described protocol, we obtained milligram quantities (3, 8, 14 mg, respectively) of the analytically pure compounds *B*, *C*, and *D*.

We have not yet determined the structure of the minor component *B*, but we have assigned the tricyclic 6.6.5 structures **3a** and **3b**, respectively, to compounds *C* and *D* on the basis of their spectroscopical data and reactivity. We have been able to get quite good crystals of **3b** but were unable to get suitable X-ray data even with a synchrotron apparatus.

Both **3a** and **3b** exhibit closely related mass spectra.[7] Their ^1H and ^{13}C NMR spectra clearly suggest[8] that the cyclization process has only involved the epoxide ring, the Δ^{6-7}, the Δ^{10-11}, and the Δ^{14-15} carbon–carbon double bonds, leaving unaffected the Δ^{18-19} *Z* and the Δ^{22-23} carbon–carbon double bonds originally present on the oxidosqualene **1b**. They furthermore imply[8,9a] the presence of an extra terminal carbon–carbon double bond on **3a**.

Their IR spectra exhibit an absorption at 3580 cm^{-1} suggesting the presence of hydroxyl moieties on each compound. This is corroborated by their polar nature (see above the description of their behavior on SiO_2 which is close to that of lanosterol) as well as by their ^1H and ^{13}C NMR spectra.[8,9b]

3a and **3b** are monoacetylated after reaction with an excess of acetic anhydride in pyridine (20 °C, 17 h; acetate of **3a**: 87% yield, R_f 0.88; acetate of **3b**: 92% yield, R_f 0.42 on SiO_2 TLC, benzene/ethyl acetate, 95:5). Under the same conditions lanosteryl acetate has an R_f of 0.91, and even under more drastic conditions (excess reagent and longer reaction time) compound **3b** cannot be diacetylated. This behavior supports the assignment of the hindered tertiary alcohol (C-14 hydroxyl group) on **3b**.

The discrimination in favor of 6.6.5 arrangements come from the NMR studies which are disclosed on the accompanying paper[8] as well as from the typical fragmentations observed on the mass spectra of the related compounds **3c** and **3d** available by chemical degradation[10] of the side chain of **3a**.

The stereochemistry of all the ring junctions of both **3a** and **3b** has been deduced from the interpretation of their 2D NMR which is presented in the accompanying paper.[8] Both **3a** and **3b** exhibit trans/syn/trans A/B/C ring junctions. However, although it has not yet been unambiguously secured, their stereochemistry at C-13 seems to be different.[8]

The β orientation of the side chain in **3a** leads us to assume that the cyclizing enzyme(s) operated normally[2,3] on **1b** insofar as possible and cyclized it from the chair–boat–chair interrupted conformation. The major biocyclized component **3b** if it possesses the α-oriented side chain would arise from an "abnormal" chair–boat–boat folding. At this point the stereochemistry at C-13 of **3a** and **3b** must be firmly established. We are therefore planning to prepare related crystalline derivatives suitable for X-ray analysis.

For the first time (i) tricyclic compounds are formed by enzymatic cyclization of an oxidosqualene analogue possessing the complete set of carbon–carbon double bonds, and (ii) the length of the hydrocarbon chain attached to the *Z* Δ^{18-19} carbon–carbon double bond of 2,3-oxidosqualene analogues is shown to have a dramatic influence on the nature of the enzymic products. This effect has never been observed[1,2,11] in the *all-trans* series of **1**.

