The Configuration at C-24 in Oogoniol (24*R*-3β,11α,15β,29-Tetrahydroxystigmast-5-en-7-one) and Identification of 24(28)-Dehydrooogoniols as Hormones in *Achlya*

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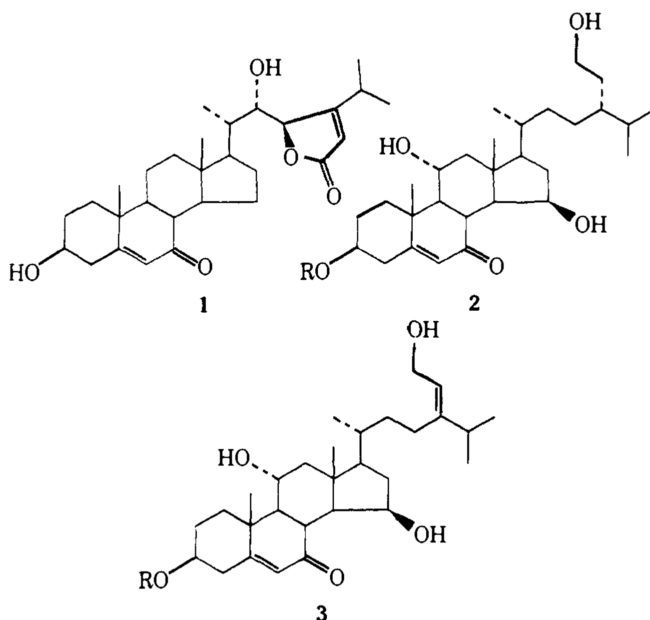
Abstract: Model steroids, 29-hydroxysitosterol and 29-hydroxyclionasterol, have been prepared. Comparison of their ^{1}H NMR spectra (220 MHz, Fourier transform), which show distinct differences, with that of oogoniol-1 indicates that the latter has the same configuration at C-24 as 29-hydroxyclionasterol. Oogoniol-1 isolated from culture liquids of *Achlya* is accompanied by a small proportion of the 24(28)-dehydro analogue, 3β,11α,15β,29-tetrahydroxystigmasta-5,24(28)(*E*)-dien-7-one 3β-isobutyrate. The two compounds can be separated by high-pressure liquid chromatography. The biological activity of the latter is considerably higher than that of oogoniol-1.

Sexual reproduction in the water mold *Achlya* is mediated by steroid hormones antheridiol (**1**) and the oogoniols (**2**, R = $(CH_3)_2CHCO$, CH_3CH_2CO, CH_3CO, H).[1] These steroids are biosynthesized from fucosterol, which is the major sterol present in the organism.[2] The structure of antheridiol, proposed 10 years ago, has been confirmed by a number of syntheses.[3] The oogoniols were isolated more recently and structures proposed for them in 1975. These have since been modified as a result of ^{13}C NMR spectral studies with model dihydroxystigmastenes, the primary hydroxyl on the side chain now being placed at C-29 instead of C-26.[4] Only one structural feature remains to be defined, namely, the stereochemistry at C-24, and we report in this paper evidence which indicates that the oogoniols possess the *R* configuration at C-24.[5] We have also succeeded in identifying a number of new steroids which are the 24(28)-dehydro analogues (**3**, R = $(CH_3)_2CHCO$, CH_3CH_2CO, H) of the oogoniols. These steroids occur as mixtures with the corresponding saturated side chain oogoniols and are separable only by high-pressure liquid chromatography.



In order to define the stereochemistry at C-24 in **2** model steroids, 29-hydroxysitosterol and 29-hydroxyclionasterol were prepared using the method developed by Sucrow and coworkers.[6] This method permits construction of the chiral center at C-24 in a predictable way from a cis allylic C-22 alcohol via Claisen rearrangement.

The starting compounds were the aldehyde **4**[7] prepared by ozonolysis of *i*-stigmasteryl methyl ether and 3-methyl-1-butyne, obtained by treatment of methyl isopropyl ketone with phosphorus pentachloride and reaction of the resulting halide mixture with potassium *tert*-amylate. The alkyne was converted to the Grignard reagent with ethylmagnesium bromide and condensed with **4** to give a mixture of acetylenic alcohols **5** and **6** which were separated by chromatography. The configuration at C-22 in the less polar alcohol **5** (22*R*) was established by catalytic hydrogenation (PtO_2) to give **7** followed by treatment with boron trifluoride–acetic anhydride, which afforded (22*S*)-22-hydroxycholesterol diacetate (**8**), mp 142–144 °C.[8] The ^{13}C NMR spectrum of the corresponding diol [52.7 (C-17), 40.3 (C-20), 11.6 (C-21), 74.0 (C-22), 33.3 (C-23), 35.7 (C-24) ppm] agreed with that expected for the authentic compound.[9] Similarly the more polar alcohol **6** (22*S*) was converted to **9** and then to (22*R*)-22-hydroxycholesterol diacetate (**10**); mp 96–98 °C; ^{13}C NMR spectrum of corresponding diol 53.2 (C-17), 42.4 (C-20), 12.5 (C-21), 74.4 (C-22), 27.8 (C-23), 36.1 (C-24) ppm.

Having established its configuration, we next partly hydrogenated the alcohol **5** (Lindlar catalyst) to give the *Z* alkene **11**. The latter was reacted with ethyl orthoacetate in a Claisen rearrangement to give in good yield the ester **12**. Catalytic hydrogenation (PtO_2) yielded **13**, which was treated with boron trifluoride–acetic anhydride and the product then converted to 29-hydroxyclionasterol (**14**) with lithium aluminum hydride. The epimeric alcohol **6** was carried through the same sequence of reactions to give 29-hydroxysitosterol (**18**).

