78.68	79 05	85.87
C-7	36.39	36.71	74 00	73.42
C-8	48.65	48 65	48.99	49 29
C-9	66.90	67 52	63.76	62.55
C-10	68 91	69.16	68.98	68 63
C-11	174.37	174 01	173.90	174 31
C-12	109.57	109.72	109.52	109.47
C-13	170 84	170 35	170 57	171 06
C-14	40.50	41.62	41.56	40.51
C-15	176.64	176.47	176 40	176 62
C-16	8 24	7 93	7.93	8 26
C-17				
C-18, 19, 20	28 95	28 96	28 95	28 99

Table 2 : ^{13}C Chemical shifts

simple since if one single carbon has been assigned, the complete carbon skeleton can be determined. All these experiments contribute to full assignment of the ^{13}C NMR spectra of the ginkgolides and the relevant data are shown in Table 2.

The analysis of these spectra shows considerable differences in the carbon chemical shifts in the different compounds. This makes significant conformational differences between the various ginkgolides conspicuous. The very small structural differences can thus induce large conformational variations which are evidenced by high variation in biological activity.

EXPERIMENTAL

All NMR experiments were performed on solutions in d6-DMSO (CEA, France) the final concentration being 20 mM and 250 mM for proton and carbon-13 NMR respectively. In all cases the sample temperature was regulated to 300 K and chemical shifts are given relative to d6-DMSO : δ (DMSO) = δ (TMS) + 2.5 ppm for proton NMR, δ (DMSO) = δ (TMS) + 39.6 ppm for ^{13}C-NMR.

Proton NMR experiments were performed at 500 MHz using a BRUKER WM 500 spectrometer. Carbon-13 measurements were carried out on a BRUKER MSL 300 spectrometer operating at 75.47 MHz for ^{13}C. Both systems were equiped with an Aspect 3000 computer and a process controller. Unless otherwise specified, all experiments were performed using standard parameters.

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