The structure of velutinol A is (15*R*,16*R*,20*S*)-14,16:15,20:16,21-triepoxy-15,16-seco-14β,17α-pregn-5-ene-3β,15-diol. A combined quantitative Overhauser effect and molecular modelling study

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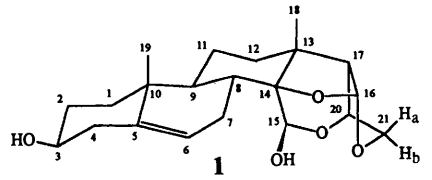
***Velutinol A*, a potent bradykinin antagonist isolated from the rhizomes of the native Brazilian plant *Mandevilla velutina*, has been confirmed to have the title structure through the combined use of quantitative ^1H–^1H nuclear Overhauser enhancement (NOE) data and molecular dynamics/energy minimisation calculations. The NOE data allowed the unambiguous selection of the structure from four possible, closely related, isomeric structures. Interproton distances from the NOE data were within 0.5 Å of those calculated from the optimised model structure for the rigid section of the molecule and within 0.6 Å when the methyl group was considered. Various models were considered for calculating the effective distance to a methyl group undergoing internal molecular motion. The most successful in reproducing the experimental data was the so-called 'pseudo-atom' approach, with a 0.3 Å correction applied to the experimental distances, and the more rigorous Rowan–Woessner approach, which considers the methyl group rotation to be by ± 120° jumps between the positions of potential minima. Through the application of field dependent ^{13}C relaxation time measurements the correlation times for overall motion of velutinol A and internal motion of the C18 methyl group were found to be 0.34×10^{-10} and 0.05×10^{-10} s, respectively. The ^{13}C spin-lattice relaxation of the sp^3 carbons is dominated by the ^{13}C–^1H dipole–dipole mechanism, however the relaxation time for the sp^2 carbon C5 is strongly field dependent, and a value of 227 ppm is obtained for the chemical shift anisotropy.**

We recently reported[1] a preliminary investigation on the structure of a novel pregnane, velutinol A, isolated from the rhizomes of the native Brazilian plant *Mandevilla velutina* (Apocynaceae). This plant is important since infusions or alcoholic extracts of the rhizomes are used in folk medicine in Brazil for the treatment of venomous snake bites, including those of *Bothrops jararaca* a variety of lancehead native to SE Brazil which is responsible for most reported snake bites. Extracts from these plants have been shown to have potent anti-inflammatory activity and to behave as selective antagonists to bradykinin, a nonapeptide generated in plasma during tissue trauma.[2–7] Velutinol A similarly showed[1] bradykinin antagonist activity. Using a combination of IR, UV and mass spectroscopic data, together with ^1H and ^{13}C NMR data the structure of velutinol A was narrowed down[1] to be one of two isomers; (15*R*,16*R*,20*S*)-14,16:15,20:16,21-triepoxy-15,16-seco-14β,17α-pregn-5-ene-3β,15-diol (**1**) or the -14,20:15,16:16,21-triepoxy-isomer (**2**). Interproton nuclear Overhauser enhancement (NOE) data which should have allowed an unambiguous choice between the two possible structures was largely lacking because it was believed that motional 'nulling' of the NOE was occurring in the 400 MHz measurements.[1,8] The choice of structure **1** was eventually made on the basis of a single NOE measured from a lower field spectrum (250 MHz) between a well resolved signal at δ 5.01 assigned to H15 and a signal in a very crowded spectral region at δ 1.37 assigned to H9. Inspection of molecular models indicated this NOE should be expected from isomer **1** with the configuration 15*R*, but not from isomer **2**. If we allowed the possibility of isomer **2** then again the 15*R* configuration would be implied by the lack of an experimental NOE between H15 and the 18-methyl protons (15*S* should give such an NOE). While these observations did point to structure **1**, the evidence clearly was not overwhelming.

Subsequent to our report on the isolation and structure of velutinol A, the structure of a closely related 14:15-seco-15-norpregnane, illustrol **3**, isolated from *Mandevilla illustris* was reported.[9] However, although alcoholic extracts of the rhizomes of the plant antagonise bradykinin[10] the purified illustrol was inactive in pharmacological tests. The relationship between velutinol isomers **1** and **2** and illustrol is that the illustrol structure can be generated from velutinol by interchange of the methylene group C21 with the adjacent oxygen and elimination of the HC15(OH) group with subsequent ring closure between the adjacent oxygen and C14. In proving the structure of velutinol A we therefore must now consider the additional possibilities which are isomers **4** and **5**. These could be generated from illustrol **3** by the insertion of a CH(OH) group between C14 and either of the two oxygens of the acetal group; α-insertion to give isomer **4** or β-insertion to give isomer **5**.

This report is of new NMR data which confirms our earlier assignment of structure **1** to velutinol A, and is based upon extensive interproton NOE data and the quantitative correlation of this data with comprehensive molecular modelling/energy minimisation and molecular dynamics simulation studies. Such an approach is widely used in the determination of the solution structure of high molecular mass biomolecules[11] and is an equally valid approach for the solution structural determination of smaller molecular systems which have not been amenable to solid-state X-ray structural analysis. Although molecular modelling was not used, Maes *et al.*[12] have recently shown that for a stereochemically rigid pregnane derivative interatomic distances derived from quantitative interpretation of interproton NOE data do



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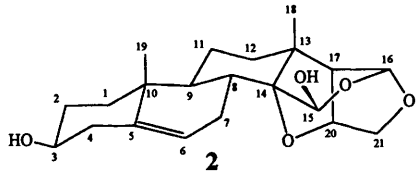

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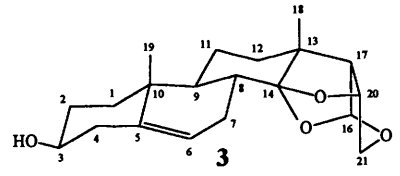

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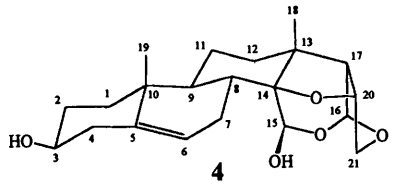

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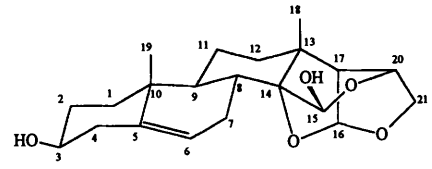

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correlate well with those distances derived from X-ray diffraction data, thus making a set of carefully measured NOEs a valuable tool for structural analysis for such rigid systems.