In order to confirm the assigned stereochemistry at C-24 in **14**, the ester **12** was reduced with lithium aluminum hydride to give the alcohol **19**. Reaction of the latter with triethylamine and methanesulfonyl chloride, then lithium aluminum hydride, gave an oily product which was converted to poriferasterol (**20**) by further reaction with *p*-toluenesulfonic acid in aqueous dioxane. The melting point and 90-MHz (^{1}H) spectrum were identical with those of an authentic sample of poriferasterol.[10] Similarly, the ester **16** was converted via the alcohol **21** to stigmasterol **22** with properties identical with those of the natural product.[10]

With the model sterols **14** and **18** in hand it was now possible to compare the 220-MHz (^{1}H) spectrum of oogoniol-1 (**2**, R = $(CH_3)_2CHCO$) with those of **14** and **18**. In the spectrum of **14** the C-26 and C-27 methyl protons appear as a triplet at δ 0.84 (J = 7 Hz) because of coincidental overlap of the low-field arm of one doublet (centered at δ 0.83) with the high-field arm

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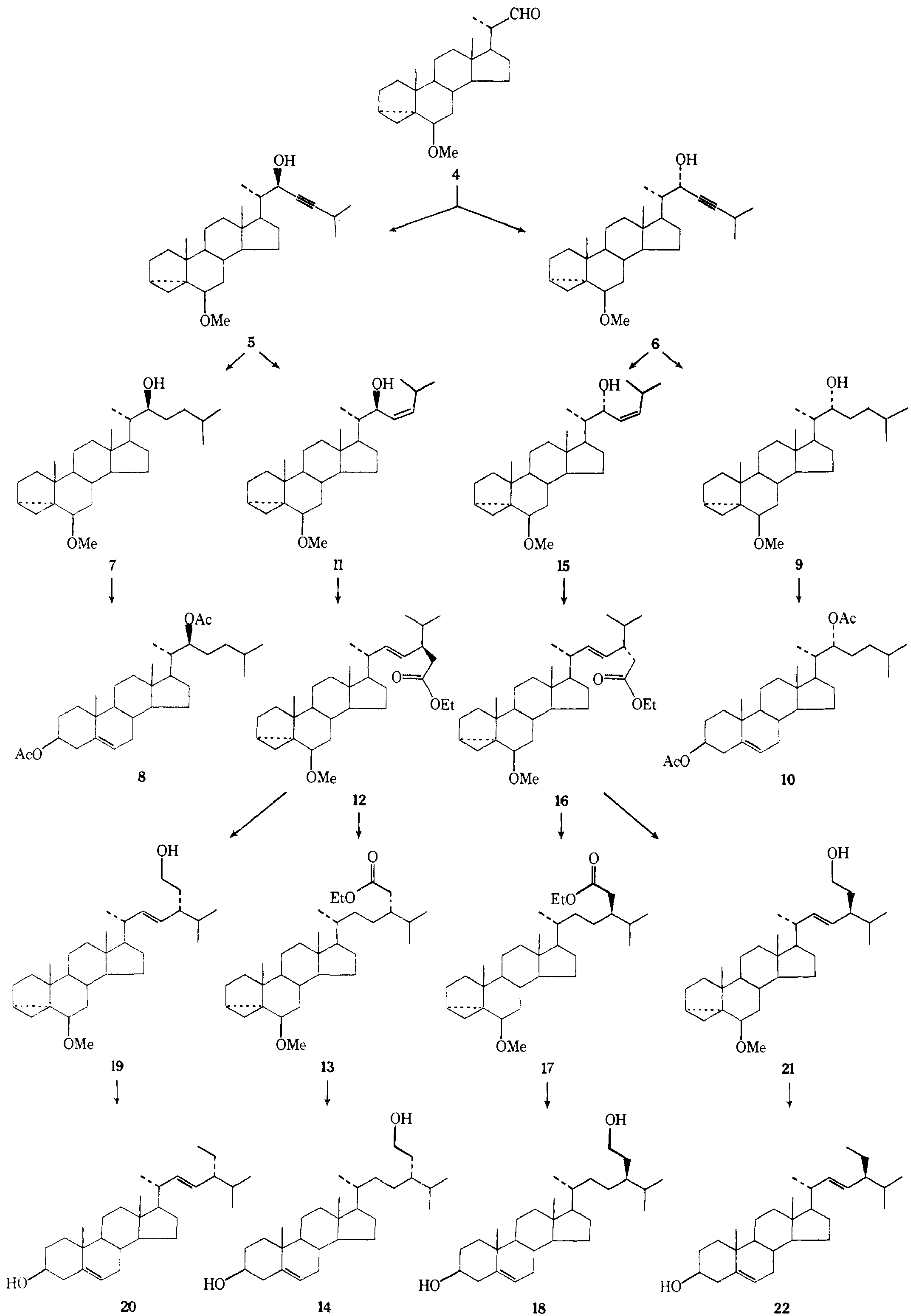
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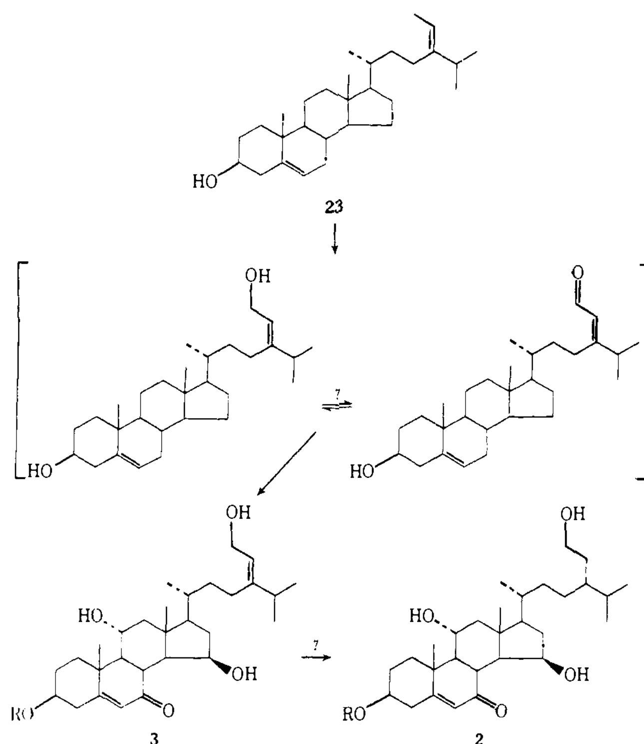
of the other (centered at δ 0.86), and the C-21 methyl protons as a doublet at δ 0.92 (J = 6.5 Hz). These peaks correspond closely with peaks in the spectrum of oogoniol-1 (triplet at δ 0.84 and doublet at δ 0.95). On the other hand, the C-26 and C-27 methyl protons appear as two doublets (δ 0.84, J = 6.6 Hz, and 0.85, J = 6.8 Hz) in the spectrum of **18.** (The C-21 methyl protons appear at δ 0.92, J = 6.4 Hz.) Thus the 220-MHz spectra clearly indicate that the configuration at C-24 in the oogoniols is the same as that in 29-hydroxyclionasterol, i.e., 24*R*. The signal for the C-29 protons in **14** appears in the form of an AB quartet centered at δ 3.65 but the C-29 protons in 29-hydroxysitosterol (**18**) give rise to a triplet at δ 3.65. The corresponding signal in the 220-MHz Fourier transform spectrum of oogoniol-1 resembles quite closely the quartet observed in the spectrum of 29-hydroxyclionasterol, thus providing additional support for the 24*R* configuration in the oogoniols.

