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Confirmation of the C-1 Stereochemistry of Ginkgolides by NMR

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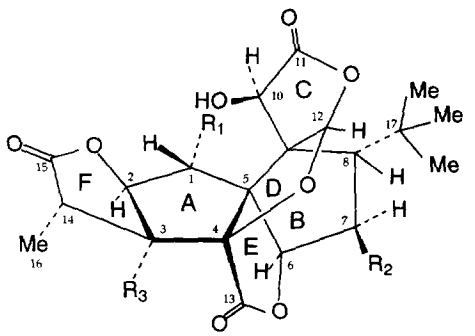
Abstract: Unambiguous evidence was obtained for the assignment of the relative stereochemistry at C-1 in ginkgolide B and C by means of ROESY NMR spectroscopy carried out at 600 MHz, chemical shift data and a conformational analysis. The 1-hydroxyl group was found to possess the α-configuration which is in accordance with earlier X-ray crystallographic and synthetic studies but in conflict with a previous detailed NMR study. In the process the ^{1}H-NMR assignments of H-1α and β in ginkgolide A were reversed and some ^{13}C-NMR assignments for ginkgolide A were corrected.

INTRODUCTION

Ginkgolides are unique, highly oxidized diterpene trilactones from the leaves and root bark from *Ginkgo biloba* (maidenhair tree). Leaf extracts from this tree are currently among the best-selling drugs in France and Germany. Ginkgolides which are potent and selective Platelet-Activating-Factor antagonists are considered to be at least partially responsible for the beneficial properties of these extracts[1]. The structures of the ginkgolides A, B and C (further abbreviated as G-A **1**, G-B **2** and G-C **3**) were independently elucidated by two Japanese groups in the sixties. Nakanishi *et al.* used a combination of chemistry and spectroscopy[2-6] while the other group relied on an X-ray study of ginkgolide A, the least substituted ginkgolide[7].

However, the relative stereochemistry at carbon atom 1 was not resolved at that time and has remained a point of some confusion up to the present day. Okabe *et al.* already remarked that the relative configuration at

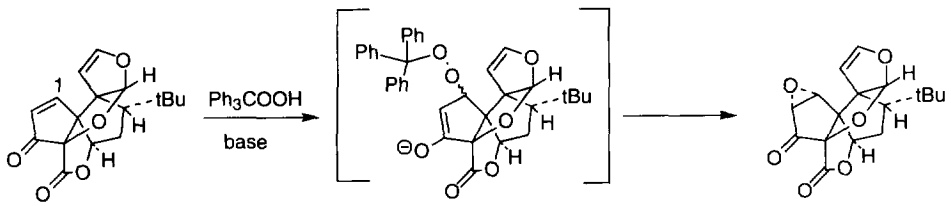


1	Ginkgolide A	$R_1 = R_2 = H$, $R_3 = OH$
2	Ginkgolide B	$R_1 = R_3 = OH$, $R_2 = H$
3	Ginkgolide C	$R_1 = R_2 = R_3 = OH$
4	Ginkgolide J	$R_1 = H$, $R_2 = R_3 = OH$
5	Ginkgolide M	$R_1 = R_2 = OH$, $R_3 = H$

C-1 in G-B and G-C was difficult to determine from coupling constants and chemical shifts. They assumed that the hydroxyl group at C-1 possessed the β-configuration because of the easy dehydration of ginkgolide B with acetic anhydride-sodium acetate (H-2α and OH-1 trans to each other)[7]. In contrast, Nakanishi *et al.* concluded that the hydroxyl group possessed the α-configuration because of the considerable deshielding experienced by H-6 when a hydroxyl group was introduced at C-1: chemical shift of H-6 in G-A, G-B and G-C 5.04, 5.73 and 5.60 ppm respectively. Their molecular models showed that only a 1α-OH is in the same plane as that of H-6[6]. Later Weinges *et al.*[8] arrived at the same conclusion as Nakanishi. They found in the ^{1}H NMR spectrum the signal for H-10 in both G-A **1** and G-J **4** at 4.92 ppm and in G-B **2** and G-C **3** at 5.01 and 4.97 ppm. They considered this evidence for a spatial anisotropic effect between a 1α-OH and H-10. Additional evidence for this anisotropic effect was obtained from the ^{13}C-NMR spectrum: C-10 resonated in G-A and G-J at 88.23 and 88.50 ppm respectively and in G-B and G-C at 92.22 and 92.45 ppm[8]. Around the same time X-ray crystallographic studies of G-B and G-C were performed by the group of Braquet[9-11]. For both compounds they found the α-configuration for the 1-OH group. This would have seemed the end of the discussion were it not for two other publications by the same group. In these detailed ^{1}H and ^{13}C NMR studies, where specific attention was given to the relative stereochemistry at C-1, it was concluded that the 1-OH group possessed the (opposite) β-configuration[12, 13].