This result let us to presume that in the enzyme, 2,3-oxidosqualenes **1** are first cyclized to tricyclic 6.6.5 intermediates **4**, which then rearrange to 6.6.6 intermediates **5** precursors of the tetracyclic pattern of steroids when an extra stabilization arising, for example, from the Δ^{18-19} carbon–carbon double bond, is available.[12] This stabilization is missing when the Δ^{18-19} carbon–carbon double bond is missing,[13-15] and in fact a compound with a 6.6.5 arrangement of the A/B/C rings is isolated suggesting that in **1b** the side chain is, for steric reasons, unable to attain the required conformation which would allow this extra stabilization. With this respect, compounds **3a** and **3b** obtained during this study would be ideal for testing this hypothesis. They will be transformed into their analogues possessing an *E* rather than a *Z* trisubstituted carbon–carbon double bond in the hope that they would lead to tetracyclic derivatives on further reaction with oxidosqualene sterol cyclase.[16]

(7) Mass spectra of **3a**: 426, 408, 365, 283, 271, 257, 247, 229, 213, 203, 189, 187, 175, 161, 147, 121, 119, 107, 95, 81, 69, 55. For **3b**: 426, 408, 393, 365, 339, 283, 271, 257, 247, 229, 204, 203, 189, 175, 161, 147, 135, 121, 119, 107, 95, 81, 69, 55. We acknowledge Dr. P. Sandra (University of Gent) for these measurements.

(8) Guittet, E.; Herve du Penhoat, C.; Lallemand, J.-Y.; Schauder, J. R.; Krief, A., following paper in this issue.

(9) (a) ^1H NMR: two singlets at 4.85 and 4.62 ppm, attributed to the hydrogen linked to the terminal carbon–carbon double bond; ^{13}C NMR: two signals at 149.20 and 109.06 ppm attributed to the two sp^2 olefinic carbon atoms. (b) ^{13}C NMR of **3a**: 79.25 (C-3); of **3b**: 79.40 (C-3) and 76.45 ppm (C-14).

(10) By ozonolysis of **3a** (O_3/CH_2Cl_2 then Me_2S), then oxidation of resulting keto aldehyde **3c** with Jones' reagent (2 equiv, acetone, 0 °C, 0.3 h), and then esterification (CH_2N_2/ether, 20 °C, 14% overall yield of **3d**).

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(15) (a) The action of the cyclase on 15′-nor-18,19-dihydro-2,3-oxidosqualene leads[15b] however to a tricyclic compound possessing a 6.6.6 arrangement. (b) van Tamelen, E. E. *J. Am. Chem. Soc.* **1982**, *104*, 6480 and references cited.

(16) We thank I.R.S.I.A. Belgium for a fellowship to J. R. Schauder and the FNRS (Belgium) for financial support.

Application of Homonuclear Chemical Shift Correlation NMR to the Study of the Cyclization Products of Unnatural Δ^{18-19}-(*Z*)-Epoxysqualene

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Interpretation of the results of the biological cyclization of 2,3-oxidosqualene analogues possessing the Δ^{18-19}-*Z* stereochemistry[1] required the structural elucidation of the products. Surprisingly enough, although NMR studies of macromolecules of biological interest is widespread, the complete structural elucidation of some comparatively small molecules such as saturated functionalized steroid-type systems remains a real challenge. This communication describes the structural determination of three tricyclic derivatives **1**, **2**, and **3** based on a combination of force-field calculations and 2D NMR, an approach easily extended to related compounds.

From $^1H^2$ and $^{13}C^3$ 1D NMR and mass spectra it appeared that compound **1** contained a tertiary and a quaternary hydroxyl group, two trisubstituted double bonds, and consequently three cycles.

A multiple quantum-filtered COSY experiment[4] revealed several distinct proton filiations (Figure 1). The intact fragment corresponding to carbons 16–24, 29, and 30 of starting epoxy-

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(1) Krief, A.; Schauder, J. R.; Guittet, E.; Herve du Penhoat, C.; Lallemand, J. Y., preceding paper in this issue.

(2) $CDCl_3$: five methyl groups at 0.878, 1.036, 1.068, 1.157, and 1.225 ppm; allylic methyl groups at 1.706 (3 H) and 1.782 (6 H) ppm; one proton α to a hydroxyl group at 3.310 ppm (d × d, 11.5 and 5.2 Hz); two ethylenic protons at 5.21 ppm (d × d, 8.0 and 8.0 Hz).

(3) C_6D_6: 26 sp3 carbons; two carbons bearing a heteroatom at 75.8 (d) and 79.2 (s) ppm.