Results and discussion

Assignment of ^{1}H and ^{13}C NMR spectra

The analysis of these spectra was repeated using new data acquired with a 14.1 T spectrometer system (^{1}H at 600 MHz and ^{13}C at 150.9 MHz). The assignments which resulted from a consideration of the interproton coupling patterns (2-D COSY spectrum and 1-D TOCSY spectra) and the one-bond ^{13}C–^{1}H couplings (2-D heteronuclear shift correlation) are given in Table 1. The assignment of the methylene proton pairs at positions 1, 2, 4, 7, 11 and 12 as α or β came from a combination of interproton coupling constants and interproton NOE data (which will be discussed below). The ^{13}C chemical shifts reported[13] for ring A of cholesterol and the ^{1}H chemical shifts[14] for rings A of 3β-hydroxypregna-5,16-diene and -5,20-diene are almost identical with those of velutinol A, and were used as a basis for our assignments. We use the H3α resonance (δ 3.53) as a starting point for the assignment of α or β stereochemistry to the methylene pairs H1, H2 and H4. It is commonly accepted that three bond axial–axial proton couplings are greater than axial–equatorial, and for the H2 methylene pair (δ 1.50 and 1.85) the coupling to H3α (axial) is greater to the lower frequency resonance, indicating this to be axial as H2β. This assignment is corroborated by NOE evidence

Table 1 ^{1}H and ^{13}C chemical shift[a] assignments for velutinol A

Position	δ_H	$\delta_{^{13}C}$
1	1.12 (α) 1.83 (β)	37.2
2	1.85 (α) 1.50 (β)	31.3
3	3.53 1.80 (OH)	71.2
4	2.30 (α) 2.23 (β)	41.9
5		139.3
6	5.38	121.3
7	2.16 (α) 1.89 (β)	25.8
8	2.01	33.4
9	1.36	45.7
10		37.6
11	1.64 (α and β)	18.6
12	2.35 (α) 1.65 (β)	26.6
13		43.5
14		87.1
15	5.01 4.75 (OH)	92.3
16	5.78	108.6
17	2.53	52.2
18	1.11	21.7
19	1.09	19.2
20	4.45	73.6
21	3.81 (a) 4.28 (b)	78.0

[a] Shifts are reported in ppm to high frequency of TMS.

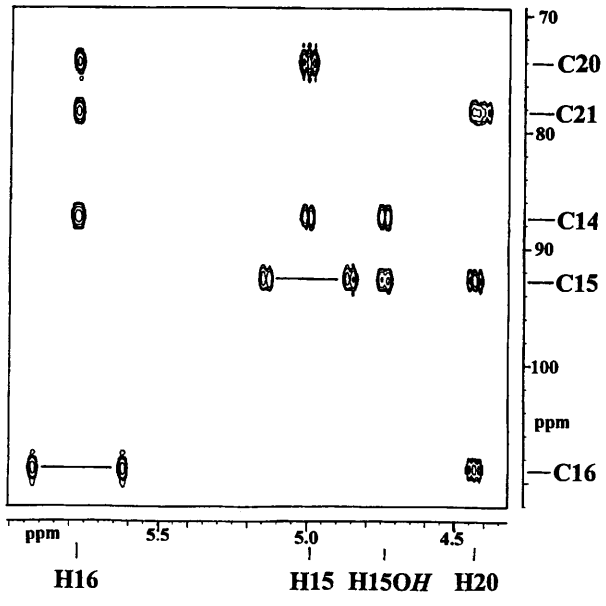


Fig. 1 Part of the 600 MHz 2-D ^{1}H–^{13}C (inverse mode, long-range coupling) shift correlation experiment on velutinol A, with the pulse sequence optimised for an assumed coupling of 7.5 Hz. The relevant correlations are detailed in Table 2, and the horizontal bars link the two components of the ^{1}H doublets with a one-bond coupling to ^{13}C.

(see below) and reverses our earlier report[1] of the relative H2α and β shifts. The coupling constant between H3α and the H4 methylene pair (δ 2.23 and 2.30) is greater to the lower frequency of the two and therefore this is assigned as axial (H4β). It will be shown below that a fairly strong NOE was found between the resonances at δ 1.50 and 2.23, indicating that these two protons are *syn* on ring A, *i.e.* both axial. For the H7 methylene pair the higher frequency of the two was assigned as H7α because of the larger coupling to H8(β) and this also reverses our earlier assignment[1] of the relative H7α and β shifts.

At this point the data do not allow any of the four possible isomers to be eliminated from consideration, however the long-range ^{1}H–^{13}C coupling heteronuclear shift correlation experiment did allow the elimination of isomers **2** and **4** as follows. The relevant region of the spectrum is shown in Fig. 1, and the important conclusions are summarised in Table 2. We use as a starting assumption for the analysis of the spectrum that one, two and three bond ^{1}H–^{13}C correlations may appear

Table 2 Long-range ^1H–^{13}C correlations[a] selecting between isomers {**1**, **5**} and {**2**, **4**}

Correlation	Occurrence	For/against
H16→C14	Present	For **1** and **5**
H20→C15	Present	For **1** and **5**
H15→C20	Present	For **1** and **5**
H20→C14	Absent	Against **2** and **4**
H16→C15	Absent	Against **2** and **4**
H15→C16	Absent	Against **2** and **4**

[a] Assumes the two and three bond correlations are observable, and the four bond are not.

