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AN EVALUATION OF THE TAUTOMERISM OF CINCHONINONE AND QUINIDINONE MADE USING A COMBINATION OF ^{1}H NMR AND ^{13}C NMR SPECTROSCOPY

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Key Word Index—*Cinchona* alkaloids; cinchoninone; biosynthesis; tautomerism; NMR spectroscopy.

Abstract—The *Cinchona* alkaloids cinchoninone and quinidinone are the substrates for the cinchoninone:NADPH oxidoreductases I and II described previously. The *in vitro* assay of this activity is complicated by the tendency of the substrates in neutral aqueous solution to undergo mutarotation owing to tautomerism. The composition of such solutions at equilibrium has been determined using a combination of ^{1}H NMR and ^{13}C NMR spectroscopy and shown to contain the keto, enol and diol forms of both the 8*S* and 8*R* steric series. All these species exist at different concentrations. Furthermore, the position of the equilibrium favours the 8*S*-isomers rather than the 8*R*-form found in both the crystalline solid and the natural biosynthetic intermediate, cinchoninone.

INTRODUCTION

The bark of *Cinchona ledgeriana* provides the quinoline alkaloids, principally quinine, cinchonidine, quinidine and cinchonine (Fig. 1), which are of value for their bittering, anti-malarial and anti-arrhythmic properties [1]. These alkaloids appear to be closely related biosynthetically, being two pairs of steric isomers at the C-8 and C-9 chiral centres, each enantiomeric series being represented by a C-6′ methoxylated and a C-6′ unmethoxylated form. Preliminary radiolabelling data [2] indicate that cinchoninone may be of key importance as the intermediate immediately prior to cinchonine and cinchonidine. The biosynthesis of quinoline alkaloids in *C. ledgeriana* cell suspension cultures [3–6] is being investigated. It has been demonstrated [7] that these cultures contain two isoenzymes, cinchoninone:NADPH oxidoreductases I and II, which reduce cinchoninone (isoenzymes I and II) to an unequal mixture of cinchonine and cinchonidine, and quinidinone (isoenzyme II only) to an unequal mixture of quinine and quinidine in NADPH-dependent reactions. These enzymes are central to determining the relative amounts of 8*S*,9*R*- and 8*R*,9*S*-enantiomers formed and to precluding the formation of the unnatural epi-series (8*R*,9*R* and 8*S*,9*S*) [8].

There are two major problems associated with the study of this reduction *in vitro*. Firstly, in cinchoninone and quinidinone the presence of a fully conjugated quinoline nucleus promotes tautomerism around the C-9 carbonyl in polar solvents [9]. This results in steric inversion of the C-8 proton, and generates a solution which potentially could contain the 8*R*- and 8*S*-keto and geminal diols and the *Z*- and *E*-enols (Fig. 2). Secondly, the species representing the true substrate(s) need to be determined. Thus, in order to interpret the kinetic parameters obtained for the oxidoreductases *in vitro* (see ref. [7]), it is necessary to describe fully the composition of solutions of the substrates. The former of these problems has been tackled using a combination of high-resolution ^{1}H NMR and ^{13}C NMR to determine the positions of enantiomeric and tautomeric isomerism, respectively. The results are reported herein.