(4) Piantini, U.; Soerensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801.

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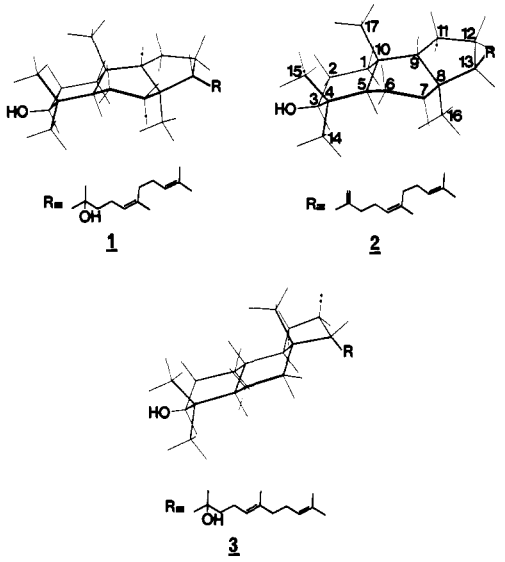

squalene was readily identified demonstrating incomplete cyclization compared to sterols. However, due to the very strong overlapping of signals, the assignment was ambiguous, and it was necessary to perform more sophisticated experiments. The ω1-decoupled COSY[5] spectrum proved to be more legible than the MQT COSY one, largely compensating for the disappearance of some cross peaks due to an unsuitable total transfer time (a phenomenom which actually enhances the legibility). The correlations within the cycle C became straightforward.

Long range W couplings have been extensively used to elucidate complex structures such as those of triterpenes.[6] In the case of **1** this approach was even more fruitful when associated with the RELAY experiment[7] which allowed edition of part of the graph of the various cycles on the well-defined and isolated methyl signals. Thus relay connectivities were established for 12α-28 through 13α-28, 2α and 2β-17 through 1α-17, 6α or 6β-16 through 7β-16 and 5β-14 through 14-15 (Figure 1).

The only structure compatible with the NMR data was **1**. The geometry at the junctions was determined by the small (unfavorable ζc) positive NOEs at 200 MHz. For instance, the cross-relaxation observed between methyl-16 and H-5 (doublet at 1.633 ppm) required a trans junction for cycles B and C. To accommodate this junction, B must adopt a boat conformation. The 3β geometry of the hydroxyl group in cycle A was based on the values of the H-3 coupling constants. It should be noted that the shifts of methyls 14, 15, and 17 are similar to those observed for analogues 3β-OH steroids.[8]

At this point the only undefined stereochemistries were those of C-13 and C-14. Precise values for the coupling constants of cycle C were extracted from high resolution 2D spectra[9] or measured from 1D spectra after establishing their relative values from 2D experiments. The inserts in Figure 1 represent the correlation peaks arising from proton 12 (positive and negative intensitieis are represented by filled and open circles, respectively) with the active and passive coupling clearly shown. However, these data did not resolve the problem at hand. Despite models based on scalar couplings and/or chemical shifts,[10,11] structural elucidation in the somewhat flexible five-membered ring systems is a hazardous task.

