0026-895X/86/030270-05$02.00/0

MOLECULAR PHARMACOLOGY, 29:270-274

The Effect of 16β-Substitution on the Structure and Activity of Digitoxigenin: Is There an Additional Binding Interaction With Na^+,K^+-ATPase?

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Received October 14, 1985; Accepted December 27, 1985

SUMMARY

We have studied the basis of the effect of 16β-substitution on the structure and activity of digitoxigenin derivatives by examining the crystal structures of these compounds and their inhibitory activity toward the receptor for these drugs, Na^+,K^+-ATPase. To understand the increase in inhibitory activity of the 16β-ester compounds and the decrease in activity of gitoxigenin (16β-hydroxydigitoxigenin), both with respect to digitoxigenin, we have compared the observed conformations of gitoxigenin, gitoxigenin 16β-formate, and other 16β-esters to that of digitoxigenin. Our data do not support the possibility of hydrogen bonding between the 16β-hydroxyl of gitoxigenin and the lactone ring, previously suggested to account for the decreased activity of gitoxigenin *vis à vis* digitoxigenin, but, rather, suggest that the decreased activity may be due to an intramolecular hydrogen bond between the hydroxyls on C-14 and C-16 and an unusual D-ring conformation which combine to alter the carbonyl oxygen of the lactone ring away from the putative active position. In contrast, the 16β-ester moiety has a preferred conformation which may serve to fix the lactone ring in the active conformation. Thus, the increased activity of the 16β-esters cannot be explained by altered carbonyl oxygen position and may be related to an additional receptor binding site for the ester moiety.

The structural features which determine the affinity of cardenolides and cardenolide glycosides toward their receptor site on the Na^+,K^+-ATPase (EC 3.6.1.3) have long been of interest. We have recently proposed a model in which the C-17 side-group carbonyl oxygen position is strongly correlated with Na^+,K^+-ATPase inhibitory activity (1–6). It is well known that gitoxigenin (16β-hydroxydigitoxigenin, **II**; see Table 1) is substantially less active than ditoxigenin (**I**), whereas the 16β-formate (**III**) and acetate (**IV**) of compound **II** are significantly more active than compound **I** (7, 8). It has been postulated by Dittrich *et al.* (7) that the variations in activity induced by the C-16 substituents result from alterations in the strength and direction of the cardenolide dipole vector. Others have proposed that hydrogen bonding between the 16β-hydroxy group and the lactone carbonyl oxygen is the cause of the reduced activity of compound **II**, whereas the increased activity of compounds **III** and **IV** is the result of the 16β-ester group binding to the same site on Na^+,K^+-ATPase as does the lactone ring (8). To test these hypotheses and our own model for cardenolide receptor binding, we have synthesized and determined the activity of a number of gitoxigenin derivatives and some of their monoglycosides, and related their biological activity to molecular fine structure as determined by X-ray crystallography (5).

Materials and Methods

Gitoxigenin analogues. The gitoxigenin analogues in Table 1 were synthesized from gitoxin by T. Hashimoto, H. Rathore, T. Satoh, and D. S. Fullerton. The details of the syntheses will be published elsewhere. Gitoxin was provided by Daisuke Satoh, Nishinomiya City, Japan.

Preparation of Na^+,K^+-ATPase. Hog kidney outer medulla Na^+,K^+-ATPase was purified as described previously (5). The specific activity of the purified enzyme was generally 1200 μmol of Pi/mg of protein/hr, with greater than 95% ouabain sensitivity.

Measurement of inhibition of Na^+,K^+-ATPase by gitoxigenin analogues. The inhibitory potency of various gitoxigenin analogues with respect to Na^+,K^+-ATPase activity was determined according to the procedures detailed previously (5). In brief, the interaction of drugs with Na^+,K^+-ATPase was allowed to take place under type I binding conditions (i.e., in the presence of Mg^{2+}, Na^+, ATP) for a period of 15 min for genins or 120 min for genin glycosides, to achieve equilibrium binding of the drugs to the enzyme (5), following which the Na^+,K^+-

This work was supported by Grant HL-21457 awarded by the Heart, Lung and Blood Institute, National Institutes of Health, Department of Health and Human Services, and by the Medical Research Fund of the United States Veterans Administration.