Meanwhile (±)-G-B has been synthesized[14]. The step where the stereochemistry of what later is to become the 1-hydroxyl group in G-B is introduced, is the enone epoxidation of intermediate **6** with triphenylmethyl hydroperoxide (5 equiv.) and benzyltrimethyl-ammonium isopropoxide (0.5 equiv.) in THF at −10° for 3 h. After reduction of excess hydroperoxide by trimethyl phosphite (10 equiv.) and column chromatography on silica peroxide **8** was isolated in 72% yield[14]. Models of the α and β isomers of the hypothetical intermediate **7** showed that an attack by the bulky triphenylmethyl hydroperoxide from the more hindered β side is unlikely. This stereochemically selective step in the total synthesis of G-B, and the fact that the synthesized (±)-G-B was identical (TLC, NMR IR, MS) to natural G-B, supports the stereochemistry at C-1 indicated by the X-ray study.

In this paper we wish to report on a new NMR study of G-A, G-B and G-C in order to solve the apparent confusion on the C-1 stereochemistry of G-B and G-C introduced by reference 13. Additionally, such knowledge might be useful for receptor-bindings studies on the PAF-antagonistic effect of ginkgolides.



6 **7** **8**

Scheme 1. Stereoselective enone epoxidation step in the synthesis of ginkgolide B which eventually leads to the α stereochemistry of the later 1-hydroxyl group[14].

RESULTS AND DISCUSSION

The first step was the confirmation of ^{1}H and ^{13}C NMR assignments published earlier. This was done by straightforward interpretation of newly recorded 600 MHz ^{1}H, ^{13}C and ^{1}H – ^{13}C correlated NMR spectra of G-A, G-B and G-C. A solvent was chosen which allowed for a total resolution of all signals in all ginkgolides.

Previous NMR studies, mostly in DMSO-d6 suffered even at 500 MHz from signal overlap, especially of H-6/H-10 and H-7α/H-7β in G-A. Acetone-d6 gave essentially first-order spectra at 600 MHz for all ginkgolides. For the observation of the hydroxyl signals the solvent had to be thorougly dried over molecular sieves prior to dissolution of the sample. The assignments for G-B and G-C were fully identical with all earlier published assignments. For G-A three different assignments of its ^{13}C NMR spectrum have been published[8, 13, 15]. Our interpretation of the spectrum is identical with the one by Llabres *et al.*[15] (see also experimental). At this stage the spectra did not allow a positive distinction between H-1α and H-1β which is important for the determination of the relative configuration of C-1 in G-B **2** and G-C **3**.

The discrimination between the NMR signals of H-1α and H-1β in ginkgolide A is not an easy task. It was realized, that coupling constants and Nuclear Overhauser effects may provide useful information for the discrimination between the two H-1 signals. However, the interpretation of such data requires precise *a priori* knowledge of the conformation of the molecule, the more so, as the experimental coupling constants of the H-1 protons to H-2 have similar magnitudes. Such information is available from the X-ray structure[10], and can also be obtained from Molecular Mechanics calculations (MM2)[16].

In a first attempt to establish the correct assignment of the two H-1 signals, the coordinates of the X-ray structure of G-A were taken from the work by Sbit *et al.*[10], and the energy was minimized, using MM2. The minimization converged rapidly, and the final coordinates were used to predict the vicinal coupling constants between the two H-1 protons and H-2, using the generalized Karplus equation[17]. These predicted coupling constants are shown in the second row of Table 1. When compared to the experimental coupling constants (first row of Table 1) it is obvious, that neither of the two possible assignments resulted in a satisfying fit between the X-ray structure derived coupling constants and the experimental values. The extremely large difference of 1.4 Hz versus 7.3 or 8.9 Hz is a strong indication that the conformation of the five-membered ring C1-C2-C3-C4-C5 (ring A) in solution differs from the solid state conformation. Therefore, the driver option of the MM2 program was used to search for another low-energy conformation that gave a better fit of experimental and predicted coupling constants.