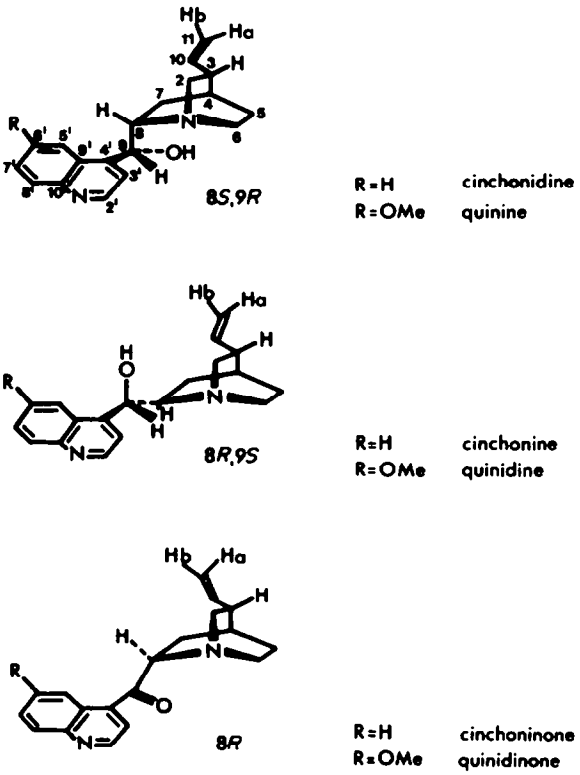

Fig. 1. Structures of *Cinchona* alkaloids.

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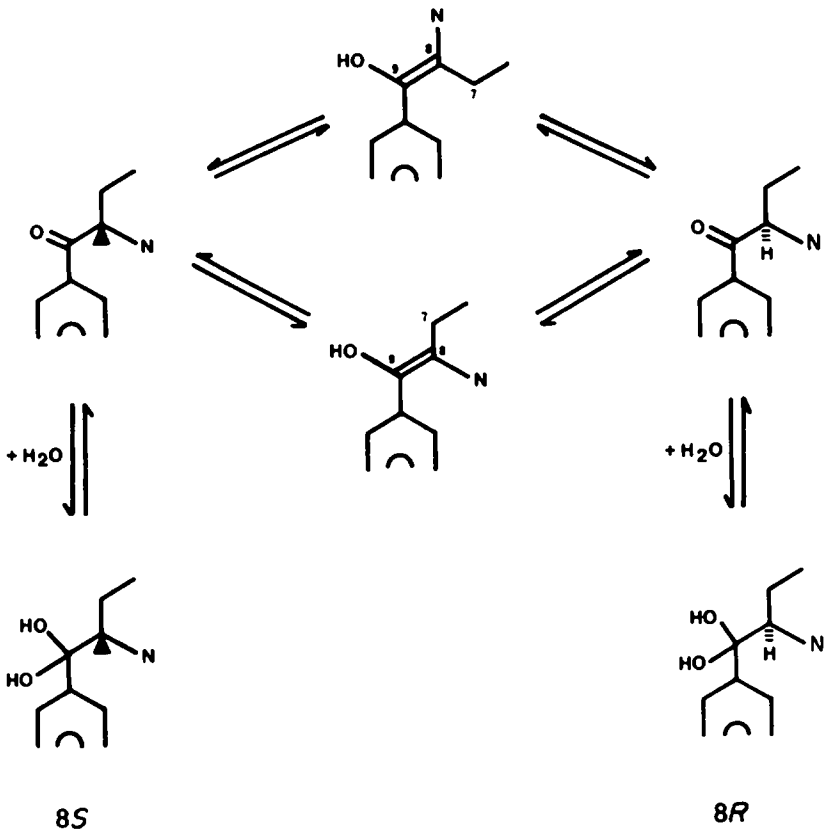

Fig. 2. Partial structures showing the various configurations generated by tautomerization of cinchoninone or quinidinone in aqueous solution.

RESULTS AND DISCUSSION

^{1}H *NMR spectra of the free bases in chloroform*

The ^{1}H NMR spectra obtained for five *Cinchona* alkaloid bases in $CDCl_3$ are summarized in Table 1. Three distinct regions are identified, corresponding to the quinoline nucleus (7.5–9 ppm), the vinyl side chain plus H-9 (4.8–6 ppm), and the quinuclidine ring (1–3.5 ppm). The first two of these regions can be fully assigned by comparison with *N*-benzoylquinotoxine [10] and quinidinone [11], and from the differences observed at C-6′ between the methoxylated and unmethoxylated species.