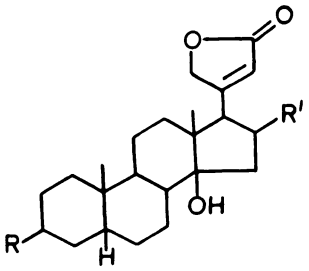
ABBREVIATION: I_{50}, the concentration required for half-maximal inhibition.

ATPase reaction was initiated. Genin analogues at varying concentrations, depending on their activity, were incubated with Na^+,K^+-ATPase in the presence of 3 mM $MgCl_2$, 3 mM ATP (Tris-salt), 110 mM NaCl, and 30 mM Tris-HCl, pH 7.45 (measured at 37°), for the periods indicated above to achieve maximal drug binding. An appropriate amount of KCl was then added to yield a final concentration of 10 mM, and the reaction was allowed to proceed for 10–20 min, so as to allow hydrolysis of less than 10% of the total ATP in the reaction. Suitable controls in the presence and absence of genin analogues with and without added KCl were included throughout. Na^+,K^+-ATPase was measured as μmol of Pi formed in the presence of Mg^{2+}, Na^+, K^+, minus that in the presence of Mg^{2+}, Na^+. I_{50} values were determined from the inhibition curves obtained in the presence of varying concentrations of a given genin analogue. Control experiments were carried out to establish that preincubation periods longer than 15 min for genins, and 120 min for glycosides, did not significantly alter the I_{50} values in various assays. When drugs were added as ethanolic solutions, suitable controls were included to establish that the amount of ethanol added (always less than 20 μl/2.0 ml) did not influence any of the above assays. Each result described is based on at least three different determinations; the values were found to vary by no more than ±5% in different experiments. The I_{50} values are listed in Table 1.

Crystallographic methods. A single crystal of compound **III**, $C_{24}H_{34}O_6$, dimensions 0.12 × 0.30 × 0.50 mm, was mounted on a Syntex P3 diffractometer equipped with a low temperature device. The 4096 data were collected at 82.(1°) K using Mokα radiation. The crystal was recentered in the X-ray beam after every filling of the Dewar with liquid N_2. Four standard reflections were monitored for decay. The data were collected using a θ-2θ scan over 2.5°. The data were processed and averaged to 3323 unique reflections of which 2982 were >2α. Cell constants were obtained from a least squares refinement of observed and calculated 2θ values of 25 reflections between 2θ = 20.14° to 24.97°.

TABLE 1

Gitoxigenin analogues studied



Analogue	R	R'	I_{50}*
			nM
I (Digitoxigenin)	OH	H	120.0
II (Gitoxigenin)	OH	OH	603.0
III	OH	OCHO	20.0
IV	OH	$OCOCH_3$	49.0
V	Digitoxose	OH	38.0
VI	$Digitoxose_2$	OH	51.9
VII (Gitoxin)	$Digitoxose_3$	OH	69.9
VIII	Digitoxose	OCHO	3.13
IX	Digitoxose	$OCOCH_3$	7.24
X	$Digitoxose_3$	$OCOCH_3$	10.2
XI	OCHO	OH	676.0
XII	$OCOCH_3$	OH	676.0
XIII	OCHO	OCHO	14.8
XIV	$OCOCH_3$	$OCOCH_3$	74.1
XV	$OCOCH_3$	OCHO	17.0
XVI	OCHO	$OCOCH_3$	95.5
XVII	$OCOOCH_3$	$OCOOCH_3$	3890.0
XVIII	$OCOOCH_3$	OH	603.0

* The I_{50} values were determined as outlined in Materials and Methods and Ref. 5.

