equation to determine the concentration of I in red blood cells ($[I]_{RBC}$):

$$[I]_{RBC} = \frac{I - [I]_p V(1 - H)}{VH} \quad \text{(Eq. 31)}$$

where I is the amount of cocaine added to a volume (V) of blood or synthetic blood with an H fraction of this volume as red blood cells. The I-values used were calculated from $I = I_0 e^{-kt}$ where I_0 is the amount of I added at t minutes before assay, and k is the apparent first-order rate constant of degradation of I in the system. The apparent red blood cell–plasma partition coefficient was $D = [I]_{RBC}/[I]_p$; values are given in Table IX. The fact that there is no significant difference among systems with and without plasma proteins is indicative of no significant protein binding of I.

Similar studies were conducted with II (Table X). Although the data are more variable than in the case for I, the reasonable consistency of red blood cell–plasma and red blood cell–buffer or plasma water coefficients (D) in parallel studies at a given concentration of II ($[II]_0$) is indicative of negligible plasma protein binding of II.

Since I has relatively high solvolysis rates in plasma and buffers, plasma protein binding studies by equilibrium dialysis or ultracentrifugation are not applicable, since they take relatively long periods of time. An attempt to use ultrafiltration through filter cones (17) was not satisfactory, since I was variable and highly bound to the filter cones. When 4 ml of plasma and 4 ml of plasma–water were filtered to the same extent through individual fresh cones, the percent of the filtered 1-μg/ml solution recovered was 32 ± 1% for plasma–water and 23 ± 1% for plasma to indicate a possible plasma protein binding of 8% for I. The filter cones were pre-equilibrated with buffer containing the same concentrations of I in plasma that were to be filtered through those same cones. The percent concentration recovered in the filtrate in the same cone from buffer filtration and subsequent plasma filtration, respectively, was 46.3 and 54.6 at 2 μg/ml and 48.8 and 54.6 at 1 μg/ml.

However, when the same two filtrations in the same cone were effected using only buffer, the percent concentration of I recovered in the same cone was 43.8 and 56.2 at 1 μg/ml and 38.8 and 61.3 at 0.5 μg/ml. This mimicked the situation when the second filtration was of I-spiked plasma to show that the loss of I in the filtrate was due to its binding to the cones on successive filtrations and that it could not be assigned to plasma protein binding.

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ACKNOWLEDGMENTS

Supported in part by Grant 2-R01-DA-00743 from the National Institute of Drug Abuse, Rockville, MD 20852.

Spectral Analysis of the Configuration and Solution Conformation of Dihydrodigoxigenin Epimers

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Received November 21, 1980, from the *College of Pharmacy, The Ohio State University, Columbus, OH 43210.* Accepted for publication May 12, 1982.

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Abstract □ The C_{20} configuration and solution conformation of each epimer of dihydrodigoxigenin has been studied by circular dichroism (CD) and NMR spectroscopy. Results from the CD spectra indicate that the two epimers have opposite orientations of the β-carbon in the lactone ring. This finding, together with X-ray crystallographic data from a separate study on the minor epimer, establishes the C_{20} configuration of the minor epimer as *S* and of the major epimer as *R*. NMR evidence indicates that the average lactone rotamer for the minor epimer has the C_{22} position located on the C_{12} side of the steroid nucleus, whereas the average lactone rotamer for the major epimer has the C_{21} position located on the C_{12} side of the steroid nucleus. Molecular models indicate that these are the least-hindered positions for the respective rotamers. Physical data characterizing the two epimers are provided.

Keyphrases □ Dihydrodigoxigenin—epimers, spectral analysis of the configuration and solution conformation □ Spectral analysis—configuration and solution conformation of dihydrodigoxigenin epimers □ Epimers, dihydrodigoxigenin—spectral analysis of the configuration and solution conformation

Since the discovery of dihydrodigoxigenin (I) in the urine of a patient requiring large doses of digoxin (1) and the discovery of the metabolite dihydrodigoxin (II) in the plasma samples of three different subjects (2), a significant amount of research has been carried out on the digoxin cardanolide metabolites that are reduced at the C_{20}—C_{22} bond. Previous studies (3, 4) found 0.2–2% of the total radioactivity in urine as II in seven subjects after an oral dose of tritiated digoxin. Excretion of 12–20% of the digoxin maintenance dose as II in the urine of nine patients has also been reported (5, 6). Others (7) have found an average of 13% (range 1–47%) of the total glycosides in the meth-