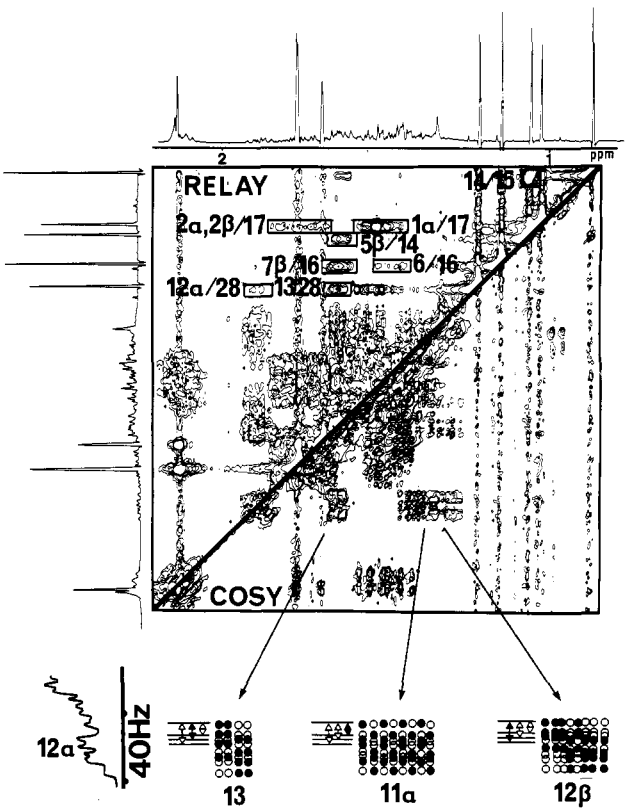

Figure 1. Contour plot of the 400.13-MHz homonuclear spin correlation maps (2 mg, $CDCl_3$, high field expansion) corresponding to a RELAY (total mixing time: 70 ms) (NW half) and a multiple-quanta filtered COSY (SE half) experiment. The corresponding 1D spectrum is provided on the abscissa and the ordinate. The 2D correlation maps consisted of 512 × 1 K data points spectra, each composed of 48 and 64 transients, respectively. A 1-s delay was allowed between each scan. The inserts show the schematic representation of different cross peaks to 12α as determined from the actual correlation peaks with the active (↔) and passive (↔) corresponding couplings, referred to the 1D expansion.

Table I. Calculated and Observed Coupling Constants for the Various Possible Geometries at C13

	H12α,Rα	H12α,Rβ	H12β,Rα	H12β,Rβ
		12β–13		
calculated	5.8	6.2	3.8	−1.0
observed	6.3	6.3	9.6	9.6
		12α–13		
calculated	9.2	13.4	10.2	(34.3)
observed	9.6	9.6	6.3	6.3

Thus, we turned to force-field calculations. Molecular parameters were calculated for the two geometrical families corresponding to the two diastereoisomers of **1** (differing only by the position of the side chain at C-13) by using this method (MM2).[12] Concomitantly, coefficients for a Karplus-type equation were estimated from the measured vicinal coupling constants between H-9 and H-11β, H-9 and H-11β, H-11α and one H-12 (assigned indifferently to H-12α or H-12β). The values for $J_{13,12\beta}$ and $J_{13,12\alpha}$ were then calculated and compared to the measured values. Best concordance was obtained for the α geometry of the side chain at C-13 (Table I). However, this approach has clear limitations, and this assignment is only tentative. The configuration of C-14 could not be determined.

The above strategy was applied to the olefin **2**, obtained with diol **1** during the biochemical cyclization of Δ^{18-19}-(*Z*)-epoxysqualene. In this case, only the configuration of chiral center C-13

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was ambiguous. The chemical shifts for all methyl groups were comparable to those of **1**. The arguments previously used for proposing the C-13 configuration of **1**, when applied to **2**, favor the β geometry of the side chain, a result that correlates well with Sharpless' structural proposals for related cyclization products.[13] Finally, we reinvestigated and confirmed structure **3** proposed by van Tamelen for the cyclization product of all *trans*-2,3-oxidosqualene under acidic conditions ($SnCl_4$, benzene).[14]

Confirmation of the configuration at C-13 in **1** and **2** as well as the determination of that of C-14 in **1** remains a necessary step in understanding the enzymic mechanism. If **1** and **2** really have different stereochemistry at C-13, a detailed discussion of the possible geometry of the transition state would shed new light on the enzyme–substrate relationship. However, the unambiguous demonstration of the formation of the 6-6-5 squeleton underlines the crucial role of the entire side chain in the cyclization process (see preceding paper).