Cell data: a = 14.226(3), b = 9.687(2), c = 8.011(2) Å, $\alpha = \gamma = 90$; β = 104.74(1)°; V = 1067.7 Å^3, Space Group = $P2_1$, Z = 2. The structure was determined using the MULTAN (9) and NQEST (10) programs. The carbon and oxygen atoms were refined with anisotropic thermal parameters and the hydrogen atoms, with isotropic thermal parameters. Full matrix least squares refinement minimizing $\Sigma\Delta^2 = (|F_o| - |F_c|)^2$ converged to an R of 0.053 for 2982 reflections having $F > 2\sigma$.[1] All bond distances and bond angles are within 3 SD of the average of 20 similar cardenolide structures. A stereo ORTEP view is given in Fig. 1.

Structural comparisons using PROPHET. The PROPHET NIH system[2] provides programming and graphics procedures for direct comparison, measurement, and manipulation of molecular structures. We have used the crystallographically determined structures for our comparisons. Previously, we have shown that the crystallographically determined structures are minimum energy conformations (1, 4, 11). The PROPHET program FITMOL (12) was used to superimpose structurally similar portions of the steroid backbones of digitoxigenin and the 16β-substituted analogues. The measured distances between relative positions of specific atoms in the two molecules are a quantitative measure of where molecules resemble one another and where they differ. The procedures used have been described in detail elsewhere (4).

The sample of 46 cardenolides studied for D-ring conformational analysis is contained in the Cambridge Crystallographic Database. The sample consists of all cardenolide-like steroids, omitting those with extra ring fusions.[3] The D-ring analysis and graphs were prepared using PROPHET software.[2]

Results

Na^+,K^+-ATPase Studies

The Na^+,K^+-ATPase inhibitory activities of the compounds in this study are shown in Table 1. Consistent with earlier reports, gitoxigenin (**II**) is less active than digitoxigenin (**I**), and the 16β-esters (**III**, **IV**) are considerably more active, the

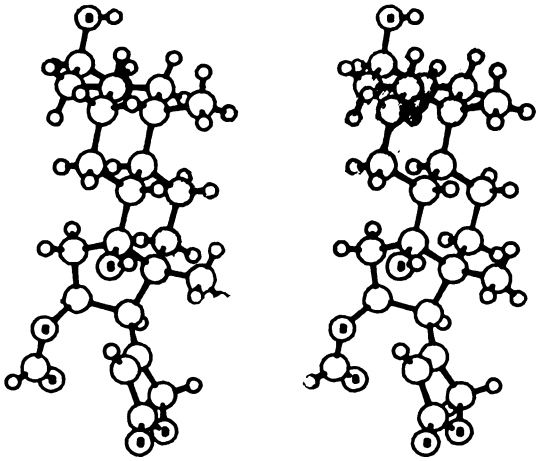


Fig. 1. Stereo ORTEP of gitoxigenin 16-formate (III) drawn from the crystallographic coordinates.

[1] The fractional atomic coordinates for compound **III** have been deposited in the Cambridge Crystallographic Database. Copies may be obtained from the Cambridge Crystallographic Data Centre, Cambridge, England, CB2 1EW, by citing this journal article.

[2] The PROPHET system is a unique national resource sponsored by the National Institutes of Health. Information about PROPHET, including how to apply for access, can be obtained from the Director, Chemical/Biological Information Handling Program, Division of Research Resources, National Institutes of Health, Bethesda, MD 20205.

[3] A list of the compounds in the sample and the literature references for them are available from one of the authors (J. F. G.).

formate being more active than the acetate, **III > IV.** This relative activity, i.e., **III > IV > I > II**, remains consistent for the 3β-formate derivatives, **XIII > XVI > XI**, and the 3β-acetate derivatives, **XV > XIV > XII**, which have activities approximately equivalent to those of the parent compounds, as well as for the 3β-monodigitoxosides, **VIII > IX > V**, which show equal enhancement of activity over the parent compounds. The enhancement of activity by a single sugar substitution at C-3 has been noted before (1, 13). The decrease in activity noted for 16β-$OCOOCH_3$ (**XVII**) indicates that formate is the optimal group to enhance activity by 16β-substitution.