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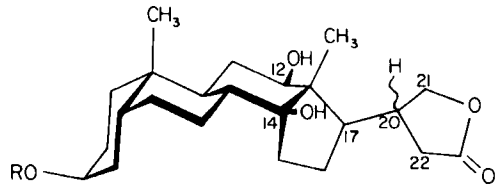

I R = H
II R = digitoxose$_3$

ylene chloride extract of urine present as II. Of the 50 subjects in this study, 48 had detectable urinary levels of II. Urine samples of 100 patients taking oral digoxin for the control of heart disease were investigated more recently (8, 9). The average percent of cardanolides present in the 0–24-hr urine was 12% (range 2–52%) based on the total methylene chloride extractable cardiac glycoside content. Out of the 100 patients, 53 excreted >10% while seven excreted >35% as the cardanolide metabolites.

Reduction of the 20,22-unsaturated lactone ring of digoxin introduces a center of asymmetry at C_{20} and generates three aspects of stereochemistry that must be investigated in order to determine the conformation of any epimers of I or II. First, the resulting saturated γ-lactone has the β-carbon out of plane, thus creating the possibility of the enantiomeric pair illustrated in Fig. 1 (10, 11). Second, the chiral center at C_{20} can have either an *R* or *S* configuration. Finally, the overall asymmetry of the steroid nucleus and the likely steric hindrance to free rotation of the lactone ring about the C_{17}—C_{20} bond results in the possibility of preferred rotamers for the lactone.

Research on the C_{20} configuration and the lactone conformation of the reduced metabolites of digoxin has been inhibited by the lack of methodology for effective separation of any epimers that may be formed either biologically or chemically. Most of the metabolic studies (1–9, 12, 13) have utilized either TLC or GC techniques which have yielded only a single peak for either reference or biologically produced I or II. In an investigation of the crystallographic conformation, C_{20} configuration, and biological activity of various cardanolides (14–17), a TLC procedure was recently reported capable of separating certain 20*R* and 20*S* cardanolide epimers (18). However, this method has not yet been applied to the epimers of I or II.

Since II is an important metabolite of digoxin, it is critical that the stereochemical properties of this metabolite be determined. Essential to this determination is the

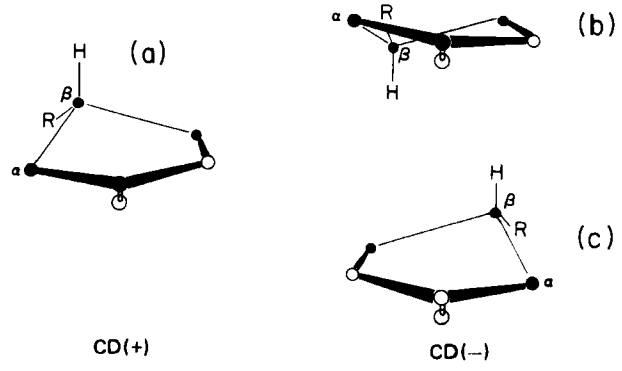

Figure 1—*Perspective representation of the two stable conformations of the γ-lactone. A positive CD spectrum is associated with conformation (a) and a negative CD spectrum with (b). Representation (c) is rotated 180° about the β carbon compared with (b) and* R *represents the steroid nucleus shown in I.*

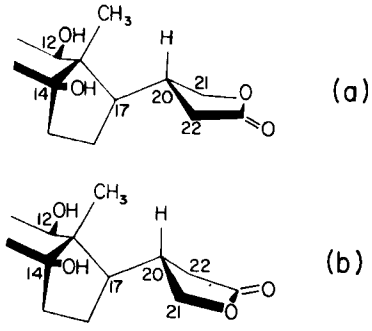

Figure 2—*Perspective representation of the relationships of the γ-lactone to the steroid nucleus wherein the 17—20 bond is pseudoequatorial, and the lactone rotamer has the oxygens oriented away from the methyl substituent at C_{13}. Structure (a) has the* R *configuration at C_{20}, and structure (b) has the* S *configuration.*

identification of suitable synthetic references with known stereochemistry so that subsequent comparisons to biologically produced metabolites may be made. Previous researchers (19) hydrogenated digoxigenin and fractionally crystallized the product yielding two epimers. However, that stereochemical assignment was based only on melting point and optical rotation values. The purpose of the present investigation was to define the C_{20} configuration and solution conformation of the epimers of synthetic I, the aglycone of II. It was not possible to carry out a definitive study on II because of interference from the sugar moieties in the interpretation of the various spectra.