Indeed, such a conformation was found, and the predicted coupling constants for this conformation are presented in the third row of Table 1. It can be seen, that the fit has improved dramatically, when the low-field H-1 signal is assigned to H-1α. Further evidence in favor of this conformation came from the MM2 steric energy, which is significantly lower in the presently derived conformation, than in the X-ray conformation (Table 1). In Table 2 the conformational characteristics of the A-ring and the adjacent F-ring (C2-C3-C14-C15-O) are summarized.

Table 1. Observed and predicted coupling constants for ginkgolide A

	E (MM2) kJ/mol	H-1α/ H-2	H-1β/ H-2	H-7α/ H-6	H-7β/ H-6	H-7α/ H-8	H-7β/ H-8
experimental		7.3	8.9	4.2	0.8	14.1	4.9
calculated (X-ray)	287	8.1	1.4	5.0	1.5	12.3	3.9
calculated (MM2)	275	7.3	9.5	5.2	1.4	12.3	4.0

Finally, strong evidence for the proposed conformation of G-A, and the currently proposed assignment of H-1α and H-β was obtained from the observed NOEs in a ROESY experiment[18]. In Figure 1 the relevant portion of the ROESY spectrum of G-A is presented. In this figure a strong NOE can be observed between H-14 and the high-field H-1, and no NOE between H-14 and the low-field H-1 proton at 2.8 ppm. The

Table 2. Conformational characteristics of the A-ring and F-ring in the solid state and in solution