Structural Studies

Effect of 16β-hydroxy-substitution. The crystallographically observed structures of gitoxigenin (**II**) (14) and 5-hydroxy-gitoxigenin (**XIX**) (15) were least squares fit to digitoxigenin (**I**) (16). The atoms fit were C-1–C-17, O-3, and O-14. A stereo view of the resulting superposition is shown in Fig. 2. In the crystal structure of compound **XIX** there is an intramolecular hydrogen bond between the 14β- and the 16β-hydroxyl groups, the lactone ring is 180° from the orientation observed in compound **I**, and the 0-23 carbonyl oxygen is displaced from that in compound **I** by 2.4 Å in approximately the same plane. In the crystal structure of compound **II**, the 16β-hydroxyl forms an intermolecular hydrogen bond, the lactone ring is moved toward the α-face of the steroid, and O-23 is displaced 2.4 Å from the O-23 of compound **I.** These motions are transmitted through a change in D-ring conformation. The D-ring conformations observed in compounds **II** and **XIX** are at the limits observed in a sample of eight 16β-substituted cardenolides, a 13,17-half-chair, and a 15-envelope (Fig. 3d).

Based on these observation, it appears that both of the observed D-ring conformations serve to move the lactone ring carbonyl oxygen away from the putative ideal position, and this may account for the decreased potency of gitoxigenin. In addition, an intramolecular hydrogen bond between the 14β- and 16β-hydroxyl groups may prevent the 14β-hydroxyl from forming a favorable interaction with the receptor site. A strong intramolecular hydrogen bond between the 14β- and 16β-hydroxyls of compound **II** would also account for the observation that compound **II** is much less soluble than compound **I** in polar solvents, even though compound **II** has one additional hydroxyl group. The formation of an intramolecular hydrogen bond between the 16β-hydroxyl group and the lactone ring, however, seems highly improbable based on modeling using the observed structures of compounds **II** and **XIX.** Thus, although pseudorotation of the D-ring can bring O-14 and O-16 within 2.7 Å, no combination of D-ring conformation and rotation of the lactone ring can bring either oxygen of the lactone ring closer than 4 Å to O-16.

Effect of 16β-substitution on D-ring conformation. It has been shown from NMR experiments (17) and from crystallographic data on a large number of progesterone compounds (11) that 16β-substitution affects the orientation of the progesterone side-chain on C-17. In the absence of a 16β-substituent, there is a highly preferred orientation for the 17β-side chain: the C-20 carbonyl is *cis* to the C-16 bond. The addition of a 16β-substituent destabilizes this conformation and two other conformers become significantly populated. The cardenolides are distinguished from progesterone by the *cis*-fusion of the C and D-rings, a 14β-hydroxyl group, and a 17β-lactone ring. We have examined the D-ring conformations and substituent conformations in a sample of 46 cardenolide structures, of which 8 had 16β-oxygen substituents, to try to understand how the D-ring transmits changes in substituent orientation, or how substituents influence D-ring conformation and lactone ring position.

Due to the rigidity imposed by the *cis*-fusion and adjacent β-substituents, the C-18 methyl group and the 14-hydroxyl, the C-18—C-13—C-14—O-14 torsion angle is restricted to a small region of conformational space (16°) and its value is not correlated with D-ring conformation (see Fig. 3a). The addition of a 16β-substituent crowds the β-face of the D-ring. The position of the C-18 methyl group, the 16β-oxygen, and C-20 of the lactone ring become correlated with each other and with the D-ring conformation. This is seen graphically in Fig. 4 (b–d), where we have plotted the torsion angles O-16—C-16—C-17—C-20 versus C-18—C-13—C-17—C-20 (R^2 = 0.96), and each angle versus Δ (R^2 = 0.98 and 0.95, respectively). Δ, the phase angle of pseudorotation, is a quantitative descriptor of D-ring conformation (18) and can be related to the qualitative description of conformation. In a sample of 46 cardenolide crystal structures, 38 have D-rings with Δ values in the range −80 to +50, with the favored conformation a 14-envelope (Δ = −37). As noted above, the two compounds with 16β-hydroxyl substitution, **II** and **XIX**, have D-ring conformations at the extreme ends of the sample, Δ = −99.2 and +70.9, representing a 15-envelope and a 13,17-half-chair, respectively (see Fig. 3).