There is one spectral feature which is not accounted for by the structure found for oogoniol-1. It is a doublet at δ 1.02 in the 220-MHz spectrum which was assigned to C-21 methyl protons.[1] We have investigated the possibility that this doublet was due to the presence of a second component in the sample of oogoniol-1. Although the latter gave a single spot on thin layer chromatography in a number of solvent systems, it has now been resolved by high-pressure liquid chromatography into two components. The major one (85%) has a mass spectrum very similar to that of the mixture. The NMR spectrum is also similar to the corresponding spectrum of the mixture. However, it lacks the doublet at δ 1.02, and the triplet at δ 0.84 is more intense. The NMR spectrum of the minor component does not have a triplet at δ 0.84 but a strong doublet at δ 1.02. There is also a vinyl proton triplet at δ 5.36 (J = 7 Hz). These signals are nicely accounted for by a structure with a side chain containing a double bond at C-24, C-28. The C-26 and C-27 methyl protons in this structure are equivalent and appear as a doublet at δ 1.02. The signal for the C-29 protons overlaps that for the C-11β proton at ~δ 4.16 and the C-28 proton gives the triplet at δ 5.36. The other peaks in the spectrum are consistent with a 24(28)-dehydrooogoniol-1 structure (**3**, R = $(CH_3)_2CHCO$) for the minor component.

The mass spectrum of the minor component does not have the intense peak at *m/e* 458 (base peak) resulting from loss of ROH from structure **2**, but rather a small peak at *m/e* 456.3250 (calcd for $C_{29}H_{44}O_4$, 456.3239) expected for the corresponding ion from the dehydro analogue **3**. There are peaks at *m/e* 438 and 420 and the base peak is at *m/e* 299.1647 (calcd for $C_{19}H_{23}O_3$, 299.1647). The latter results from cleavage of the C-17–C-20 bond with further loss of two hydrogen atoms from the tetracyclic nucleus. Fragmentations of this type have been shown to be characteristic of sterols containing a side-chain double bond.[11]

The stereochemistry of the side-chain double bond in **3** is more likely to be the same as that in fucosterol (**23**) rather than in 28-isofucosterol on biogenetic grounds. We have attempted to substantiate this with evidence from NMR spectra. The C-25 methine proton in the *E* isomer would be expected to give a signal (septet) at ~δ 2.2 while that in the *Z* isomer would absorb at ~δ 2.8, similar to that in fucosterol and 28-isofucosterol, respectively.[12] The spectrum of oogoniol-1 (saturated side chain) shows two broad singlets at δ 2.82 and 2.88 which integrate for at most two protons and are due to two of the three protons, H-4 (α and β) and H-8β. One of these presumably absorbs at higher field (<δ 2.68). (The methine proton of the isobutyrate moiety absorbs at ~δ 2.6.) The same peaks occur in the spectrum of 24(28)-dehydrooogoniol-1 but there does not appear to be a multiplet in this region which would be expected for H-25 in the *Z* isomer. However, the region around δ 2.2 in the spectrum does appear to be more complex than the corresponding region in the spectrum of the saturated side



chain analogue. A fucosterol skeleton is therefore indicated for (24)28-dehydrooogoniol-1.

Analysis of a sample of oogoniol-2 by high-pressure liquid chromatography indicates that the corresponding dehydro analogue (**3**, R = CH_3CH_2CO) is also present. This is consistent with the NMR spectrum, which has a doublet at δ 1.01 and a weak triplet at δ 5.4 expected for the presence of about 15% of **3**. The NMR spectrum of the mixture of oogoniol-3 and the C-15 ketone corresponding to oogoniol-2 also shows a weak triplet at δ 5.4 indicating the presence of one or possibly two more dehydro steroids. Finally, analysis of a sample of oogoniol shows the presence of a small amount of **3** (R = H). Thus all samples of oogoniols so far isolated by thin layer chromatography appear to be mixtures of steroids possessing saturated and unsaturated side chains. It does not matter whether the source is the hermaphroditic strain *Achlya heterosexualis*, which secretes the oogoniols under normal conditions, or the strong male *Achlya ambisexualis* E87, which secretes oogoniols only in the presence of exogenous antheridiol. The proportion of 24(28)-dehydro steroid varies with different isolates, being as high as 25% of the mixture in some cases.