	ring F	ring A
X-ray	C_3endo	C_3exo
NMR	C_{14}endo, C3exo	C_1endo, C_2exo

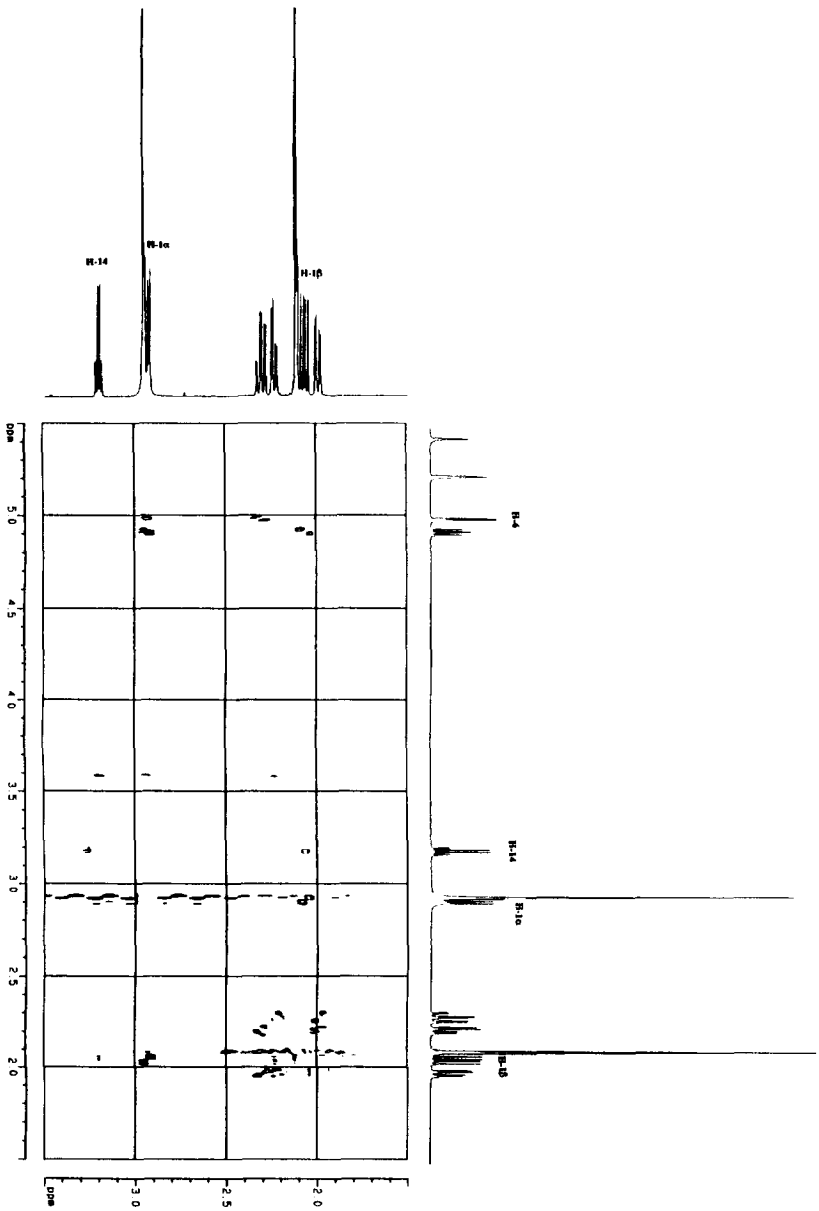


Figure 1. Part of the ROESY NMR spectrum of ginkgolide A.

Table 3. Proton-proton distances (d) in the solid state conformation, and in the proposed solution conformation. Both sets of distances were obtained after MM2 minimization. Last column: observed NOEs for ginkgolide A in acetone-d6 solution

	d (Å) solid state	d (Å) solution	NOE observed
H-1α/H-6	2.2	2.4	+
H-1β/H-6	3.5	3.7	–
H-1α/H-14	5.1	4.1	–
H-1β/H-14	5.0	2.5	+

Table 4. ^{1}H-NMR data of ginkgolides A, B and C in acetone-d6 (= 2.11 ppm)

	Chemical shift (δ) in ppm		
proton	G-A	G-B	G-C
H-1α	2.92	—	—
H-1β	2.06	4.29	4.24
OH-1	—	n.o.	4.80
H-2	4.94	4.73	4.72
OH-3	5.30	5.54	n.o.
H-6	5.00	5.44	5.17
H-7α	2.30	2.18	4.37
H-7β	2.24	2.32	—
H-8	2.00	2.01	1.86
H-10	5.23	5.33	5.34
OH-10	6.11	n.o.	n.o.
H-12	6.12	6.17	6.19
H-14	3.20	3.09	3.07
H-16 (Me)	1.32	1.31	1.33
t-Bu	1.21	1.22	1.31
	Coupling constants (J) in Hz		
J (1α-1β)	−15.2	—	—
J (1α-2)	7.3	—	—
J (1β-2)	8.9	8.0	7.2
J (6-7α)	4.2	4.3	4.2
J (6-7β)	0. 8	0.3	—
J (7α-7β)	−13.6	−14.0	—
J (7α-8)	14.1	14.2	12.4
J (7β-8)	4.9	4.8	—
J (14-16)	7.2	7.1	7.2

Abbreviations: — = does not exist, n.o. = not observed.

corresponding proton-proton distances in the X-ray derived conformation are collected in Table 3. It is immediately clear from this table, that the distances H-1α/H-14 and H-1β/H-14 in the solid state conformation are large and have equal magnitudes. These distances can not explain the observation of a large NOE between H-14 and one of the two H-1 protons, irrespective of the assignment of the two H-1 protons. On the other hand, the proposed conformation in solution has proton-proton distances, that match very well the observed NOEs of both H-1 protons to H-14, as well as to H-6, as long as the low-field H-1 signal is assigned to H-1α.