(c) 16β-Ester conformation. When the 16β-hydroxyl of compound **II** is esterified either to a formate or an acetate, the D-ring is observed in the normal range between a 14α-envelope and a 13β,14α-half-chair (Fig. 3). We have fit the observed structure of compound **III** to the observed structure of the

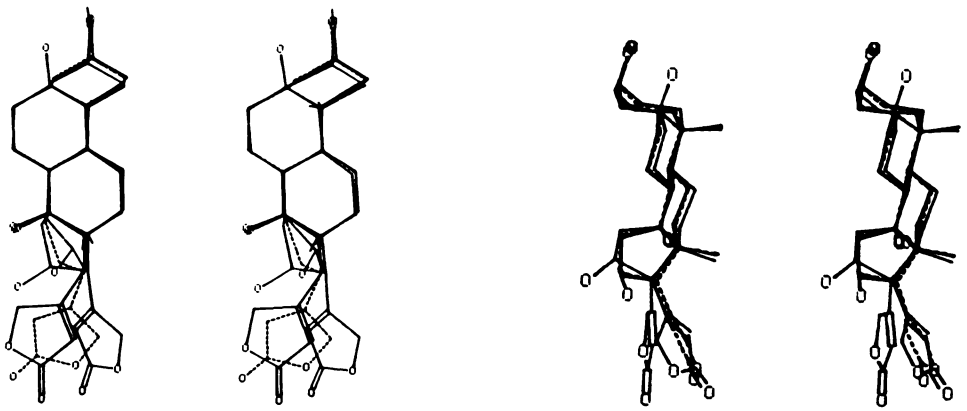


Fig. 2. Two stereo views showing the effect of 16β-OH substitution on conformation. Digitoxigenin (**I**) (*dashed line*), gitoxigenin (**II**), and 5β-OH gitoxigenin (*solid line*) have been fit (C-1–C-17, O-3, O-14). 5-OH gitoxigenin has an intramolecular H-bond between the 16β-OH and the 14β-OH, the lactone ring is flipped 180°, and the O-23 carbonyl oxygen is displaced from digitoxigenin by 2.40 Å in approximately the same plane. Gitoxigenin forms an intermolecular H-bond, the lactone ring is moved toward the α-face of the steroid, and the carbonyl oxygen is displaced 2.42 Å from digitoxigenin. These motions are transmitted through a change in D-ring conformation.