In the region 4.8–6 ppm (Fig. 3), the resonances show differing chemical shifts characteristic of the steric series to which the compound belongs, though, as expected, the same pattern of coupling is shown by all the bases (Table 1). Thus, in cinchonine (Fig. 3a), H-10 shows an octet at 5.97 ppm with three coupling constants (J = 7.3, 11.3 and 16.2 Hz). The two doublets at 5.00 (J = 10.2 Hz) and 4.99 ppm (J = 16.0 Hz) are assigned therefore to H-11b and H-11a, respectively, on the basis of their coupling with H-10. The remaining doublet at 5.67 ppm (J = 4.6 Hz) is due to H-9 and, as would be predicted, is absent in cinchoninone. In cinchonidine (Fig. 3b), H-10 resonates further upfield at 5.71 ppm. H-11b and, to a lesser extent, H-11a are also found at higher field. These differences in these three well-resolved proton resonances make it possible to determine the amounts of 8*R*- and 8*S*-isomer in an unknown mixture, and this property has been exploited to measure the 8*R*:8*S* ratio in an equilibrium mixture of cinchoninone (see below).

The high-field region is complex and largely unassigned but also shows differing shifts for the two steric series. Some assignments can be made on the basis of coupling constants; the complex resonances are too closely juxtaposed to be analysed by conventional irradiation methods. Two well-resolved octets due to *t*-protons occur in cinchonine. That at 3.29 ppm has J = 7.9 Hz, consistent with this being coupled to H-10, making it H-3. Similarly, the octet at 3.08 ppm may be assigned to H-8 on the basis of J = 4.6 Hz, as found for H-9, and its shift in cinchoninone to 4.16 ppm. A quartet at 2.86 ppm may be assigned to H-4 on the basis of apparent coupling (J = 13.4 Hz) to H-3. The other protons have not been assigned.

In the 8*S*,9*R*-series, protons resonate in this region at slightly differing chemical shifts, indicating small changes in the shielding environment, but the coupling pattern is closely analogous and assignments may be similarly made (Table 1).

^{1}H *NMR spectrum of cinchoninone in chloroform*

Woodward *et al.* [12] argued, on the basis of high positive specific optical rotations, that the 8*R* (quinidine) configuration should be assigned to the crystalline ketone they prepared from quinine. In polar solution, pure

Table 1. ^{1}H NMR spectral data from the spectra of *Cinchona* quinoline alkaloids in $CDCl_3$