The identification of dehydrooogoniols in culture liquids of *Achlya* raises interesting questions about the biosynthesis and the biological activity of this group of steroids. It is known that *Achlya* possesses a sterol composition which is typical of many brown algae (Phaeophyta).[13] Thus the major sterol in the mycelium of *Achlya* is fucosterol (**23**), which is accompanied by smaller amounts of 24-methylenecholesterol and cholesterol. These sterols are presumably biosynthesized via the cycloartenol route.[14] Carbons 28 and 29 in fucosterol produced by *Achlya* are derived from methionine[15] probably by way of *S*-adenosylmethionine as in other organisms.[16] Fucosterol appears to be the substrate for enzymatic conversion to all polyhydroxylated sterols found in *Achlya*.

For the biosynthesis of antheridiol, dehydrogenation at C-22, C-23 may be the first step in the modification of the side chain and it may precede oxidation at C-29.[17] To our knowledge metabolites of fucosterol oxidized at C-29 have not been reported from any other organisms besides *Achlya*, though the isomer of 29-hydroxyfucosterol, saringosterol (24-hydroxy-24-vinylcholesterol), has been isolated from several brown algae.[13] Oxidation to the level of carboxyl, hydroxylation, and

lactonization lead to the side chain of antheridiol. The stage at which oxidation at C-7 occurs is not known.

In the biosynthesis of the oogoniols oxidation at C-29 and C-7 also occurs but in addition there is hydroxylation at C-11 and C-15 on the steroid nucleus. No metabolites have yet been isolated which would indicate that dehydrogenation at C-22, C-23 is required. Oxidation at C-29 up to the level of aldehyde appears to occur in strains of *Achlya* which produce the oogoniols. When isolated from cultures grown in the presence of [CD_3]-methionine the oogoniols were found to contain two deuterium atoms, one at C-28 and the other at C-29.[15] Since the oogoniols possess a C-29 hydroxyl a reversible dehydrogenase ($CH_2OH \rightleftharpoons CHO$) may be operating. The identification of 24(28)-dehydrooogoniols suggests that hydroxylation at C-11 and C-15 and oxidation at C-7 may take place before reduction of the C-24, C-28 double bond. This reductive step might then be correlated with the biological activity of the compounds.

Thus 24(28)-dehydrooogoniol-1 has been found to induce formation of oogonial initials in *A. ambisexualis* 734 (♀) at a concentration of approximately 50 ng/mL while oogoniol-1 (fully saturated side chain) does not show activity at concentrations below about 5 μg/mL. These results suggest that the dehydrooogoniols may be the true hormones and they are metabolized to the saturated analogues with great loss of activity. The reductase which effects this reaction in *Achlya* does not act on fucosterol itself, for neither sitosterol nor clionasterol could be detected in the mycelium of *A. heterosexualis*.[2] Hydroxylation of fucosterol may first be required before reduction of the double bond can occur. The stereochemical outcome of the reduction, i.e., production of a clionasterol rather than sitosterol skeleton, is the same as that observed in certain algae, e.g., *Ochromonas danica*.[18]

There are several unanswered questions concerning structure–function relations among the oogoniols. They include the role of the C-11 and C-15 hydroxyl groups and the isobutyrate, propionate, and acetate functions. The presence of oogoniols with a C-15 keto group may also be significant. There is evidence of additional polyhydroxy-7-ketofucosterol derivatives in culture liquids of *Achlya* and their identification may provide further insight into the biosynthetic pathways to the hormones of *Achlya*. Feeding and trapping experiments with plausible synthetic intermediates should also prove useful.

Experimental Section

Melting points were determined on a Kofler hot-stage microscope and are uncorrected. Spectra were obtained on the following instruments: Varian EM-390 and HR-220 with Fourier transform (^{1}H NMR), Varian CFT-20 (^{13}C NMR), Perkin-Elmer spectrophotometer 550 (UV), Beckman IR 18A-X (IR), LKB 9000 (low-resolution mass spectra). NMR spectra were taken in $CDCl_3$ with Me_4Si as internal standard. The ionizing voltage for mass spectra was 70 eV. Infrared spectra were taken of KBr pellets. High-resolution mass spectra were obtained from the Department of Chemistry, University of California, Los Angeles. Elemental analyses were performed by Pascher Laboratories, Bonn, West Germany.

Column chromatography was carried out with silica gel 60 (E.M. Laboratories, Elmsford, N.Y.) and thin layer chromatography with silica gel 60 F 254.

3-Methyl-1-butyne. This compound was prepared essentially by the method reported in the literature.[19] 3-Methyl-2-butanone (39.5 g, 0.459 mol) was carefully added to PCl_5 (90.7 g, 0.436 mol) at 0 °C. The mixture was allowed to warm to room temperature, kept overnight with continuous stirring, then poured onto 500 g of crushed ice and stirred for a further 1 h. Ether (100 mL) was added and the organic phase separated, dried ($MgSO_4$), and distilled to give a mixture of dichloro and unsaturated monochloro products (23 g, bp 90–115 °C). A portion of the mixture (6.6 g) was added to 45 mL of 2.3 M potassium *tert*-amylate in toluene at 110 °C in an apparatus equipped with a reflux condenser and a dry ice–acetone trap. A drying tube containing Drierite was attached to the other side of the trap. The temperature at the top of the reflux condenser was maintained at 30–35 °C, at which temperature the 3-methyl-1-butyne distilled over (2.0 g): NMR (^{1}H) δ 2.37 (1, septet of d, J = 7, 2.5 Hz), 1.86 (1, d, J = 2.5 Hz), 0.99 (6, d, J = 7 Hz).