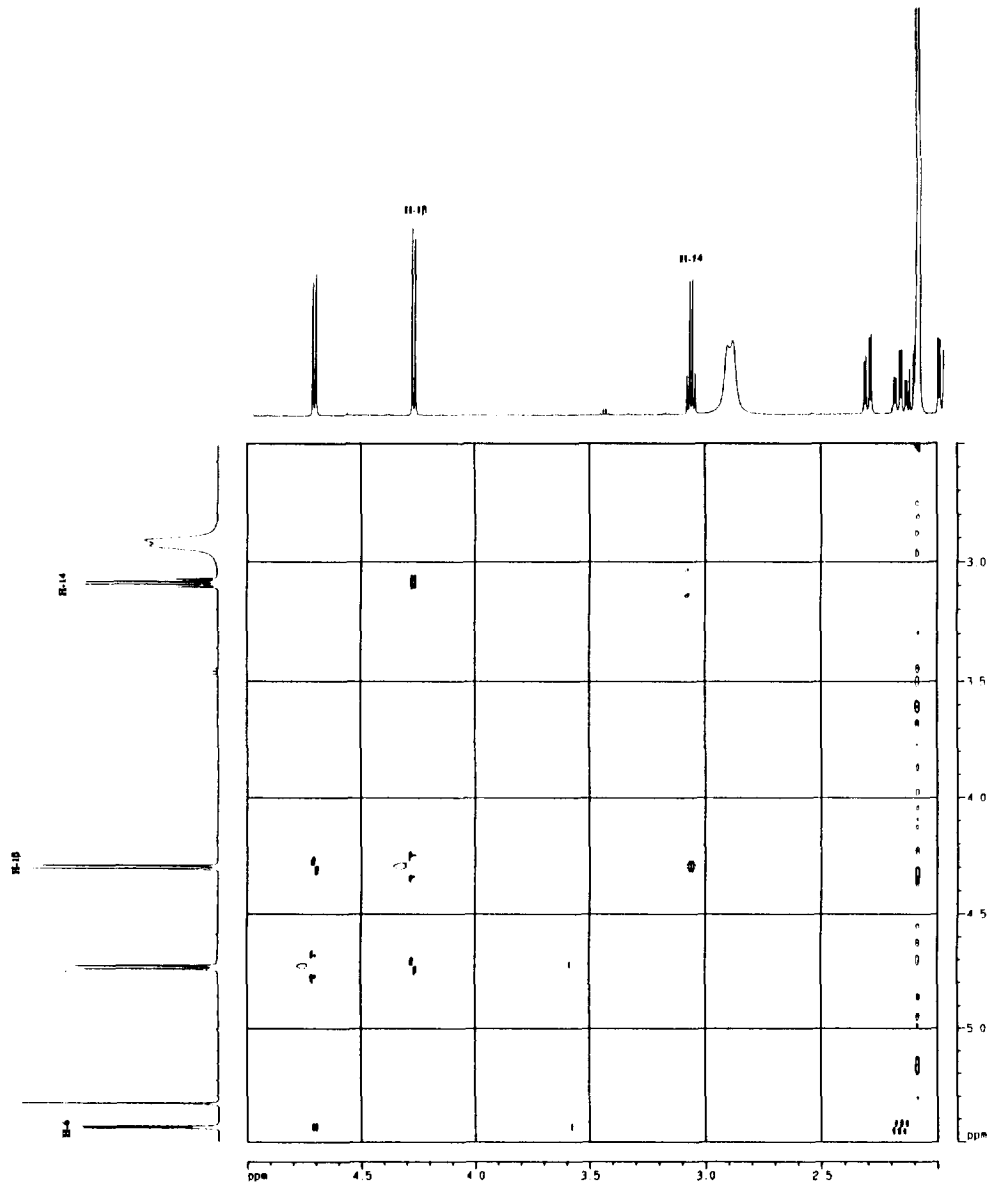


Figure 2. Part of the ROESY NMR spectrum of ginkgolide B.

We are aware that this is in contrast with all other NMR studies of G-A[6-8, 13, 15]. However, apart from Okabe *et al.* nobody has ever made a remark on the assignment of these two protons and to our knowledge this has never been investigated in detail. The complete assignment of the G-A spectrum together with all coupling constants is given in Table 4. Because the spectrum is essentially first order for the first time the coupling constants between H-6, H-7α, H-7β and H-8 could be accurately measured. They deviate significantly from values so far published[8, 13, 15]. The spectra of G-B and G-C posed less problems and our shifts and coupling constants are similar to values published earlier.