H	Cinchonine	Quinidine	Cinchoninone	Cinchonidine	Quinine
2′	8.86	8.67	8.97	8.82	8.70
	(*d*; 4.6)	(*d*; 4.3)	(*d*; 4.4)	(*d*; 4.6)	(*d*; 4.6)
3′	7.58	7.51	7.65	7.55	7.49
	(*d*; 4.6)	(*d*; 4.6)	(*d*; 4.6)	(*d*; 4.8)	(*d*; 4.6)
5′	8.08	7.17	8.20	8.09	7.22
	(*d*; 8.6)	(*s*; *meta* = 2.4)	(*d*; 8.5)	(*d*; 8.5)	(*s*; *meta* = 2.7)
6′	7.66	—	7.72	7.65	—
	(*t*; 7.7, 7.7)		(*t*; 7.7, 7.7)	(*t*; 7.6, 7.6)	
–OMe	—	3.87	—	—	3.90
7′	7.45	7.31	7.58	7.44	7.33
	(*t*; 7.7, 7.7)	(*d*; 9.2, *meta* = 2.7)	(*t*; 7.7, 7.7)	(*t*; 7.6, 7.6)	(*d*; 9.2, *meta* = 2.7)
8′	7.98	7.97	8.12	7.96	7.99
	(*d*; 8.2)	(*d*; 9.2)	(*d*; 8.5)	(*d*; 8.5)	(*d*; 9.2)
2	n.a.	n.a.	n.a.	n.a.	n.a.
3	3.29	3.21	3.15	3.41	3.36
	(*o*; 1.5, 7.9, 13.7)	(*o*; 1.5, 7.9, 13.1)	(*d*; 10.3, 14.1)	(*h*; 2.7, 5.4, 10.6, 13.3)	(*h*; 2.8, 5.6, 10.6, 13.5)
4	2.86	2.88	n.a.	3.05	3.07
	(*q*; 10.1, 13.4)	(*q*; 9.9, 13.0)		(*q*; 10.3, 13.6)	(*q*; 10.1, 13.7)
5	n.a.	n.a.	n.a.	n.a.	n.a.
6	n.a.	n.a.	n.a.	n.a.	n.a.
7	n.a.	n.a.	n.a.	n.a.	n.a.
8	3.08	3.07	4.16	3.11	3.15
	(*o*; 4.6, 9.2, 9.2)	(*o*; 4.4, 8.6, 9.2)	(*q*; 9.8, 18.4)	(*o*; 4.6, 8.1, 8.1)	(*o*; 4.9, 8.8, 8.8)
9	5.67	5.50	—	5.62	5.55
	(*d*; 4.6)	(*d*; 4.3)		(*d*; 4.3)	(*d*; 4.3)
–OH	3.34 *s* (*br*)	n.a.	—	n.a.	3.39 *s* (*br*)
10	5.97	5.96	5.92	5.71	5.74
	(*o*; 7.3, 10.3, 16.2)	(*o*; 7.5, 10.1, 16.4)	(*o*; 7.3, 10.0, 17.3)	(*o*; 7.4, 10.2, 17.3)	(*o*; 7.7, 10.1, 17.4)
11a	4.99	4.98	5.08	4.93	4.95
	(*d*; 16.0)	(*d*; 16.6)	(*d*; 16.2)	(*d*; 15.9)	(*d*; 16.7)
11b	5.00	4.99	5.05	4.89	4.91
	(*d*; 10.2)	(*d*; 10.8)	(*d*; 11.1)	(*d*; 10.8)	(*d*; 9.9)

Multiplicity of couplings (in Hz) are given in parentheses.
n.a. = Not assigned.

crystalline quinidinone undergoes slow mutarotation [9], forming a mixture of 8*R*- and 8*S*-epimers. In non-polar solution, epimerization does not occur. As shown in Fig. 3c, the ^{1}H NMR spectrum, obtained from a solution of pure crystalline cinchoninone in $CDCl_3$, of the ketone derived from cinchonidine closely correlates with that of cinchonine (Fig. 3a), confirming that it is the 8*R*-species which crystallizes. The 8*S*-isomer cannot be obtained as a crystalline solid. The assignment of H-8 of cinchoninone to the resonance at 4.16 ppm is confirmed by its being fully exchangeable in D_2O.

^{1}H *NMR spectra of cinchoninone and quinidinone in aqueous solution*

The 8*R*:8*S* ratio was obtained from the ^{1}H NMR spectra of the DCl salts of these bases, which are freely soluble in D_2O. As it was desired to determine the ratio in conditions as close as possible to those used for the enzymological studies [7], the pD of the solutions was adjusted to between 6.4 and 6.5 with NaOD. Above pH 6.5–7.0, the free base precipitates and is too insoluble for spectral determinations to be made. As it takes less than 3 hr for enantiomeric equilibrium to be established [9], spectra were obtained from such solutions approximately 3 and 17 hr after solubilization. These spectra were identical, indicating that equilibration was complete, and showed resonances due to both steric series. Figures 3d and 3e show those obtained after 17 hr for cinchoninone and quinidinone. From the areas of the peaks due to the different steric isomers the 8*R*:8*S* ratio was determined as 7.74:2.26 for cinchoninone and 6.74:3.26 for quinidinone. Thus, in solution, it is the opposite steric series (8*S*) to the crystalline form (8*R*) which dominates.