in the spectrum, but not four bond correlations. The C14 resonance at δ 87.1 shows correlations to H15 (δ 5.01), H15O*H* (δ 4.75) and H17 (δ 2.53), which are all two or three bond correlations for all four isomers. There is also a correlation to H16 (δ 5.78) which is a three bond correlation for isomers **1** and **5**, but would be a four bond correlation for isomers **2** and **4**, and therefore is evidence against **2** and **4** and in favour of **1** and **5**. Support for this choice (albeit of a somewhat negative nature) comes from the lack of a correlation between C14 and H20 (δ 4.45) which might be expected for isomers **2** and **4** (three bond), but not for **1** and **5** (four bond). Similar reasoning applied to the correlation observed between C15 (δ 92.3) and H20 (δ 4.45), and the lack of a correlation between C15 and H16 (δ 5.78) also points to isomers **1** and **5**, but against **2** and **4**. Further supportive evidence is from the observed correlation between C20 (δ 73.6) and H15 (δ 5.01), and the lack of a correlation between C16 (δ 108.6) and H15. This interpretation of the spectrum depends upon the assumption of no four bond correlations, and that possible three bond correlations are not absent through a misset of the appropriate delay in the experimental pulse sequence (see Experimental section). Therefore the selection of isomers **1** and **5** against **2** and **4** is not conclusive, but the consistency of the interpretation is strong evidence for the selection.

Quantitative NOE data

For a rigid molecule undergoing isotropic reorientation in solution the cross relaxation rate (σ) due to the dipole–dipole interaction between a pair of protons (I and S) is given by eqn. (1)[15] where the symbols have their usual meaning, and $f(\omega_H, \tau_c)$

$$\sigma = Kr_{IS}^{-6} \quad (1)$$

$$K = \left(\frac{\mu_0}{4\pi}\right)^2 \left(\frac{h}{2\pi}\right)^2 \gamma_H{}^4 f(\omega_H, \tau_c) \quad (2)$$

is a function of the ^1H resonance frequency (ω_H) and of the correlation time (τ_C) for the molecular motion. The value of σ for a particular proton pair may be estimated as follows.[11] A set of 2-D NOESY spectra is measured with the mixing time (τ_m) varied between spectra. The volume, $v_{ij}(\tau_m)$ of the off-diagonal peak, giving the NOE correlation between the selected proton pair (i,j), is then measured as a function of the mixing time. For short mixing times there is an increase in the volume with mixing time due to the cross relaxation (σ_{ij}), while at longer mixing times spin diffusion (a relaying of magnetisation throughout the dipolar coupled spin system) affects the volumes, and eventually the volumes are attenuated by spin-lattice relaxation. The analysis of the experimental data can proceed either *via* the so-called full relaxation matrix approach[16,17] or more simply by taking the initial slope of the v_{ij} *vs.* τ_m curve to be proportional to σ_{ij}. We have used this latter approach. In order to extract interatomic distances it is necessary to find a value for the constant of proportionality [eqn. (1)] between the initial slope and the cross relaxation rate, and this is achieved by measuring the initial slope for a proton pair with a known internuclear separation. For velutinol A we used the average of the initial slopes for the methylene pairs H21a–H21b and H7α–H7β, taken to be 1.79 and 1.78 Å apart, respectively. These reference distances were taken from the SYBYL structures (see below), and the resulting interproton distances are given in Table 3. Molecular models of the four possible velutinol A structures (**1**, **2**, **4**, **5**) were constructed using the SYBYL software (see Experimental section) and these were subjected to molecular dynamics/energy minimisation to provide the best calculated structures. The relevant calculated interproton distances from these models are also included in Table 3. The correspondence between the experimental (NOESY-derived) interproton distances and the calculated (SYBYL-derived) for each of the four possible structures is given in Fig. 2 (excluding data on the methyl group protons, see below). As shown in Fig. 2, only one of the calculated structures (**1**) has all the computed interproton distances within 0.5 Å of the corresponding experimental distance, in keeping with the results of Keepers and James[16] who estimated that a set of NOESY experiments (*i.e.* multiple τ_m values) should lead to interproton distances with an error of ±0.5 Å. Therefore we conclude that the data are consistent with the assignment of structure **1** to velutinol A. In considering structure **2** it can be seen [Fig. 2(*b*)] that there are severe discrepancies (> 1.0 Å) between the experimental and calculated distances for H9–H15 and for H12α–H15, and these discrepancies would not be removed by a change in configuration from 15*R* to 15*S*. Structure **4** is eliminated from consideration because of the large difference (~ 1.2 Å) in the experimental and calculated distances for H12α–H20 [Fig. 2(*c*)], and likewise structure **5** is eliminated [Fig. 2(*d*)] because of the large experimental–calculated distances for H12α–H20, H9–H15 and H12α–H15 (and again a change from 15*R* to 15*S* would not improve the situation).

Table 3 Calculated (SYBYL structures **1**, **2**, **4** and **5**) and experimental (NOESY) interproton distances

Protons	NOESY distances/Å	Distances/Å from SYBYL structures			
		1	**2**	**4**	**5**
21a–20	2.1_6	2.5_2	2.1_4	2.5_2	2.6_4
17–16	2.4_4	2.4_4	2.3_8	2.3_3	2.3_5
4α–6	2.1_5	2.3_2	2.2_6	2.3_3	2.3_3
7β–6	2.4_6	2.4_7	2.4_8	2.4_9	2.4_0
1α–3α	2.3_6	2.5_3	2.5_9	2.5_3	2.5_3
8–7α	2.6_4	3.0_6	3.1_3	3.0_5	3.0_9
7β–8	2.2_7	2.3_6	2.3_6	2.3_6	2.4_3
17–20	2.3_6	2.4_3	2.6_4	2.1_1	2.0_8
12α–20	2.5_7	3.0_3	2.4_5	3.7_9	3.7_5
2β–4β	3.0_0	2.6_2	2.6_3	2.5_9	2.6_7
21b–20	2.7_6	2.7_1	2.6_7	2.9_1	2.9_8
4α–3α	2.4_6	2.4_5	2.3_9	2.4_8	2.4_4
7α–15	2.1_4	2.3_6	2.2_8	2.3_9	2.2_1
9–15	1.9_6	2.2_4	3.1_2	2.1_9	3.0_6
12α–15	2.5_8	2.6_1	3.7_8	2.9_0	3.7_6
17–21a	2.7_3	2.7_5	2.7_6	2.8_7	2.7_0
21a–16	3.0_7	2.6_2	3.0_7	2.9_8	2.9_2