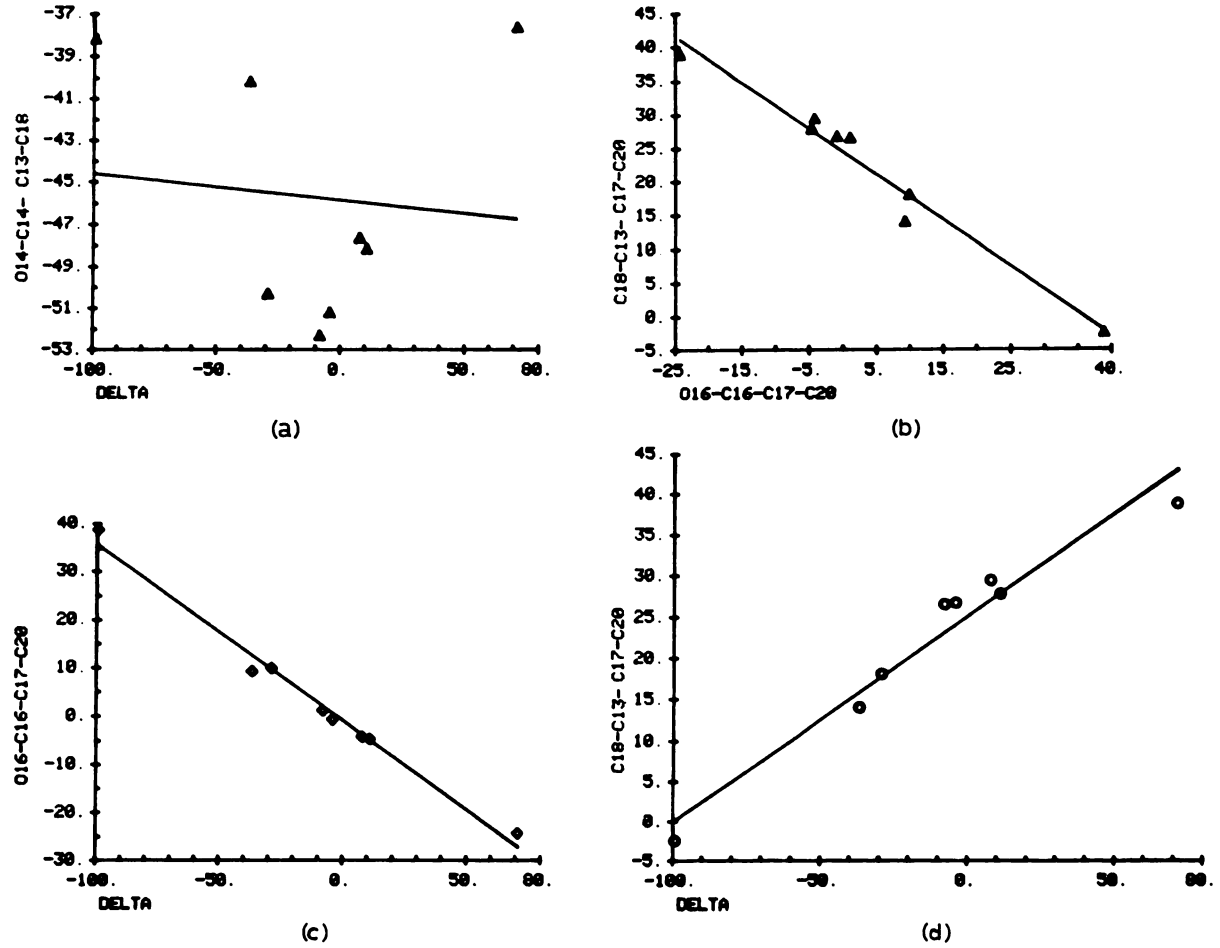


Fig. 3. Correlation between D-ring conformation and orientation of substituents on the D-ring in the sample of eight 16β-substituted cardenolides obtained from the crystallographic coordinates. Δ is a quantitative measure of the conformation of a five-membered ring (18). a, There is no correlation between D-ring conformation and the C-18–C-13–C-14–O-14 torsion angle. b–d, There is a strong correlation between D-ring conformation and the orientation of the substituents on C-16, C-17, and C-13 as revealed in the correlation between Δ and the O-16–C-16–C-17–C-20 and C-18–C-13–C-17–C-20 torsion angles, and between the torsion angles themselves. The two crystallographic observations with 16β-hydroxy substitution have unusual D-ring conformations with Δ values at the extremes (*left* and *right*) of the lines in b, c, and d.