^{13}C *NMR spectra of cinchoninone and quinidinone in aqueous solution*

Preliminary experiments showed by ^{13}C NMR that tautomeric equilibrium was rapidly established, there being no difference between the spectra obtained 2, 18 and 170 hr after dissolution. The resonances in the ^{13}C NMR spectra of cinchoninone and quinidinone in D_2O after approximately 18 hr are given in Table 2. The majority of the carbon atoms within these structures have a chemical shift close to the reduced forms and can readily be assigned on that basis [13]. As expected, the resonance around 71 ppm from C-9 has been lost and replaced by a carbonyl resonance at about 198 ppm, and C-8 shows the

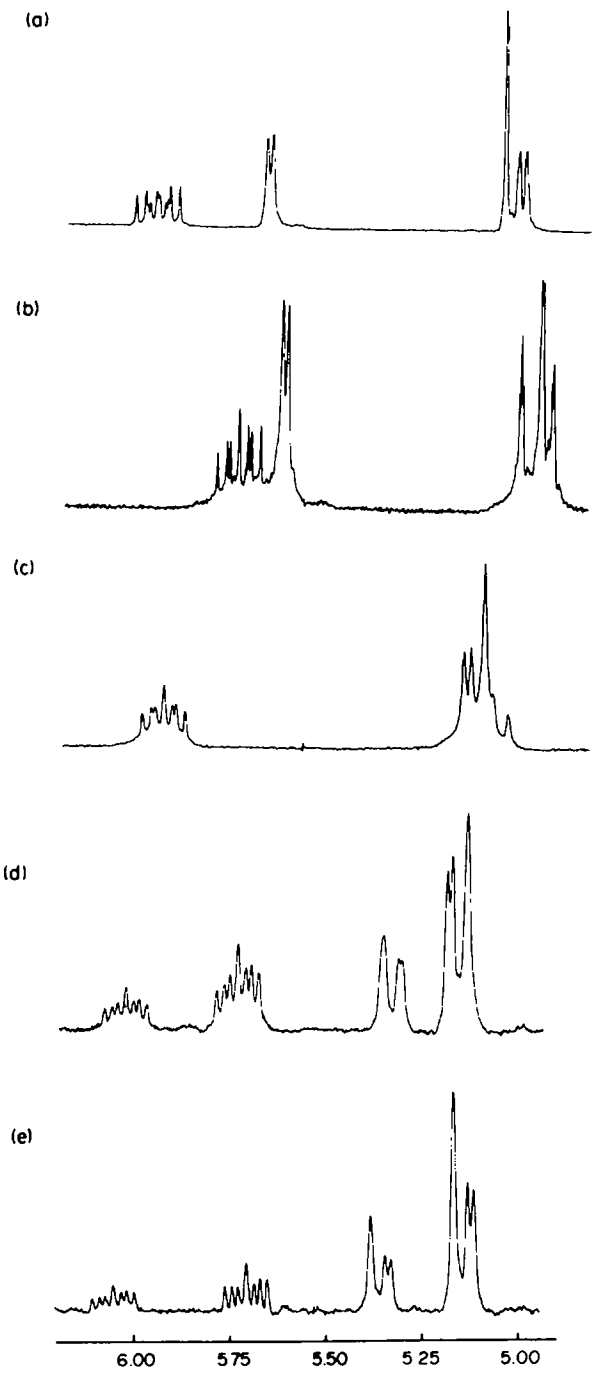

Fig. 3. ^{1}H NMR spectra of *Cinchona* quinoline alkaloids in the region 6.2–4.8 ppm. (a) Cinchonine in $CDCl_3$; (b) cinchonidine in $CDCl_3$; (c) cinchoninone in $CDCl_3$; (d) cinchoninone in D_2O (pD 6.5); (e) quinidinone in D_2O (pD 6.5). For acquisition conditions, see Experimental.

expected small downfield shift (*ca* 3 ppm) as a result of this change.