Internal motion and the NOE

The 18-methyl group protons show distinctive correlations in the NOESY spectra with two other protons, H16 and H17, in the novel tricyclic sub-structure, however quantitative interpretation of these NOEs is complicated by the internal rotation of the methyl group. The complication arises because the estimation of interproton distances by the initial slope method described above, strictly requires that the internuclear vector describing the reference distance undergoes the same detailed motion as does the vector describing the internuclear distance to be measured. The internal rotation of a methyl group means that the internuclear vector of interest has additional motion,

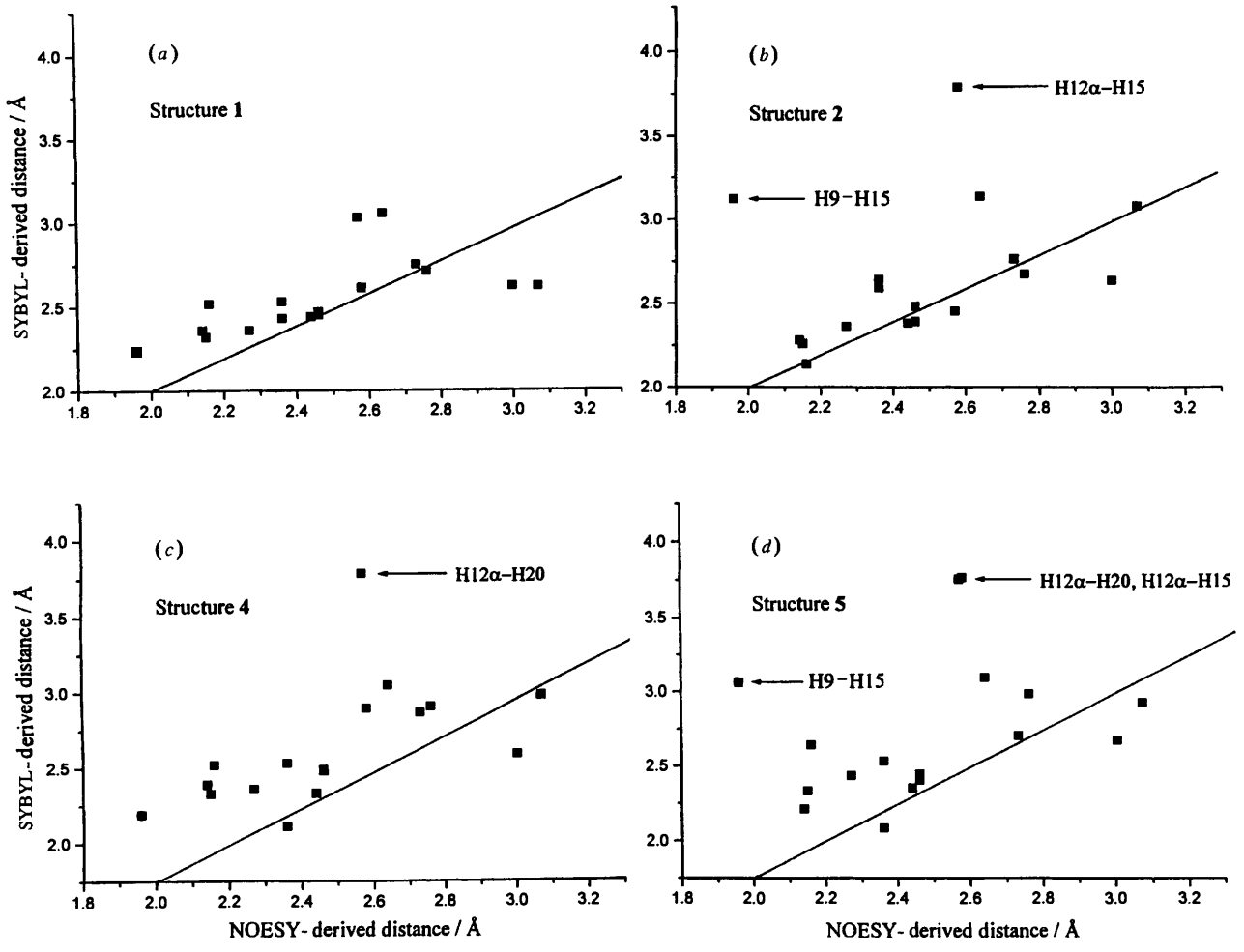


Fig. 2 Correlations between the NOESY-derived interproton distances and those from the optimised SYBYL structures **1**, **2**, **4** and **5** for velutinol A. The straight lines correspond to an ideal 1 : 1 correspondence between NOESY and SYBYL distances.

and there have been several approaches to approximating the effective distance between the protons of a methyl group undergoing rapid internal rotation, and another intramolecular proton. These approaches have been summarised by Koning *et al.*[18] The simplest approximation is to consider the methyl protons to be effectively at the centre of the circle described by the group rotation (the so-called pseudo-atom approach), giving the effective distance to the other proton as r_{ps}. In this case corrections in the range 0.3–1.0 Å are sometimes made [18] to the experimental distance before comparison with a distance involving the pseudo-atom. Several other approaches require a knowledge of the position of the methyl protons themselves (obtainable here from the energy minimised SYBYL structures), and may take a direct average of the distances, r_{ix}, from proton X to each of the three methyl protons (r_{av1}), the cube root of the average of the distances to the third power (r_{ix}^3), *i.e.* $r_{av3} = \sqrt[3]{\langle r_{ix}^3 \rangle}$ or the sixth root of the average of the distances to the sixth power (r_{ix}^6), *i.e.* $r_{av6} = \sqrt[6]{\langle r_{ix}^6 \rangle}$. Another approach due to Pegg *et al.*[19] does not require specific positions for the methyl protons and assumes rapid internal rotation of the methyl group such that the correlation time for the internal motion (τ_{int}) is much less than that for the overall molecular motion, *i.e.* $\tau_{int} \ll \tau_c$, this gives an effective distance between proton X and the methyl group which we call r_{Pegg}. Finally we consider the method due to Rowan *et al.*[20] which is based upon the general approach of Woessner.[21,22] This method takes into account the internal motion of the methyl group, with the methyl protons making 120° jumps around the internal rotation axis, but does not require the starting assumption that $\tau_{int} \ll \tau_c$.