For the unambiguous determination of the relative stereochemistry at C-1 in G-B and G-C on the basis of NMR data a 3 pronged approach was used: (1) NOE data obtained from ROESY spectra of G-B and G-C, (2) chemical shift data, and (3) comparison of experimentally derived coupling constants with values predicted by molecular modelling. The ROESY spectrum of G-B (for the relevant part of this spectrum see Figure 2) showed an intense cross peak between H-1 and H-14. In analogy with the NOE's observed for G-A where H-1α did not and H-1β did show a cross peak with H-14, it is concluded that the hydroxyl group at C-1 must have the α-configuration. Confirmation for this configuration is found in the absence of an NOE between H-1 and H-6. Also in G-A only H-1α shows an NOE to H-6. The ROESY spectrum recorded for G-C (not shown) gave exactly similar results so it is concluded that the 1-OH group in G-C has the same relative configuration as in G-B.

Secondly, we have looked at chemical shift evidence. When methylene groups are substituted with a hydroxyl the remaining hydrogen experiences a considerable downfield shift. For instance, in the pairs cyclopentane/cyclopentanol the substituent effect is 2.64 ppm in DMSO and 2.72 ppm in acetone. In the pair cyclohexane/cyclohexanol these numbers are 2.02 ppm and 2.08 ppm respectively. In G-A there are apart from C-1 two other methylene carbons which can be substituted by a hydroxyl, i.e. at C-7 and C-10. In the case of C-7 the substituent parameters can be calculated by substracting the chemical shift of H-7α in G-A and G-B from the shift value of the identical proton in G-C, G-J and G-M. The latter three compounds all possess a hydroxyl at the 7β position. In the case of C-10 the chemical shifts of H-10α in G-A, G-B, G-C, G-J and G-M

Table 5. Substituent effects in ginkgolides for a methylene to secondary alcohol conversion*. Shift (δ) values in ppm.

Proton	Ginkgolide	Mean δ	Ginkgolide	Mean δ	Substituent effect
H-7α	A	2.03	C	4.06	
	B	1.93	J	4.18	
			M	4.02	
Average	A and B	1.98	C, J and M	4.08	2.10 ppm
H-10α	A	4.91	Ginkgolide	2.83 or 3.12**	
	B	5.01	skeleton		
	C	4.98			
	J	4.93			
	M	4.95			
Average	all	4.96			2.13 or 1.84 ppm

* All values used[8, 13, 15, 19] have been recorded in DMSO-d6.

** No assignment of H-10α and H-10β has been given in the original publication[19].

were averaged. These compounds all possess a hydroxyl at C-10 and the average was compared to the shift of H-10α of a recently synthesized ginkgolide derivative lacking this hydroxyl group. The results are summarised in Table 5. If the difference in chemical shift between H-1α in G-A and H-1α in G-B – assuming a β-configuration for the hydroxyl group – is calculated a value of 4.25 – 2.90 = 1.35 ppm is found which is unusually low. When the same calculation is carried out with the hydroxyl group in the α-position a value of 4.25 – 2.10 = 2.15 ppm is found which gives a much better fit with the substituent parameter for secondary alcohols reported above. This should be considered as supportive evidence for the α-configuration of the hydroxyl group in ginkgolide B and C.

Finally, some further evidence for the α-configuration of the 1-OH group was obtained from vicinal coupling constants. First of all, it was verified by means of MM2 calculations, whether in ring A of G-B the C_1endo, C_2exo conformation prevails, analogous to the situation in G-A. This was found to be true, irrespective of the stereochemistry of the 1-OH group. Next, the vicinal coupling constants of the C1-C2 fragment were predicted, using the generalized Karplus equation[17]. Both situations were considered, i.e. the 1-OH in the α-position, and the 1-OH in the β-position, and the resulting values are compared to the experimental coupling constants in Table 6. It can be seen that a better fit is obtained, when it is assumed, that the 1-OH group occupies the α-position.

Table 6. Observed and predicted coupling constants for ginkgolide B

	H-1/H-2	H-7α/H-6	H-7β/H-6	H-7α/H-8	H-7β/H-8
experimental	8.0	4.3	0.3	14.2	4.0
calculated for 1-α OH	5.4	5.1	1.4	12.3	4.0
calculated for 1-β OH	3.7	5.0	1.5	12.3	3.9

Unfortunately, for reasons not clear at this moment, predicted coupling constants in the ginkgolides differ significantly from the experimental values. This is for example true for the H-1/H-2 coupling constants in G-B presented in Table 6, and also for J(H7α-H8) in G-A (see Table 1). In the latter case, a better prediction of the coupling constant was obtained (13.1 Hz), when the Karplus equation recently developed by Altona *et al.* was used[20]. Unfortunately, the results for other proton-proton coupling constants were then less satisfactory.