RESULTS AND DISCUSSION

Catalytic hydrogenation of either digoxin or digoxigenin yielded two components that were separable as the 3,5-dinitrobenzoyl esters on high-performance liquid chromatography (HPLC). The component with the longer retention time on a normal phase system was present in larger amount and is designated the major fraction. Isolation of the derivatized major and minor fractions of I was accomplished by collecting the HPLC eluate. The corresponding underivatized fractions were then obtained by base hydrolysis. It was also possible to isolate the major underivatized fraction of I by fractional crystallization, but not the minor underivatized fraction. These major and minor fractions were each then subjected to spectral analysis for information concerning the conformation of the lactone ring, the configuration at C_{20}, and the preferred average rotamer of the lactone in relation to the steroid backbone.

Lactone Conformation and C_{20} Configuration—Associated with the optically active lactone is the $n \rightarrow \pi^*$-absorption band in the region of 214–219 nm. Circular dichroism studies have shown that a lactone ring will have a CD curve whose sign and magnitude are determined by the interactions within the lactone ring structure (10). Previous evidence (10, 11, 20, 21) has shown that the sign of the lactone CD curve for γ-lactones is associated with the location of the β-carbon relative to the planar lactone ring. The orientation of the plane is such that the α- and γ-carbons and the lactone moiety form the plane, the carbonyl function faces forward, and the ring oxygen is located to the right (Fig. 1a and b). The CD curve is positive when the conformation of the γ-lactone has the β-carbon above the plane and negative when the β-carbon is below the plane.

Results of the CD study of I revealed that the underivatized major component had a positive CD curve ($[\theta]_{218} = 136$) and the underivatized minor component had a negative CD curve ($[\theta]_{216} = -206$). These results indicate that the β-carbon of the lactone (C_{20} of I) is oriented as in Fig. 1a for the major component of I and as in Fig. 1b for the minor component. The energetically preferred position for the steroid substituent on the β-carbon would appear to be pseudoequatorial as shown in Fig. 1. In support of this viewpoint, the 22-methylene analogue of dihydrodigitoxigenin has been shown by X-ray crystallographic data (16) to have the steroid part of the molecule in a pseudoequatorial position at C_{20}. If this is the case for dihydrodigoxigenin, then the configuration at C_{20} (β-carbon of the lactone) is 20*R* for the major component and 20*S* for the minor component of I. This interpretation of the spectral data with respect to lactone conformation and C_{20} configuration has been confirmed by direct

X-ray crystallographic determination of the structure of the minor epimer (22).

Preferred Rotamer For Lactone—When either the 20*R* or 20*S* epimer of I is illustrated with Dreiding models and the molecule is rotated about the C_{17}—C_{20} bond, the conformation having the least steric hindrance with the C_{18}-methyl is that rotamer in which the lactone is oriented away from the C_{18}-methyl. This is illustrated for the 20*R* epimer of I in Fig. 2a. To orient the lactone away from the C_{18}-methyl in the 20*S* epimer it is necessary to rotate the lactone conformation shown in Fig. 1b by ~180° to that shown in Fig. 1c. The result for the dihydrodigoxigenin 20*S* epimer is shown in Fig. 2b.

A strategy was developed for testing the hypothesis that the two epimers of I have the lactones oriented as shown in Fig. 2. Advantage was taken of the fact that the tertiary 14-hydroxyl group does not form esters (23). Thus, esterification with 3,5-dinitrobenzoyl chloride at the 3 and 12 positions of I yields a derivative that should have the added aromatic moiety at the 12 position sufficiently close to the C_{21} protons in the *R* epimer (Fig. 2a) to influence the NMR spectrum of these protons. For the *S* epimer (Fig. 2b) the NMR of the C_{22} protons should be influenced by their proximity to the C_{12} aromatic moiety.

The NMR spectra of the underivatized major and minor components of I lacked similarity in the regions of the C_{21} protons (3.9–4.5 ppm) and the C_{22} protons (2.1–2.7 ppm, Fig. 3, spectra b and d). Differences in the chemical shifts occurring in these two regions were compared in the underivatized and derivatized forms of each component. A comparison of the NMR spectrum of the underivatized major component of I with that of the derivatized major component (Fig. 3, spectra b and a) reveals that the 3,5-dinitrobenzoate ester at the C_{12} position induces significant chemical shift changes in the C_{21} protons (underivatized major component of I apparent triplets at 4.00 and 4.40 ppm, derivatized major component at 3.76 and 4.53 ppm). However, the results are different with respect to the C_{22} protons. Overlap of the apparent triplets in the region of 2.2–2.5 ppm makes this segment of the NMR spectrum difficult to interpret, but only minor differences exist in this region when the underivatized major component of I is compared with the derivatized major component (suspected location of apparent triplets for C_{22} protons, underivatized major component at 2.34 and 2.48 ppm, derivatized major component at 2.35 and 2.57 ppm).