The evaluation of the effective interproton distances (r_{ps}, r_{av1}, r_{av3}, r_{av6} and r_{Pegg}) between the 18-methyl protons and H16 or H17 depends solely upon the coordinates of the atoms in the energy minimised SYBYL structures **1**, **2**, **4** and **5**. These values are given in Table 4. The determination of the effective distances by the Rowan–Woessner method is more complicated. First, a value for the overall correlation time for molecular reorientation (τ_c) must be obtained and this is achieved conveniently by measurement of the ^{13}C spin-lattice relaxation times for the backbone carbons of velutinol A. For the backbone carbons (no internal motion) the dipole–dipole contribution to the ^{13}C spin-lattice relaxation rate is given by eqn. (3) where N is the number of hydrogens attached to a given

$$\frac{1}{T_1^{DD}} = \frac{N\gamma_H^2\gamma_C^2h^2f(\omega_H,\omega_C,\tau_c)}{4\pi^2r_{CH}^6} \quad (3)$$

carbon and the other symbols have their usual meanings. In the 'extreme narrowing' limit, the function $f(\omega_H, \omega_C, \tau_c)$ is simply equal to τ_c. This was checked for velutinol A by measurement of the ^{13}C T_1 values and the ^{13}C–$\{^1H\}$ NOEs at two different ^{13}C resonances frequencies, 68 and 150 MHz (spectrometer field strengths 6.4 and 14.1 T, respectively). The results, collected in Table 5, show that the T_1 values are effectively independent of the operating frequency (except for C5), and that the ^{13}C–$\{^1H\}$ NOEs for the proton-bearing carbons are close to the theoretical maximum value 2.98. These observations confirm that velutinol A is in the extreme narrowing region and that the experimental T_1 values may be reasonably set equal to T_1^{DD} [eqn. (3)]. For the carbons with directly bonded protons

(excluding the methyl carbons because of internal motion, and C6 because of possible contribution to the relaxation from the chemical shift anisotropy mechanism, see below) the average values for the quantity (NT_1) are $1.3_8 \pm 0.1_4$ and $1.4_7 \pm 0.1_4$ s, respectively at 6.4 and 14.1 T. From eqn. (3) these give 0.34×10^{-10} and 0.33×10^{-10} s for τ_c, and the average of these two values was used in subsequent calculations. The second step in the Rowan–Woessner method is the determination of the correlation time for the internal methyl group rotation. The model has the protons of the methyl group jumping between potential minima separated by 120°, and (assuming tetrahedral geometry of the methyl carbon) the correlation time of eqn. (3) is replaced by an effective correlation time τ_{eff} [eqn. (4)]:

$$\tau_{eff} = \frac{\tau_c}{9} + \frac{8\tau_1}{9} \quad (4)$$

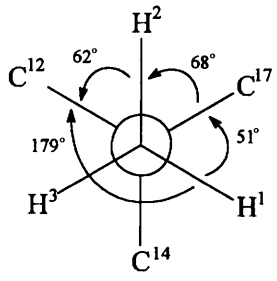
where

$$\frac{1}{\tau_1} = \frac{1}{\tau_c} + \frac{1}{\tau_j} \quad (5)$$

and $[2/(3\tau_j)]$ is the jump rate for a given methyl proton. The NT_1 values for the C18 methyl carbon are 5.8_5 and 6.0_9 s at 6.4 and 14.1 T, respectively, and from eqns. (3)–(5) these values give an average value for $\tau_j = 0.053 \times 10^{-10}$ s. The correlation time function appearing in eqn. (2) $f(\omega_H, \tau_c) = \tau_c$ (since $\omega_H^2\tau_c^2 \approx 0.01$, this is the extreme narrowing regime for the proton resonance), and the Rowan–Woessner formulism generates an effective distance, r_{RW}, between the methyl group and another proton [eqn. (6)] where the coefficients A and B

$$\frac{1}{r_{RW}^6} = A + B\frac{\tau_j}{\tau_c + \tau_j} \quad (6)$$

are functions of the direction cosines of the vectors joining the proton X and each of the three methyl proton positions and the lengths of these three vectors (see Appendix). The final positions for the C18 methyl protons obtained from the molecular dynamics simulations (see Experimental section) is that they are staggered with respect to the carbon substituents at C13, as shown by the Newman projection along C18→C13. The relevant values for r_{RW} are given in Table 4.



The NOESY-derived distances H18–H16 and H18–H17 are 2.8 and 2.9 Å, respectively, and comparison with the data in Table 4 gives additional evidence for the rejection of structures **4** and **5** (H18–H16 is calculated to be > 5 Å by all six methods).

Table 4 Calculated distances (Å) from the methyl protons[a] (H18) to H16 and to H17

Structure		r_{ps}	r_{av1}	r_{av3}	r_{av6}	r_{Pegg}	r_{RW}
1	H18–H16	3.5_5	3.6_3	3.7_4	3.8_7	3.4_8	3.3_8
	H18–H17	2.8_6	2.9_9	3.0_8	3.2_2	2.8_5	2.9_6
2	H18–H16	3.6_3	3.7_0	3.8_1	3.9_3	3.5_7	3.4_2
	H18–H17	3.2_9	3.4_1	3.4_6	3.5_4	3.3_1	3.4_3
4	H18–H16	5.2_0	5.2_8	5.3_1	5.3_3	5.2_5	5.3_2
	H18–H17	3.2_6	3.3_8	3.4_5	3.5_4	3.2_8	3.2_8
5	H18–H16	5.0_0	5.1_0	5.1_0	5.1_2	5.0_8	5.1_7
	H18–H17	3.4_5	3.5_8	3.6_2	3.6_7	3.5_3	3.6_3

[a] The NOESY-derived distances were (see text) H18–H16 = 2.8 Å and H18–H17 = 2.9 Å.