The equilibrium mixture could potentially contain up to six species resulting from tautomerism at the C-8 to C-9 bond (Fig. 2), viz. the 8*S*- and 8*R*-ketones, the 8*S*- and 8*R*-diols and the *Z*- and *E*-8,9-enols. A strong resonance at 198 ppm indicates that, like oxaloacetate [14], the keto form is the predominant species at neutral pD. Minor resonances, indicative of the presence of the two enol and the diol species, are also apparent. Thus, minor resonances at 107.38 ppm in cinchoninone and at 103.39 ppm in quinidinone are assigned to the C-9 geminal diol on the basis of analogy with other dioxy-substituted carbons such as those in sucrose (104.4 ppm) [15], tazettine derivatives (101.2 ppm) [16] and *exo*-brevicomin (107.6 ppm) [15].

The enol could be present as one or both of the *Z*- and *E*-isomers. From a consideration of the structures involved, it is found that both these forms are subject to steric interference since the 8,9-double bond causes the two rings of the molecule to be brought into closer proximity than in the keto or diol species. Nevertheless, the ^{13}C NMR spectra contain a number of minor resonances which can satisfactorily be assigned to C-7, C-8 and C-9 of the enolic species. In cinchoninone, resonances at 139.32, 138.56, 125.14 and 117.94 ppm may be assigned to the two C-8 and the two C-9 nuclei of the *Z*- and *E*-enols. The assignment of the higher field resonances to C-8 is based on comparison with a number of other alkaloids such as the cryptoechinulines (144.4 and 111.7 ppm) [17] and the staphidines of *Delphinium* (146.7 and 101.2 ppm) [18], which contain similar partial structures. It has not proved possible to distinguish the pairs of resonances representative of the *Z*- and *E*-forms. C-7 also, as expected, shows small changes due to the *Z*- and *E*-configurations, and two minor resonances at 24.42 and 24.19 ppm may be assigned to this nucleus. In quinidinone, similarly, resonances are found with the correct chemical shifts for the enolic species, at 138.97, 136.81 and 117.77 ppm. The other C-9 resonance is lost, presumably as it underlies a signal from another nucleus, possibly C-11. Two resonances at 24.89 and 24.78 ppm may be assigned to C-7.

CONCLUSIONS

By using a combination of high-resolution ^{1}H NMR and ^{13}C NMR it has proved possible to resolve the mixture of structures present in solutions of the alkaloids cinchoninone and quinidinone. The steric isomer which crystallizes is shown, by ^{1}H NMR, to be the opposite to that predominant in aqueous solution, the 8*S*-conformer being the more favoured species with both compounds. The 8*R*:8*S* ratio found (23:77) for cinchoninone in aqueous solution is similar to the cinchonine:cinchonidine ratio obtained (39:61) when cinchoninone (8*R*) is reduced *in vitro* by the cinchoninone:NADPH oxidoreductases from *C. ledgeriana* cells [7]. While this may be coincidental, it could indicate that the affinities of the isoenzymes for cinchoninone and cinchonidinone are similar.

The ^{13}C NMR spectra show that in neutral solution, although the keto is the dominant species, significant amounts of both the enol and the geminal diol are present. Although an accurate quantitative determination of the concentration of each of these cannot be obtained from broad-band ^{1}H-decoupled ^{13}C NMR spectra, the keto:enol:diol ratio for oxaloacetate obtained by ^{13}C NMR [14] (pH 7.0) from the peak heights of the C-3 resonances is 75:14:11, which agrees well with the values 74:18:8 (pH 7.4) obtained by rapid-reaction analysis of the enzymatic reduction of oxaloacetate [20]. The peaks due to the C-9 resonances in the ^{13}C NMR spectra of

Table 2. ^{13}C chemical shifts for cinchoninone and quinidinone in D_2O (pD 6.4)