In comparing the NMR spectrum of the underivatized minor component of I with that of the derivatized minor component, the reverse effect was observed for the C_{21} and C_{22} protons (Fig. 3, spectra d and c). Minimal differences occurred in the chemical shift locations of the C_{21} protons when the underivatized minor component (4.05 and 4.35 ppm) was compared with the derivatized minor component (4.02 and 4.40 ppm). Again, the NMR spectra are difficult to interpret in the C_{22} region; however, significant differences do exist in this region between the underivatized minor component of I and the derivatized minor component (Fig. 3). The suspected location of the C_{22} protons for the underivatized minor component is 2.29–2.53 ppm and for the derivatized minor component 2.02–2.64 ppm.

The results of the NMR studies indicate that the major and minor components of I are opposite rotamers at the C_{17}—C_{20} bond. The influence of derivatization on the chemical shift of the C_{21} protons of the major component of I but not the C_{22} protons confirms the predictions from the Dreiding model of the preferred average rotamer for the *R* epimer, as illustrated in Fig. 2a. The opposite effect of derivatization on the chemical shift of the C_{21} and C_{22} protons of the minor component is consistent with the predicted average rotamer for the *S* epimer, as shown in Fig. 2b.

Determination of the Influence of the Isolation Procedure on the Spectral Observations—The major and minor components of I were separated by derivatization with 3,5-dinitrobenzoyl chloride, HPLC of the derivatives yielding separate peaks for the two components and then base hydrolysis of the separated components. As a control to determine the influence of the isolation procedure on the structure of I, the major component was also prepared by fractional crystallization. Attempts to purify the minor component by fractional crystallization were not successful. Comparison of the NMR spectrum of the recrystallized major component with the NMR of the same component isolated by derivatization, HPLC, and base hydrolysis revealed that the spectra were essentially the same. Thus, the derivatization and base hydrolysis procedures required for the isolation of the two components of I did not alter the starting material, dihydrodigoxigenin (I).

Conclusion—The agreement for the minor epimer of I between the CD results and the results of a separate X-ray crystallography study (22) establishes the C_{20} configuration as *S* and the lactone conformation as shown in Fig. 2b. NMR evidence suggests that the average lactone rotamer in solution is approximately as shown in Fig. 2b for the minor epimer. The CD results for the major epimer indicate the opposite conformation of the β-carbon in the lactone compared with the minor epimer. This result, together with the X-ray crystallography data for the minor epimer, establishes the *R* configuration at C_{20} for the major epimer. NMR evidence suggests that the average lactone rotamer for the major epimer is approximately as shown in Fig. 2a.