Effective distance to the methyl protons

The NMR data have allowed the selection of structure **1** for velutinol A and this may be used as the basis to test which of the methods for effective distance determination to the methyl group gives best agreement with the NOESY-derived distance. For the H18–H17 interaction, all six methods for the effective distance give values which are less than 0.4 Å from the NOESY-derived value. However for the H18–H16 interaction, it is only the Rowan–Woessner method which gives a value ($r_{RW} = 3.3_8$ Å) within a reasonable range of the experimental value (2.8 Å). The pseudo-atom approach, as described by Wüthrich *et al.*,[23] compares r_{ps} with the experimental value plus a correction

Table 5 ^{1}H and ^{13}C spin-lattice relaxation times (s) and ^{13}C–{^{1}H} nuclear Overhauser enhancements for velutinol A

		^{13}C T_1		^{13}C–{^{1}H} NOE	
Position	^{1}H T_1	67.9 MHz	150.9 MHz	67.9 MHz	150.9 MHz
1		0.7_7	0.8_4	2.6	3.1
2	0.6_3 (β)	0.7_2	0.7_9	2.7	3.0
3	1.3_5	1.3_9	1.4_6	2.5	2.7
4	0.6_7 (α) 0.6_8 (β)	0.7_6	0.7_9	2.8	2.9
5		8.6_8	3.5_7	1.8	1.3
6	1.2_9	1.3_0	1.1_1	2.6	2.2
7	0.6_4 (α) 0.6_5 (β)	0.7_6	0.7_9	2.9	3.1
8	1.1_3	1.4_0	1.5_6	2.7	3.0
9	0.7_2	1.4_4	1.5_0	2.7	2.9
10		10.9_3	10.9_3	2.1	2.6
11		0.7_0	0.7_7	2.6	2.9
12	0.6_5 (α)	0.7_0	0.7_6	2.7	2.6
13		11.6_5	10.2_2	2.5	2.5
14		14.9_9	12.3_0	2.2	2.3
15	1.0_2 1.9_1 (OH)	0.9_5	1.2_7	2.6	2.6
16	1.9_9	1.3_9	1.1_7	2.7	2.6
17	1.4_4	1.2_8	1.3_9	2.7	2.8
18	0.8_4	1.9_5	2.0_3	2.6	2.8
19		1.5_9	1.6_7	2.8	3.1
20	1.5_3	1.3_5	1.4_8	2.5	2.7
21	0.8_0 (a) 0.8_7 (b)	0.6_4	0.6_4	2.8	2.4

distance of 1 Å, while Koning *et al.*[18] recommended a correction distance of 0.3 Å. Using this latter correction the H18–H16 'experimental' distance becomes 3.1 Å which compares favourably with the value for $r_{ps} = 3.5_5$ Å (Table 4).

Chemical shift anisotropy (CSA) and ^{13}C spin-lattice relaxation

The ^{13}C T_1 value for C5 (Table 5) is strongly dependent upon the spectrometer magnetic field, and to a lesser extent, so is that for the other sp^2 carbon C6. This observation suggests that the chemical shift anisotropy mechanism is important for these, and the contribution from this mechanism to the ^{13}C relaxation is given by eqn. (7) where B_0 is the spectrometer magnetic field

$$\frac{1}{T_1^{CSA}} = \frac{2}{15}\gamma_C^2 B_0^2(\Delta\sigma)^2\tau_c \qquad (7)$$

strength, $\Delta\sigma$ is the anisotropy in the nuclear shielding and τ_c is the same correlation time for molecular rotation as described above for dipole–dipole relaxation [eqn. (3)]. If the change in the C5 relaxation time between 6.4 and 14.1 T is due solely to the CSA mechanism, then with the above value for $\tau_c = 0.335 \times 10^{-10}$ s, a value of 227 ppm is obtained for the anisotropy in the shielding. This is in good agreement with the values of *ca.* 200 ppm found by Zilm *et al.*[24] for the sp^2 carbons in some simple alkenes and cycloalkenes. In that same study the ^{13}C shielding anisotropies for sp^3 methyl and methylene carbons were found to be much smaller in the range 20–40 ppm, which explains why the CSA mechanism is not effective for the sp^3 carbons in this study on velutinol A. There is a slight decrease in the relaxation time for C6 at the higher field, but this change is within the RMS error limits for the other proton bearing carbons and so may not be significant.

Experimental

Velutinol A

The isolation and purification of velutinol A has been described previously. The NMR spectra described below were obtained in a solution of *ca.* 25 mg velutinol A in 0.5 ml $CDCl_3$, contained in a 5 mm od NMR tube.

NMR spectroscopy

^{1}H and ^{13}C NMR spectra were measured at 600 and 150.9 MHz, respectively, using a Bruker AMX-600 spectrometer and additional ^{13}C spin-lattice relaxation time measurements at 67.9 MHz were made using a JEOL GX-270 spectrometer. Typical 90° pulse widths for data acquisition were ^{1}H *ca.* 7 μs and ^{13}C *ca.* 9 μs (AMX-600) and ^{13}C *ca.* 8 μs (GX-270). The ^{1}H and ^{13}C spin-lattice relaxation times were measured using the standard inversion-recovery sequence. ^{13}C–{^{1}H} NOE factors were determined from a comparison of ^{13}C peak intensities in fully ^{1}H-decoupled and ^{1}H inverse gated decoupled spectra. The COSY spectra were measured using the double-quantum filtered, phase sensitive mode and the NOESY spectra were similarly measured in a phase sensitive mode. The NOESY spectra were acquired with 2 K data points in the t_2 domain, 512 experiments with 16 transients (each having acquisition time, $AQ = 0.2$ s) per experiment and a relaxation delay, $D1 = 1.0$ s. The spectrum was repeated with mixing times $\tau_m = 0.5, 1.0, 1.5, 2.0$ and 2.5 s. The data were zero-filled and convoluted with sine-bell squared, shifted by $\pi/2$, window functions before Fourier transformation, giving a final digital resolution of 2.6 Hz/point in f_2 and 10.5 Hz/point in f_1. The off-diagonal peak volumes were corrected, as described by Barsukov, Lian and Sutcliffe,[11] for incomplete relaxation of the ^{1}H longitudinal magnetisation, using the factor $1 - \exp[-(AQ + D1)/T_{1j}]$ where T_{1j} is the spin-lattice relaxation time of the proton (j) whose chemical shift is in the f_1 domain. For a given proton pair the corrected peak volumes from either side of the diagonal were averaged. The final distances derived in this way are given in Table 3. It is worth noting that distances derived without use of the ^{1}H T_1 correction factor were almost all longer than when the correction was used, however the differences between corrected and uncorrected distances were all small (≤ 0.2 Å). The 1-D selective TOCSY experiments[25] used selective Gaussian-shaped pulses[26] with the MLEV-17 spin-lock sequence[27] and were repeated with mixing periods 35, 70 and 140 ms in order to monitor the transfer of magnetisation through the spin systems.