	Cinchoninone			Quinidinone		
C	Keto	Enol*	Diol*	Keto	Enol*	Diol*
2	49.08			49.20		
3	36.28			36.40		
4	27.30			27.36		
5	23.54			24.01		
6	48.73			48.61		
7†	23.60	24.19		23.84	24.78	
		24.42			24.89	
8†	63.26	138.56		63.64	136.81	
		139.32			138.97	
9†	197.97	117.94	107.38	198.32	117.77	103.39
		125.14			n.r.	
10	138.91			136.73		
11	124.05			125.46		
OMe	—			56.42		
2′	147.89			144.25		
3′	121.59			122.35		
4′	150.59			147.66		
5′	117.48			102.98		
6′	129.40			159.69		
7′	130.10			117.06		
8′	131.69			130.69		
9′	124.82			123.11		
10′	137.09			137.67		

*As for keto unless shown otherwise.

†Values for both *Z*- and *E*-enol species are shown but not assigned to one or the other form.

n.r., Not resolved due to overlapping peaks.

Table 3. Relative composition of an equilibrium mixture of cinchoninone or quinidinone in D_2O at pD 6.4

	Cinchoninone		Quinidinone	
Tautomeric species	8*S*	8*R*	8*S*	8*R*
Keto	49	14	47	22
Enol*	14		15	
	10		n.r.	
Diol	10	3	11	5

**Z* and *E*, not assigned

n.r., Not resolved.

cinchoninone and quinidinone give ratios for keto:enol:diol of 76:13:11 and 69:15:16, respectively, very similar to those obtained for oxaloacetate by two independent methods [14, 20]. Hence, the equilibrium mixtures of cinchoninone and quinidinone may be concluded to contain species in the ratios shown in Table 3. Using these values, the real substrate concentrations may be predicted and the apparent kinetic constants of the cinchoninone:NADPH oxidoreductases corrected [7].

EXPERIMENTAL

Materials. D_2O and $CDCl_3$ from Aldrich: gold label 99.996 atom % for ^1H NMR; 99.8 atom % for ^{13}C NMR. NaOD was from Fluorochem Ltd. as a 30 % (w/v) soln in 99.8 atom % D_2O. Cinchoninone and quinidinone were prepared by oxidation of cinchonidine and quinine (Sigma), respectively, with potassium *t*-butylate [12].

Preparation of alkaloids for NMR spectroscopy. The DCl salts of the keto bases were obtained by D_2O substitution of H_2O from a soln (300 mg) in dilute HCl. For ^1H NMR, *ca* 10 mg was dissolved in 2 ml D_2O and the pD adjusted to 6.5 with 1 M NaOD in D_2O. For ^{13}C NMR, *ca* 250 mg dissolved in 1 ml D_2O was similarly treated. ^1H NMR spectra were also recorded for solns of the free bases with *ca* 5 mg in 1 ml $CDCl_3$.

Recording of NMR spectra. ^1H NMR were recorded on a Bruker CXP 300 spectrometer (300 MHz) at normal probe temp. Spectra were recorded in $CDCl_3$ with a spectral width of 5000 Hz, a 32K data table, a 10 sec pulse repetition rate and a 10 μsec pulse width. Spectra were recorded in D_2O (pD 6.5) using a spectral width of 3500 Hz, a 32K data table, a 6 sec repetition rate and a 20 μsec pulse width. In both solvents a pulse angle of 90° was used. The signal from D_2O was used as the external lock and chemical shifts were determined relative to a TMS standard in $CDCl_3$ or to the H_2O resonance (4.80 ppm) in D_2O.

^{13}C NMR spectra were determined on a Jeol JNM-FX100 Fourier transform NMR spectrometer at 25.05 MHz. Spectra

were recorded at ambient temp. using the deuterium signal of D_2O as the internal lock signal with full broad-band proton decoupling of 2500 Hz from a 99.55 MHz source. Chemical shifts were measured on 6024 Hz sweep width spectra relative to an internal capillary standard of TMS, accumulated with a pulse angle of 30°, a pulse width of 5 μsec and a pulse repetition of 3 sec. Approximately 20 000 data accumulations were obtained on each sample.

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