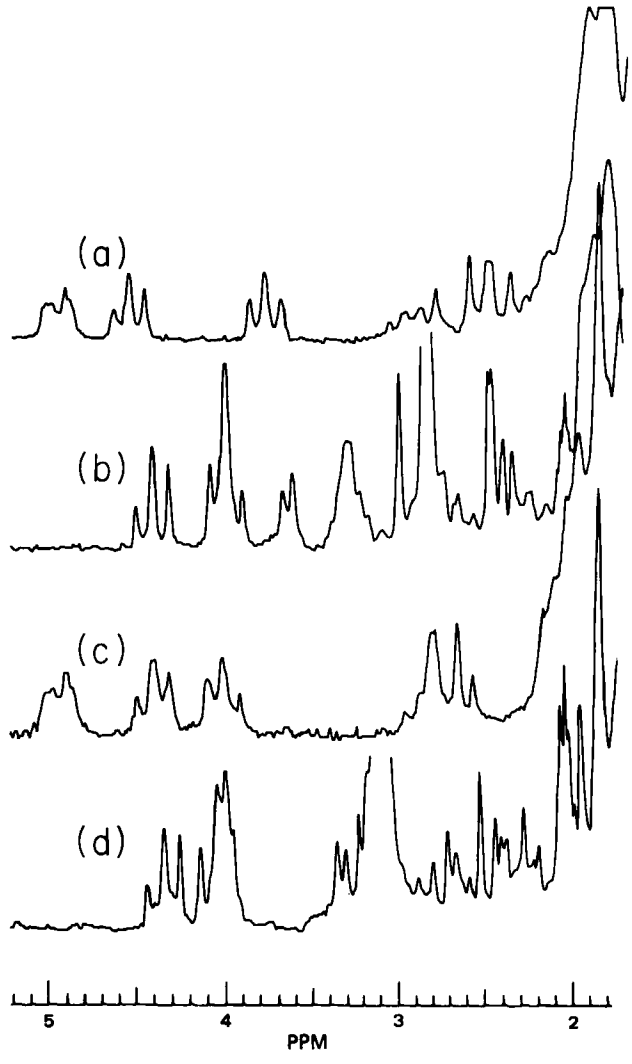

Figure 3—*Partial proton NMR spectrum of (a) derivatized major component of I (in deuterated chloroform), (b) underivatized major component of I (in deuterated acetone), (c) derivatized minor component of I (in deuterated chloroform), and (d) underivatized minor component of I (in deuterated acetone). The derivatizing agent was 3,5-dinitrobenzoyl chloride.*

EXPERIMENTAL

Hydrogenation of Digoxigenin to Dihydrodigoxigenin (I)—Digoxigenin[1] (500 mg) was added to a hydrogenation bottle containing 60 ml of ethanol and 85 mg of platinum oxide catalyst. The digoxigenin was hydrogenated for 12 hr at 30 psi. The platinum oxide was removed by filtering the ethanol solution through a fritted glass filter, and the ethanol was evaporated under a stream of nitrogen at 50°.

Fractional Crystallization of I—Five hundred mg of I was recrystallized from ethyl acetate. The resulting crystals were recrystallized four additional times from ethyl acetate to yield 23 mg. *Less than 4% contamination of the major component of I with the minor component was* detected based on high-performance liquid chromatography (HPLC): mp 189–213°; NMR (CD_3COCD_3) integration (35.4 protons, theoretical 36 protons) δ 4.40 (t J = 8 Hz, 1, C_{21}—H), 4.00 (s, 1, C_3—H), 4.00 (t, one

[1] Boehringer Mannheim Biochemicals, Indianapolis, Ind.

peak under the 4.00 s, J = 8 Hz, 1, C_{21}—H), 2.26–2.65 (m, with major peaks at 2.26, 2.34, 2.39, 2.46, 2.65, 2, C_{22}—H), 0.90, and 0.93 (each s, 6, C_{18}—H, and C_{19}—H).

Derivatization and Liquid Chromatography of (I) to Isolate the Major and Minor Components—One g of I was derivatized with 3,5-dinitrobenzoyl chloride by taking 10-mg aliquots of I and dissolving in 13 ml of pyridine which had been distilled and stored over sodium hydroxide pellets. Next, 1.077 g of 3,5-dinitrobenzoyl chloride[2] (recrystallized from petroleum ether) was added and the solution was heated for 30 min in a water bath (50°). After the major portion of pyridine was removed under vacuum (60°), 10 ml of 5% $NaHCO_3$ solution containing 20 mg of 4-dimethylaminopyridine[2] was added to hydrolyze the excess derivatizing agent and 10 ml of chloroform was added to solubilize and extract the derivatized I. Following the removal of the aqueous phase, the organic phase was washed with 10 ml of 5% $NaHCO_3$ followed by four 30-ml washings with a 0.05 N HCl solution containing 5% NaCl. The chloroform solution from each aliquot was concentrated to a total of 10 ml by evaporating the chloroform under a nitrogen stream at 50°. This solution was washed eight times with the acid wash to remove any residual pyridine, and the final volume of 2 ml was achieved by evaporation under nitrogen (50°). Aliquots of this solution were chromatographed by HPLC using a mobile phase of hexane–methylene chloride–acetonitrile (3:1:1) and a silica gel stationary phase[3]. Appropriate fractions were collected to isolate the derivatized major and minor components of I. Purity with respect to the opposite component was determined by HPLC. Derivatized major component of I, <1% cross-contamination from derivatized minor component; NMR[4] ($CDCl_3$) δ 9.12–9.28 (m, with major peaks at 9.12, 9.14, 9.21, 9.23, 9.25, 9.28, 6, aromatic H), 5.49 (s, 1, C_3—H), 4.88 (m, 1, C_{12}—H), 4.52 (t, J = 8 Hz, 1, C_{21}—H), 3.75 (t, J = 8 Hz, 1, C_{21}—H), 2.25–2.76 (m, with major peaks at 2.34, 2.44, 2.56, 2, C_{22}—H), 1.22 (s, 3, C_{19}—H), 1.09 (s, 3, C_{18}—H). Derivatized minor component of I, <1% cross-contamination from derivatized major component; NMR ($CDCl_3$) δ 9.12–9.24 (m, with major peaks at 9.12, 9.14, 9.24, 6, aromatic-H), 5.48 (s, 1, C_3—H), 4.90 (m, 1, C_{12}—H), 4.40 (t, J = 8 Hz, 1, C_{21}—H), 4.02 (t, J = 8 Hz, 1, C_{21}—H), 2.64 (t, J = 8 Hz, 1, C_{22}—H), 2.02 (t, J = 8 Hz, 1, C_{22}—H), 1.23 (s, 3, C_{19}—H), 1.10 (s, 3, C_{18}—H).