The one-bond C–H correlation experiment used an assumed value for the ^{1}H–^{13}C coupling of 135 Hz and the long-range coupling C–H correlation was repeated using assumed values of 5 and 7.5 Hz for the coupling. Both these heteronuclear experiments were run in 'inverse' mode, *i.e.* with direct ^{1}H detection. All the above 2-D experiments have been described in detail by Sanders and Hunter.[28] In addition, the one-bond C–H correlation was measured with ^{13}C detection, based on the DEPT pulse sequence as described by Bendall and Pegg.[29]

Computational methods

The molecular modelling, energy minimisation and molecular dynamics simulations were all carried out using the SYBYL programme, version 6.1, from Tripos Inc. on a Silicon Graphics R3000 workstation. In order to try for the best correlation between NOESY-derived interproton distances and those measured from the SYBYL models for the four structures **1**, **2**, **4** and **5**, and to obtain the best positions for the energy minima for the C18 methyl protons, the following procedure was employed. A molecular structure was created using the Sketch option of the SYBYL programme. The structure was then submitted to conjugate gradient energy minimisation,[30] *in vacuo*, to relieve unfavourable contacts between non-bonded atoms and to optimise bond lengths and geometries (this minimisation used the Tripos force field[31]). Subsequent molecular dynamics simulations were run for the molecule contained in a box of solvent molecules, which was built by successively adding molecules of $CHCl_3$ (a total of 228) to the isomer of velutinol A to generate a box of dimensions 29.6 × 29.6 × 29.6 Å. The Molecular Silverware option was used as the model for solvation.[32] At this point the NOESY-derived interproton distances were entered as structural constraints. The errors on these distances are quite large and therefore the 'weak, medium and strong' approximation[33] was used with 'strong' corresponding to distances ≤ 2.5 Å and 'medium' to distances ≤ 3.2 Å. These were assigned the allowed ranges in the simulations, 1.7–2.8 and 1.8–3.5 Å, respectively. The resulting system (velutinol A isomer plus solvent) was submitted to conjugate gradient energy minimisation and then entered into a molecular dynamics simulation. In the simulation the system was rapidly heated to 1000 K, slowly cooled to 300 K and maintained at 300 K for 300 fs. The lowest energy structure was selected from this final period at 300 K, 'extracted' from the solvent box and subjected to 2000 steps of conjugate gradient energy minimisation. In this final minimisation step a value[34] of 4.8 for the relative permittivity was entered into the force field to simulate the presence of the solvent $CHCl_3$.

All calculations to give the effective distances to the methyl protons (see Table 4) were made using programmes written in Fortran for a PC-486, using as input the Cartesian coordinates of the atoms obtained from the final SYBYL structures.

Conclusions

Four structures (**1**, **2**, **4**, **5**) were considered possible for velutinol A. A combination of one-dimensional ^{1}H and ^{13}C NMR spectra and the two-dimensional experiments ^{1}H COSY and ^{1}H–^{13}C heteronuclear shift correlations allowed the elimination of **2** and **4**, but did not allow an unambiguous selection between

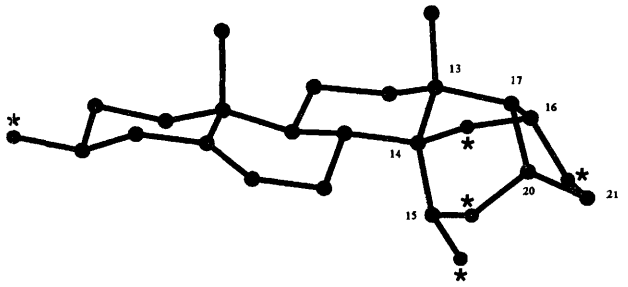


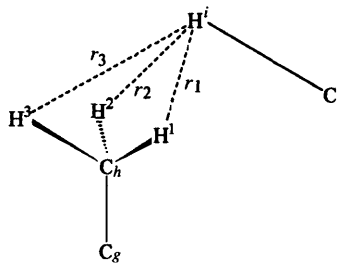
Fig. 3 Optimised SYBYL structure for velutinol A showing the proximity of the four oxygen atoms (labelled with asterisks) associated with the tricyclic fragment

1 and **5**. Structure **1** was assigned to velutinol A through quantitative interproton distance determination using ^{1}H–^{1}H NOEs (excluding methyl protons subject to internal motion), and a comparison of these experimental distances with distances calculated from energy minimised models generated from the SYBYL package. Structures **2**, **4** and **5** all gave one or more crucial experimental distances which were more than 1.0 Å different from the calculated (model) distance. Structure **1** however gave distances which were all within 0.5 Å of the experimental distances. For the rigid structural fragment of velutinol A the application of constrained molecular dynamics simulations probably does not give significantly better model structures than the application of the energy minimisation method alone. However for flexible systems, *e.g.* when the methyl groups are included, or when the molecule includes other modes of intramolecular motion,[35] then the combination of constrained molecular dynamics/energy minimisation is clearly the preferred option. Various methods were investigated for estimating effective interproton distances to the protons of a rapidly rotating methyl group. Two of these methods gave satisfactory correlations with the experimental distances. The pseudo-atom approach which replaces the methyl protons by a 'pseudo-atom' at the centre of the circle described by the rotating methyl protons, gave a discrepancy < 0.5 Å with the experimental distance corrected by the addition of 0.3 Å. The Rowan–Woessner approach considers the methyl group to be rotating by 120° jumps, with the jump rate estimated from ^{13}C nuclear relaxation data. This approach gave a discrepancy < 0.6 Å with the experimental distance. The pseudo-atom approach has the advantage of relative simplicity, but does involve the application of a 'correction' to the experimental distance. The Rowan–Woessner approach is more rigorous, but does require the additional measurement of ^{13}C spin-lattice relaxation times. Structure **1** has all four oxygen atoms (see Fig. 3) of the tricyclic ring system grouped in closer proximity than in structures **2**, **4** or **5**, and it is possible that this grouping is responsible (in part) for the high level of *in vivo* activity of this compound.