(22*R*- and 22*S*-) 6β-Methoxy-3α,5-cyclo-5α-cholest-23-yn-22-ol (5 and 6). A solution of ethylmagnesium bromide was prepared from 1.2 mL of ethyl bromide (16 mmol) and 0.5 g of Mg in 6 mL of ether. The solution (3.5 mL) was added to 2.0 mL of 3-methyl-1-butyne in 5 mL of dry tetrahydrofuran at −15 °C (argon atmosphere). The resulting mixture was stirred at −15 °C for 30 min, then at room temperature for 45 min. Dry benzene (5 mL) was next added followed by a solution of 640 mg (1.9 mmol) of the aldehyde **4**[7] in 15 mL of dry tetrahydrofuran at 0 °C. The mixture was stirred for 45 min, then poured into ice-water. The product was isolated by extraction with ether, washing the extract (10% NH_4Cl), drying it ($MgSO_4$), and removing the ether. The residue was chromatographed on silica gel (75 g) with petroleum ether–ethyl acetate (10:1) to give **5** (less polar epimer, 22*R*, 190 mg): mp 119–122 °C (recrystallized from ethyl acetate); IR 3450, 2235 cm^{-1}; NMR (^{1}H) δ 0.3–0.6 (3, m), 0.72 (3, s), 1.00 (3, s), 1.05 (6, d, J = 6 Hz), 1.14 (3, d, J = 7 Hz), 2.56 (1, septet of d, J = 6, 1 Hz), 2.73 (1, m), 3.29 (3, s), 4.42 (1, m); MS *m/e* (rel intensity) 412 (M$^+$, 51), 397 (31), 380 (37), 315 (M$^+$ − $CH(OH)C{\equiv}CCH(CH_3)_2$, 18), 283 (100). The more polar epimer **6** (22*S*, 217 mg) was also recrystallized from ethyl acetate: mp 47–49 °C; IR 3400, 2235 cm^{-1}; NMR (^{1}H) δ 0.3–0.6 (3, m), 0.73 (3, s), 1.01 (3, s), 1.01 (6, d, J = 6 Hz), 1.15 (3, d, J = 7 Hz), 2.57 (1, septet of d, J = 6, 1.5 Hz), 2.74 (1, m), 3.30 (3, s), 4.40 (1, m); MS *m/e* 412 (45), 397 (29), 380 (32), 315 (19), 283 (100).

(22*S*- and 22*R*-) 22-Hydroxycholesterol Diacetate (8 and 10). The acetylene **5** (70 mg, 0.17 mmol) dissolved in methanol (5 mL) was stirred with 50 mg of PtO_2 in a hydrogen atmosphere at room temperature and pressure for 2 h. The catalyst was separated by filtration and solvent removed from the filtrate leaving a noncrystalline residue whose spectral properties indicated loss of the triple bond. This product was dissolved in the ether (1 mL) and acetic anhydride (1 mL) and 0.2 mL of freshly distilled boron trifluoride etherate added at 0 °C. The mixture was stirred for 30 min, ice added, and the stirring continued for 30 min, then the product was extracted with ether. The ether solution was washed with saturated $NaHCO_3$ solution, water, and saturated NaCl solution and dried ($MgSO_4$) and the solvent was removed. The residue was purified by preparative TLC (silca gel plates, 2 mm thick, developed twice with petroleum ether–ethyl acetate, 15:1) yielding 36 mg of the diacetate **8** which was recrystallized from methanol: mp 142–144 °C (lit. 146 °C[8]); NMR (^{1}H) δ 0.69 (3, s), 0.87 (6, d, J = 6 Hz), 1.01 (3, s), 2.02 (6, s), 4.59 (1, m), 4.94 (1, broad t, J = 6 Hz) 5.38 (1, m); NMR (^{13}C) of diol from **8**: 37.3 (C-1), 31.7 (C-2), 71.8 (C-3), 42.4 (C-4), 141.2 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.2 (C-9), 36.6 (C-10), 21.2 (C-11), 39.9 (C-12), 42.4 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 52.7 (C-17), 11.8 (C-18), 19.4 (C-19), 40.3 (C-20), 11.6 (C-21), 74.0 (C-22), 33.3 (C-23), 35.7 (C-24), 27.8 (C-25), 22.7, 22.6 (C-26, C-27) ppm. Acetylene **6** (45 mg) was converted to the corresponding diacetate **10** in the same way as described above, yielding 36.3 mg of product which was recrystallized several times from methanol: mp 96–98 °C (lit. 102 °C[8]); NMR (^{1}H) δ 0.68 (3, s), 0.88 (6, d, J = 6 Hz), 1.01 (3, s), 2.02 (6, s), 4.57 (1, m), 4.85 (1, m), 5.33 (1, m); NMR (^{13}C) of diol from **10**: 37.4 (C-1), 32.0 (C-2), 71.9 (C-3), 42.7 (C-4), 141.3 (C-5), 121.6 (C-6), 32.0 (C-7), 32.2 (C-8), 50.2 (C-9), 36.6 (C-10), 21.2 (C-11), 39.9 (C-12), 42.4 (C-13), 56.5 (C-14), 24.5 (C-15), 28.3 (C-16), 53.2 (C-17), 11.9 (C-18), 19.5 (C-19), 42.4 (C-20), 12.5 (C-21), 74.4 (C-22), 27.8 (C-23), 36.1 (C-24), 28.0 (C-25), 22.6, 23.0 (C-26, C-27) ppm.

(22*S*- and 22*R*-) 6β-Methoxy-3α,5-cyclo-5α-cholest-(23*Z*)-en-22-ol (11 and 15). The acetylene **5** (126.3 mg, 0.31 mmol) dissolved in absolute ethanol (5 mL) containing synthetic quinoline (2 drops) was stirred with 40 mg of Pd on $BaSO_4$ in a hydrogen atmosphere at room temperature and pressure for 2 h. Most of the solvent was removed in a stream of nitrogen and the remaining mixture filtered through a short column of silica which was further eluted with dichloromethane–ethyl acetate. Removal of solvent from the eluate yielded 127 mg of **11** which was crystallized from methanol, mp 42–45 °C, MS *m/e* 414 (M$^+$). Similar hydrogenation of acetylene **6** (121.7 mg, 0.29 mmol), but for 6 h, gave 102 mg of **15** after chromatography on silica with petroleum ether–ethyl acetate (7:1), mp 52.5–54 °C, MS *m/e* 414 (M$^+$).