Base Hydrolysis of the Derivatized Major and Minor Components of I—A mixture of chloroform (1.5 ml), methanol (13.5 ml), and 5% $KHCO_3$ (1.5 ml) containing either 100 mg of the derivatized major component of I or 131 mg of the derivatized minor component was refluxed for 90 min. After increasing the volume of the aqueous phase with 3 ml of distilled water, the aqueous phase was extracted three times with methylene chloride (40 ml). To ensure against opening of the lactone ring from the base hydrolysis procedure (24), the aqueous phase was acidified to ~pH 2 with 0.2 N HCl. Twelve hours later the solution was adjusted to pH 10 with 5% $KHCO_3$ and extracted three times with methylene chloride (40 ml). After combining like fractions and evaporating the methylene chloride, each component was crystallized from ethyl acetate. Purity was determined by HPLC. Major component of I (37.3 mg, 37.3% yield) <1% contamination from minor component; mp 235–237°; NMR (CD_3COCD_3) integration (35.1 protons, theoretical 36 protons), δ 4.41 (t, J = 8 Hz, 1, C_{21}—H), 4.00 (s, 1, C_3—H), 4.00 (t, one peak under the 4.00 s, J = 8 Hz, 1, C_{21}—H), 2.24–2.54 (m, with major peaks at 2.24, 2.34, 2.40, 2.46, 2.48, 2.54, 2, C_{22}—H), 0.90 and 0.93 (each s, 6, C_{18}—H and C_{19}—H); $[\alpha]_D^{21}$ + 20.2° (C 0.436); CD $[\theta]_{218}$ 136[5].

Anal.—Calc. for $C_{23}H_{36}O_5$: C, 70.38; H, 9.24. Found: C, 70.02; H, 9.46.

Minor component of I (41.6 mg, 31.8% yield), <1% contamination from major component; mp 209.5–211.5°; NMR (CD_3COCD_3) integration (35.9 protons, theoretical 36 protons), δ 4.35 (t, J = 8 Hz, 1, C_{21}—H), 4.01 (s, 1, C_3—H), 4.05 (t, J = 8 Hz, 1, C_{21}—H), 2.20–2.62 (m, with major peaks at 2.20, 2.29, 2.38, 2.45, 2.53, 2.62, 2, C_{22}—H), 0.92 (s, 6, C_{18}—H and C_{19}—H); $[\alpha]_D^{21}$ + 17.9° (C 0.29); CD $[\theta]_{216}$–206.

Anal.—Calc. for $C_{23}H_{36}O_5$: C, 70.38; H, 9.24. Found: C. 70.10; H, 9.38.

[2] Purum grade, Fluka A.G., Buchs, Switzerland.
[3] Partisil 10, Whatman Inc., Clifton, NJ.
[4] Bruker HX-90 NMR spectrometer.
[5] Durrum-Jasco ORD-CD spectrometer.

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ACKNOWLEDGMENTS

Presented before the Medicinal Chemistry Section at the 26th National Meeting of the APhA Academy of Pharmaceutical Sciences, Anaheim, Calif., April 1979.

The financial assistance of Central Ohio Heart Chapter Inc. (grant 78-41) and NIH (Training grant no. GM07622-HNB) is gratefully acknowledged. The authors wish to thank Susan B. Ashcraft and Brent E. Morrison for technical assistance and Drs. Raymond W. Doskotch, Duane D. Miller, and H. B. Bhat for their encouragement and expert advice.