Appendix

Rowan *et al.*[20] developed the Woessner[21,22] approach for the relaxation of a proton H*i* due to a nearby intramolecular methyl group undergoing random 120° jumps about its carbon–carbon axis, between the positions of potential minima for the hydrogens. The model further assumed extreme narrowing (which is the case here) and that overall tumbling of the molecule was isotropic (also assumed here) by small-step diffusion. However the final expressions derived by Rowan *et al.*[20] for the constants A and B of eqn. (6) (above) are only applicable for one symmetric conformation of the methyl group (in crotonaldehyde). Therefore we present here the more general expressions for A and B which are applicable for any particular conformation of the methyl group with any molecule-fixed Cartesian coordinate system.

An important step in the Rowan–Woessner approach is



averaging the quantity Q over the internal motion [eqn. (A1)]

$$Q(\mu,\nu) = [3(l'l'' + m'm'' + n'n'')^2 - 1]\mathbf{r}_{ij'}^{-3}\,\mathbf{r}_{ij''}^{-3} \qquad \text{(A1)}$$

where l, m and n are direction cosines of the vector ($\mathbf{r}_{ij}$) joining the molecule-fixed proton H*i* and a methyl proton *j* undergoing internal motion. The single and double primes refer to the vector $\mathbf{r}_{ij}$ at some time t (single prime) and at a later time $t + \tau$ (double prime), and the indices μ and ν refer to the allowed positional vectors $\mathbf{r}_{ij}$. The chosen model is for ± 120° jumps about the C_g–C_h axis with $2/(3\tau_j)$ jumps per second in random directions between the three minima (positions 1, 2 and 3) of the torsional potential. The average is given by eqn. (A2) where the

$$\langle Q(\mu,\nu)\rangle = \sum_{\mu'',\nu''=1}^{3} \frac{1}{3} p_{\nu''}^{\mu''}(\tau) Q(\mu'',\nu'') \qquad \text{(A2)}$$

p-factors are conditional probabilities for a methyl proton at a given site (*e.g.* 1) at time t to be at sites 1, 2 or 3 at time τ later [eqn. (A3)]. The summation [eqn. (A2)] has nine terms

$$p_1^1(\tau) = \frac{1}{3} + \frac{2}{3}\exp(-\tau/\tau_j)$$
$$p_2^1(\tau) = p_3^1(\tau) = \frac{1}{3} - \frac{1}{3}\exp(-\tau/\tau_j) \qquad \text{(A3)}$$

corresponding to probabilities 1→1, 1→2, 1→3, 2→2, 2→3, 2→1, 3→3, 3→2, 3→1. The distances from the molecule-fixed proton H*i* to the methyl protons at the positions of the potential minima are r_1, r_2 and r_3, and the corresponding direction cosines are (l_1, m_1, n_1), (l_2, m_2, n_2) and (l_3, m_3, n_3). The first three terms in the summation (A2) are for the conditional probabilities 1→1, 1→2, 1→3 [eqns. (A4)–(A6)] and eqn. (A4) simplifies to eqn. (A7).

$$\left[\frac{1}{3} + \frac{2}{3}\exp(-\tau/\tau_j)\right][3(l_1^2 + m^2 + n_1^2)^2 - 1]r_1^{-6} \qquad \text{(A4)}$$

$$\left[\frac{1}{3} - \frac{1}{3}\exp(-\tau/\tau_j)\right][3(l_1l_2 + m_1m_2 + n_1n_2)^2 - 1]r_1^{-3}r_2^{-3} \qquad \text{(A5)}$$

$$\left[\frac{1}{3} - \frac{1}{3}\exp(-\tau/\tau_j)\right][3(l_1l_3 + m_1m_3 + n_1n_3)^2 - 1]r_1^{-3}r_3^{-3} \qquad \text{(A6)}$$

$$2\left[\frac{1}{3} + \frac{2}{3}\exp(-\tau/\tau_j)\right]r_1^{-6} \qquad \text{(A7)}$$

In their application to crotonaldehyde, Rowan *et al.*[20] were able to simplify the computation by choosing an appropriate molecule-fixed Cartesian axis system in which the methyl rotation axis (C_g–C_h) was the z-axis, C_g, C_h, H^1 and H*i* were all in the yz-plane, and H^2 and H^3 were symmetrically disposed either side of the yz-plane. In the general case it is not possible to define the yz-plane in this manner since the positions of the

methyl hydrogens in their potential minima will not result in one of the hydrogens being co-planar with C_g, C_h and H*i*. The general expression for the effective distance between the hydrogen H*i* and the methyl protons, using an arbitrary molecule-fixed axis system, still has the form of eqn. (6), but the constants A and B are now given by eqns. (A8) and (A9)

$$A = C + D \tag{A8}$$

$$B = 2C - D \tag{A9}$$

where

$$C = \tfrac{1}{9}(r_1^{-6} + r_2^{-6} + r_3^{-6}) \tag{A10}$$

$$\beta = \tfrac{1}{9} = \left\{ \begin{array}{l} [3(l_1l_2 + m_1m_2 + n_1n_2)^2 - 1]r_1^{-3}r_2^{-3} + \\ [3(l_1l_2 + m_1m_3 + n_1n_3)^2 - 1]r_1^{-3}r_3^{-3} + \\ [3(l_2l_3 + m_2m_3 + n_2n_3)^2 - 1]r_2^{-3}r_3^{-3} \end{array} \right\} \tag{A11}$$

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