In our view the above findings unambiguously prove that the C-1 hydroxyl group in G-B and G-C, and probably also in G-M, possesses the α-configuration. This result is in accordance with an X-ray study carried out some years ago which we consider solid evidence[9-11]. The result is also in accordance with steric arguments in a total synthesis[14] and with most earlier chemical and NMR papers on ginkgolides[6-8]. However, some of the earlier authors of these NMR papers admitted themselves that their NMR data should not be considered convincing evidence. It is in contradiction with the outcome of the most recent detailed NMR study[12, 13]. Assuming that the outcome of all other studies including the X-ray one and ours is correct, this means that either the recorded data or the conclusions in the papers by Roumestand *et al.* are incorrect[13].

Giving their study a closer look we believe that the latter is the case. They observed by saturation transfer experiments that the C-10 hydroxyl function must be in close proximity of the C-1 hydroxyl in G-B and G-C. They concluded from this that the hydroxyl group at C-1 thus has the β-configuration. However, according to our models and MM2 calculations both an α-hydroxyl and a β-hydroxyl group are in close proximity to the C-10 hydroxyl. The distance between O-1 and O-10 in G-B with the 1-OH group in the α- and β-position were

calculated as 2.78 and 2.77 Å respectively, thus making the information from the saturation transfer experiments unintelligible for this specific problem.

EXPERIMENTAL

Terpene standards. Ginkgolide A, B and C were a gift from Prof. Dr. K. Weinges.

NMR Spectroscopy. All NMR spectra of ginkgolides were recorded on a Bruker AMX-600 spectrometer operating at a ^{1}H frequency of 600 MHz. All spectra were recorded on a 5 mm inverse triple resonance probe, equipped with self-shielded gradients. Samples were prepared containing 10 mg of the ginkgolide in 0.5 mL acetone-d6 solution. All chemical shifts were expressed relative to TMS, using the central peak of acetone as an internal standard (δ ^{1}H (acetone) = 2.11 ppm and δ ^{13}C (acetone) = 30.2 ppm). The ^{1}H- and ^{13}C-NMR spectra were assigned by a combination of two-dimensional NMR techniques. A ^{1}H detected ^{13}C-^{1}H correlation spectrum (HSQC)[21] was recorded, employing pulsed field gradients for coherence selection[22]. The length of the sinusoidal gradient pulses was 1 ms and the gradient strength was 0.5 T $\cdot$ m^{-1}. 256 spectra of 1K datapoints each were recorded with 8 scans per increment.

A ^{1}H detected ^{13}C-^{1}H correlation spectrum optimized for two- and three-bond coupling constants (HMBC)[23] was also recorded, employing pulsed field gradients[24]. Sinusoidal gradient pulses were employed of 1 ms length and the gradient strength was 0.5 T $\cdot$ m^{-1}. 256 spectra of 1K datapoints each were recorded with 16 scans per increment. A delay of 60 ms was chosen for evolution of long range coupling constants.A 2D ROESY spectrum[18] was recorded, using the TPPI method. The spectra of cyclohexane, cyclohexanol, cyclopentane and cyclopentanol were recorded on a Bruker AC-E 200.

All molecular mechanics calculations were carried out on an IBM compatible PC using the MM2 program[16].

^{13}C-NMR data of ginkgolide A (acetone-d6): δ 177.4 (C-15), 174.9 (C-11), 171.9 (C-13), 111.4 (C-12), 101.7 (C-4), 88.9 (C-2), 87.9 (C-3), 86.8 (C-6), 70.5 (C-10), 69.7* (C-5), 68.5* (C-9), 50.3 (C-8), 42.1 (C-14), 37.9 (C-1), 37.5 (C-7), 33.3 (C-17), 29.8 (C-18), 29.8 (C-19), 29.8 (C-20), 8.8 (C-16) (* means assignments may be interchanged).

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