(24*R*- and 24*S*-) 6β-Methoxy-3α,5-cyclo-5α-stigmastan-29-oic

Acid Ethyl Ester (13 and 17). The cis allylic alcohol **11** (130 mg, 0.31 mmol) was dissolved in 6 mL of triethyl orthoacetate, 3 drops of propionic acid added, and the mixture heated at 130–140 °C with stirring overnight in an argon atmosphere. Most of the liquid was removed in a stream of nitrogen, ether added, and the solution washed twice with saturated sodium bicarbonate solution, once with water, twice with dilute HCl solution, twice with saturated NaCl solution, and dried ($MgSO_4$). Removal of the solvent and silica gel chromatography (medium pressure) of the residue with hexane-ethyl acetate (15:1) as eluent gave 47 mg (25%) of the ester **12** as an oil: NMR (^1H) δ 0.3–0.7 (3, m), 0.72 (3, s), 0.83 (3, d, J = 7 Hz), 0.87 (3, d, J = 7 Hz), 0.96 (upper arm of H-21 doublet), 1.02 (3, s), 1.22 (3, t, J = 7 Hz), 2.76 (1, m), 3.30 (3, s), 4.04 (2, q, J = 7 Hz), 5.16 (2, m).

The sample of **12** was used directly for the next step. It was dissolved in methanol (6 mL) and stirred with 30 mg of PtO_2 in an atmosphere of hydrogen at room temperature and pressure for 2 h. The catalyst was separated by filtration and the solvent removed from the filtrate leaving 46 mg of the saturated ester **13** as an oil: NMR (^1H) δ 0.3–0.7 (3, m), 0.70 (3, s), 0.84 (3, d, J = 6.5 Hz), 0.87 (3, d, J = 6.5 Hz), 0.89 (3, d, J = 6.5 Hz), 1.01 (3, s), 1.25 (3, t, J = 7 Hz), 2.77 (1, m), 3.30 (3, s), 4.12 (2, q, J = 7 Hz).

The cis allylic alcohol **15** (121 mg, 0.29 mmol) on similar treatment with triethyl orthoacetate gave 114 mg (80%) of the oily ester **16:** NMR (^1H) δ 0.3–0.7 (3, m), 0.71 (3, s), 0.83 (3, d, J = 6 Hz), 0.87 (3, d, J = 6.5 Hz), 0.90 (3, d, J = 6 Hz), 1.01 (3, s), 1.22 (3, t, J = 7 Hz), 2.73 (1, m), 3.29 (3, s), 4.06 (2, q, J = 7 Hz), 5.16 (2, m). Hydrogenation of **16** as above gave the saturated ester (quantitative yield): NMR (^1H) δ 0.3–0.7 (3, m), 0.69 (3, s), 0.82, 0.87, 0.89, 0.93 (four peaks discernible for overlapping signals of H-21, H-26, and H-27), 1.28 (3, t, J = 7 Hz), 2.80 (1, m), 3.34 (3, s), 4.2 (2, q, J = 7 Hz).

29-Hydroxyclionasterol and 29-Hydroxysitosterol (14 and 18). The ester **13** (46 mg, 0.09 mmol) was dissolved in 1 mL of ether and 1 mL of acetic anhydride. Boron trifluoride etherate (0.2 mL) was added at 0 °C and the mixture stirred for 30 min, then cold water added and the mixture set aside for 2 h. It was extracted with ether, the extract was washed twice with saturated $NaHCO_3$ solution, water, and saturated NaCl solution and dried ($MgSO_4$), and the solvent was removed leaving the oily acetate (39 mg, 82%): NMR (^1H) δ 0.67 (3, s), 0.83 (3, d, J = 6 Hz), 0.87 (3, d, J = 6 Hz), 0.91 (3, d, J = 6 Hz), 1.01 (3, s), 1.24 (3, t, J = 7 Hz), 2.01 (3, s), 4.11 (2, q, J = 7 Hz), 4.61 (1, m), 5.37 (1, m). The acetate was dissolved in 2 mL of dry ether, 25 mg of lithium aluminum hydride added, and the mixture stirred at room temperature for 90 min. It was diluted with ethyl acetate and filtered through silica gel. Removal of the solvent in vacuo yielded 30 mg of crystalline **14** which was recrystallized twice from methanol: mp 169–171 °C; IR 3335, 1020 cm^{-1}; NMR (^1H, 220 MHz FT) δ 0.68 (3, s), 0.84 (6, t, J = 7 Hz, from coincidental overlap of H-26 and H-27 doublets), 0.92 (3, d, J = 6.5 Hz), 1.03 (3, s), 3.53 (1, m), 3.65 (H-29, m, "AB" pattern partly overlapping multiplet at 3.53), 5.35 (1, m); MS *m/e* (rel intensity) 430 (M^+, 24), 412 (53), 397 (18), 255 (36), 213 (39), 55 (100). A sample for analysis was dried at 60 °C in vacuo to constant weight. Anal. Calcd for $C_{29}H_{50}O_2 \cdot 0.25CH_3OH$: C, 80.10; H, 11.72. Found: C, 80.10; H, 11.64.