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Protein Nitrogen Coordination to the FeMo Center of Nitrogenase from *Clostridium Pasteurianum*

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Nitrogenase is a two-component enzyme system consisting of an iron (Fe) and an iron–molybdenum (FeMo) protein.[1] Together these proteins catalyze the ATP-driven six-electron reduction of N_2 to ammonia with concomitant evolution of H_2. In its resting state the FeMo protein exhibits a unique EPR spectrum that is associated with the FeMo cofactor (FeMoco or M center) which is believed to be a key part of the active site for N_2 fixation.[2,3] The EPR signal arises from an $S = {}^3/_2$ center consisting of 6–8 iron atoms and 8–10 sulfur atoms per molybdenum atom.[3–5] Information concerning the cofactor structure has been obtained from X-ray absorption,[6] EXAFS,[6,7] Mössbauer,[8] and EPR[9] data

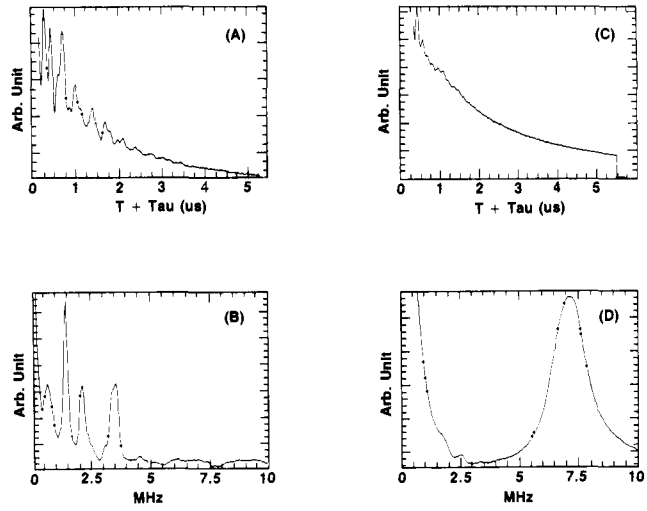

Figure 1. The three pulse stimulated echo envelope waveform is shown for the FeMo protein in (A) and its Fourier transform in (B) and for the FeMo cofactor in (C) and its Fourier transform in (D). The time axis in (A) and (C) is $\tau + t_1$. Conditions are $\tau = 0.12$ μs, B_0, 1740 G, microwave frequency, 9.0595 GHz; temperature, 4.2 K; $\pi/2$ pulse width, 0.02 μs. Fourier transformation was facilitated by using a procedure described by Mims.[21]

and, more recently, from ^1H, ^{57}Fe, ^{95}Mo, and ^{33}S ENDOR studies.[10,11] Indirect evidence for nitrogen[12] or oxygen donor ligands to Mo[6,7a] and, somewhat less definitively,[7c] to Fe was previously suggested in the analysis of EXAFS data. However, no conclusive evidence for the type of ligand nor its definition as part of FeMoco or as part of the protein was presented. In this communication we report electron spin echo (ESE) experiments on the resting state of FeMo protein isolated from *Clostridium pasteurianum* and of FeMoco isolated in *N*-methyl formamide (NMF) from that protein. The ESE modulation spectrum of the protein contains lines characteristic of nuclear quadrupole transitions for nitrogen coordinated to the paramagnetic metal center. These frequencies are clearly absent from the spectrum of FeMoco isolated in NMF suggesting that the FeMo cofactor is coordinated

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(11) ^{14}N couplings were not observed in recent ENDOR studies on the FeMo protein reported by Hoffman, Orme-Johnson, and co-workers. Two possibilities exist to account for the observation of ^{14}N couplings in the present ESE experiments but not in the ENDOR experiment. First, the pure quadrupole transitions, $\Delta M_I = \pm 1$, 2, observed in the ESE experiment are forbidden in ENDOR while the allowed ENDOR transitions, $\Delta M_S = \pm 1$, $\Delta M_I = \pm 1$, may be too broad to be detected. Second, the magnitude of the ENDOR enhancement depends on a delicate balance between nuclear and electronic spin relaxation rates. The ratio of these relaxation rates may not be favorable for ENDOR at low temperatures.

(12) Preliminary two-pulse electron spin echo data, in which low-frequency modulation was observed, was sited as an unpublished result in ref 10.

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