The ester **17** (86 mg, 0.18 mmol) was treated with boron trifluoride etherate in the same way as **13** to give the corresponding acetate (67 mg, 73%): NMR (^1H) δ 0.64 (3, s), 0.87 (9, broad triplet from overlap of H-21, H-26, and H-27), 0.99 (3, s), 1.21 (3, t, J = 7 Hz), 1.98 (3, s), 4.08 (2, q, J = 7 Hz), 4.58 (1, m), 5.33 (1, m). The acetate (58 mg) was similarly converted to crystalline **18** (39 mg) which was recrystallized four times from methanol: mp 167–169 °C; IR 3300, 1055, 1018 cm^{-1}; NMR (^1H, 220 MHz, FT) δ 0.68 (3, s), 0.84 (3, d, J = 6.6 Hz), 0.85 (3, d, J = 6.8 Hz), 0.92 (3, d, J = 6.4 Hz), 1.01 (3, s), 3.53 (1, m), 3.65 (2, t, J = 6.7 Hz), 5.35 (1, m): MS *m/e* (rel intensity) 430 (M^+ 35), 412 (43), 397 (18), 273 (18), 255 (30), 213 (41), 55 (100). A sample for analysis was dried at 80 °C in vacuo to constant weight. Anal. Calcd for $C_{29}H_{50}O_2 \cdot CH_3OH$: C, 77.86; H, 11.76. Found: C, 77.83; H, 11.48.

(24*R*- and 24*S*-) 6β-Methoxy-3α,5-cyclo-5α-stigmast-22-en-20-ol (19 and 21). The ester **12** (370 mg, 0.76 mmol) was dissolved in 110 mL of dry ethyl ether and lithium aluminum hydride (550 mg, 14.9 mmol) was added. The mixture was refluxed for 1 h, then 0.55 mL of water, 0.55 mL of 15% sodium hydroxide solution, and 1.65 mL of water were added sequentially. Filtration of the mixture and removal of the ether from the filtrate gave 294 mg (87.0%) of the alcohol **19** as an oil: NMR (^1H) δ 0.3–0.7 (3, m), 0.73 (3, s), 0.81 (3, d, J = 6 Hz), 0.86 (3, d, J = 6 Hz), 1.01 (3, d, J = 6.5 Hz), 1.02 (3, s), 2.73 (1, m), 3.32 (3, s), 3.59 (2, dt, J = 7, 2.5 Hz), 5.14 (2, m).

Similar treatment of the ester **16** (41 mg, 0.085 mmol) with lithium aluminum hydride gave the alcohol **21**: NMR (^1H) δ 0.3–0.7 (3, m), 0.74 (3, s), 0.83 (3, d, J = 6 Hz), 0 .87 (3, d, J = 6 Hz), 1.01 (3, d, J = 5 Hz), 1.03 (3, s), 2.77 (1, m), 3.33 (3, s), 3.62 (2, broad t), 5.16 (2, m).

Poriferasterol (20) and Stigmasterol (22). To a stirred solution of alcohol **19** (40 mg, 0.09 mmol) and freshly distilled triethylamine (55 μL, 0.40 mmol) in 1.3 mL of dry CH_2Cl_2 at 0 °C under nitrogen was added freshly distilled methanesulfonyl chloride (22 μL, 0.29 mmol). After 30 min at 0 °C the liquid was removed in vacuo. Dry tetrahydrofuran (6.5 mL) and lithium aluminum hydride (220 mg, 5.79 mmol) were added and the mixture was stirred at room temperature for 2 h. The product was isolated in the same way as that employed for alcohol **19.** The yield was 38 mg (99%): NMR (^1H) δ 0.3–0.7 (3, m), 0.73 (3, s), 0.84 (6, broad d, J = 6 Hz), 1.00 (3, d, J = 6.5 Hz), 1.01 (3, s), 2.74 (1, m), 3.30 (3, s), 5.07 (2, m). The product was dissolved in 4 mL of dioxane and water added until the solution became cloudy. A small crystal of *p*-toluenesulfonic acid was added and the mixture was heated at 100–105 °C for 3 h. Water was added until cloudiness appeared and the mixture allowed to cool. The resulting white precipitate was filtered off, washed with water, and recrystallized twice from methanol to give poriferasterol (**20**), mp 154–156 °C (lit. 157–158 °C),[10] NMR (^1H) identical with that reported for the natural product.

Similar treatment of alcohol **21** gave the cyclosteroid with the stigmasterol side chain (18 mg): NMR (^1H) δ 0.3–0.7 (3, m), 0.73 (3, s), 0.78–0.92 (9, m), 1.00 (3, d, J = 6 Hz), 1.01 (3, s), 2.77 (1, m), 3.33 (3, s), 5.10 (2, m). It was converted to stigmasterol (**22**) in the same way as described for the preparation of poriferasterol. The product was recrystallized from methanol, mp 167–169 °C (lit. 169–170 °C),[10] NMR (^1H) identical with that reported for the natural product.

Separation of (24*R*)-3β,11α,15β,29-Tetrahydroxystigmast-5-en-7-one 3β-Isobutyrate (2) and 3β,11α,15β,29-Tetrahydroxystigmasta-5,24(28)(*E*)-dien-7-one 3β-Isobutyrate (3). A sample of "oogoniol-1" (3 mg) purified by thin layer chromatography followed by recrystallization from methanol[1] was dissolved in methanol (~0.25 mL) and this solution used for high-pressure liquid chromatography. The separation was performed on a Waters Associates Model 6000A instrument, using a 30 cm × 4 mm i.d. prepacked μ-Bondapak C_{18} column. The mobile phase was 70% MeOH in water with a flow rate of 2.6 mL/min at 2000 psi. The amount of solution for each injection was 25 μL. Three peaks with retention times of 10.2, 11.9, and 14.1 min were observed. The relative areas were ~1:13:77, respectively. The quantity of material corresponding to the major peak (14.1 min) was 1.6 mg. (Reinjection of a portion of this fraction showed that <0.3% of the peak with retention time of 11.9 min was present.) It crystallized from MeOH, mp 154–156 °C. Spectral properties confirmed its identity as **2** (R = $(CH_3)_2CHCO$): NMR (^1H, 220 MHz FT) δ 0.84 (6, t, J = 7 Hz), 0.94 (high-field arm of doublet for H-21. The other arm is partly concealed by the H-18 signal), 0.98 (3, s), 1.17 (6, d, J = 7 Hz, isobutyrate CH_3), 1.36 (3, s), 3.65 (H-29, broad peak), 4.13 (1, m, H-11), 4.69 (2, m, H-3 and H-15), 5.83 (1, s); MS *m/e* (rel intensity) 458 (100), 440 (31), 425 (9), 422 (11), 407 (6), 301 (6), 299 (3), 283 (37), 265 (14), 245 (9), 227 (11), 161 (54).

The compound corresponding to the fraction with retention time of 11.9 min was identified as **3** (R = $(CH_3)_2CHCO$) and amounted to 0.4 mg. It was crystalline and had mp 124–131 °C but because of the minute amount it could not be recrystallized. The broad melting point range is almost certainly a result of impurities introduced in the separation: NMR (^1H, 220 MHz FT) δ 0.99 (3, s), 1.02 (6, d, J = 7 Hz), 1.17 (6, d, J = 7 Hz, isobutyrate CH_3), 1.36 (3, s), 4.16 (3, m, H-11 and H-29), 4.70 (2, m, H-3 and H-15), 5.36 (1, t, J = 7 Hz), 5.84 (1, s); MS *m/e* (rel intensity) 456 (14), 438 (91), 423 (36), 420 (9), 395 (18), 342 (9), 341 (14), 339 (23), 299 (100), 283 (18), 245 (27), 161 (68); MS (high resolution) *m/e* 456.3250 ($C_{29}H_{44}O_4$), 299.1647 ($C_{19}H_{23}O_3$).

The third peak had the same retention time as **2** (R = CH_3CH_2CO). A sample of "oogoniol-2" obtained by thin layer chromatography and recrystallization[1] was resolved by high-pressure liquid chromatography into components corresponding to **2** (R = CH_3CH_2CO) and **3** (R = CH_3CH_2CO). They were present in a similar ratio as the isobutyrate analogues. This agreed with the NMR spectrum of the mixture which showed a weak triplet at ~δ 5.4 ex-

pected for H-28. Similarly, "oogoniol" was resolved into two components, presumably **2** (R = H) and **3** (R = H), but complete characterization was not possible because of insufficient material.

Biological activity of **2** (R = $(CH_3)_2CHCO$) and **3** (R = $(CH_3)_2CHCO$) was determined by the method of Barksdale and Lasure.[20] Addition of a dilute solution of the compound in MeOH-water (proportion of MeOH, 1% or less) to a culture of the female strain *Achlya ambisexualis* 734 caused the formation of oogonial initials. These became visible after about 12 h and reached a maximum number in 24–48 h. Solutions of **3** were active at a concentration as low as 50 ng/mL but a higher concentration of **2** (50 μg/mL) was required to induce formation of oogonial initials.

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Paramagnetic Ion Induced Perturbations in the ^1H NMR Spectrum of Lysozyme: A Reassignment of the Tryptophan Indole NH Resonances[1a]

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Abstract: In H_2O the 360-MHz ^1H NMR spectrum of the egg white lysozyme, an enzyme which has six tryptophan residues, contains only five resolvable indole NH resonances. These peaks have been assigned to specific tryptophan residues on the basis of the shift perturbations induced by Co^{2+} and line broadenings induced by Gd^{3+}. Since each of these perturbations obeys a different geometric relationship, the agreement of the two sets of assignments provides a check of the overall method. The results of inhibitor binding studies, chemical modification experiments, and deuterium isotope exchange rates are discussed in terms of the new assignments. Apart from any specific conclusions reached on HEW lysozyme it is clear that for many macromolecules the line broadenings induced by Gd^{3+} can be analyzed in terms of absolute metal proton distances. In making these assignments we have made use of the crystallographic data for HEW lysozyme. In general, the application of the methodology presented here is restricted to those biomolecules whose structure has been determined by an independent method.

The increased resolution and sensitivity available through the use of superconducting nuclear magnetic resonance spectrometers have made possible a wide variety of ^1H NMR investigations into the structure and dynamics of proteins in solution.[2] An essential first step in any such study is the assignment of a particular resonance in the complex ^1H NMR spectrum of the protein to a given hydrogen atom in the molecule. This process is usually accomplished in two steps. The first step is the assignment of the resonance to a particular kind of amino acid. The second is the identification of the particular peak with a specific residue in the sequence of the protein. Methods for making these assignments have been reviewed in the literature.[2-6] This communication describes an approach to the problem of making such assignments which is based on the stereospecificity of electron-nuclear interactions present in paramagnetic complexes.[7]

There is an extensive literature which deals with the applications of paramagnetic ion effects to the study of configurations and conformations of molecules in solution.[8] Strategies for employing these paramagnetic effects in conjunction with structural information available from X-ray crystallographic techniques to determine the solution structure of proteins have also been described.[5,6,9-11] Previous efforts in our laboratory have been directed at the development of a quantitative, statistically valid, computer-based method for the analysis of the chemical-shift perturbations and relaxation rate enhancements induced by paramagnetic metal ions.[9,12] The present study illustrates an application of this approach to the assignment of the tryptophan indole NH resonances of lysozyme.

Lysozyme is a low molecular weight enzyme (~14 700) with a known amino acid sequence.[13,14] The X-ray crystallographically determined structure for the native enzyme[15-17] as well as Fourier difference maps for various metal complexes[18,19] have been reported. This enzyme has been shown to bind polyvalent cations in solution[5,6,9,10,20-30] with side-chain carboxyl groups of Glu-35 and Asp-52 forming at least part of the metal binding site.[26] Although this enzyme contains six tryptophan residues at sequence positions 28, 62, 63, 108, 111,

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