parent compound **I** (least squares fit of 21 atoms, average distance between atoms fit = 0.09 Å), and the O-23 position is 0.55 Å from its position in compound **I**. The lactone rings in three crystallographic determinations of 16β-acetate-substituted cardenolides, oleandrin (19), the 21-bromo derivative of compound **XIV** (20), and compound **XIV** (21) are observed in the putative active conformation, with the carbonyl oxygen within 0.5 Å of the carbonyl oxygen of digitoxigenin when the steroid rings are fit. A stereo view of the superposition of the formate, compound **III**, and the three acetates fit to compound **I** is shown in Fig. 4. The ester groups are all observed in the same conformation; the four-atom plane of the ester is almost parallel to the lactone ring plane and the carbonyl of the ester and the carbonyl of the lactone ring make an angle of approximately 130°. The lactone rings all lie in the same orientation and the carbonyl oxygens all lie within 0.5 Å of the carbonyl oxygen of compound **I**. The distance between the carbonyl oxygen of the lactone ring and the carbonyl oxygen of the ester carbonyl is 5.3 ± 0.1 Å for the four esters. The distance between the carbonyl groups and the fact that they are pointing in opposite directions would seem to preclude their forming hydrogen bonds to the same functional moiety on the enzyme.

Discussion

The observed activities of the 16β-formate and acetate derivatives of gitoxigenin are comparable to those previously reported (8). The enhanced activity of these compounds is not due to gross solubility effects, since esterification of the 3β-hydroxyl by formate or acetate does not alter activity (Table 1). In contrast, monoglycoside substitution at the 3-hydroxyl site does substantially potentiate activity. This potentiation has been observed previously by Yoda *et al.* (13) and our group (1). There appears to be less potentiation when digitoxose is substituted to a highly active cardenolide such as the 16β-formate or acetate than when it is attached to gitoxigenin. As we have previously shown that 3β-monoglycoside attachment does not appear to have structural effects on C-16 substituents and D-rings of the steroid or carbonyl oxygen position (1), the reasons for the difference in potentiation are unclear.

The explanation for the effects of 16β-OH addition seems more clear. Analysis of the published cardiac steroid structures with a 16β-hydroxy substituent suggests that an intramolecular hydrogen bond between 16β-hydroxyl and the lactone ring is not possible. What is seen is either an intramolecular H-bond between the 14β- and the 16β-hydroxyl groups or an intermolecular H-bond from the 16β-hydroxyl group. Both influence

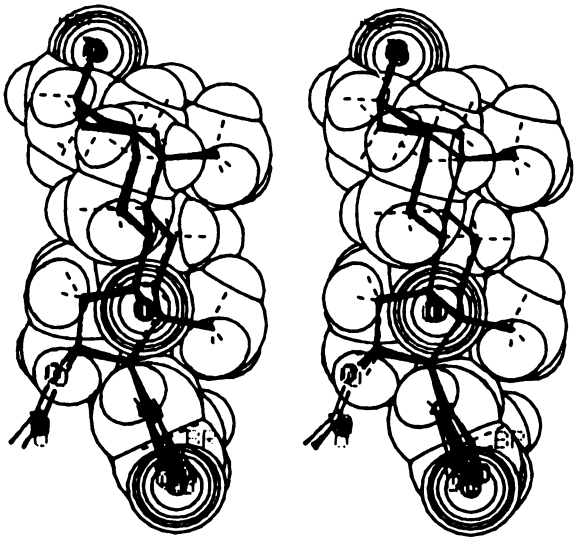


Fig. 4. Comparison of digitoxigenin (**I**) and four 16β-substituted esters, compound **III**, oleandrin, compound **XIV**, and 21-br**XIV**. Compound **I** is shown *dashed* and *spaced-filling*, and the esters are drawn in *solid lines*. The O-23 position in all four esters is within 0.5 Å of its position in compound **I**, and the lactone rings are all oriented similarly. The structures of gitoxigenin 16β-formate and 16β-acetates show that the formate/acetate carbonyl has a preferred conformation. The ester carbonyl oxygen is an average of 5.3 Å from the lactone ring carbonyl and points in the opposite direction. This observed geometry would seem to preclude the interaction of both carbonyls to the same moiety at the enzyme-binding site.

the D-ring conformation so as to move the lactone ring carbonyl oxygen away from the putative ideal position, thus accounting for the decreased potency of gitoxigenin.

As indicated above, the enhanced activity of the short-chain 16β-esters cannot be explained on the basis of altered position of the C-17 side-group carbonyl oxygen. Analysis of the structural data on C-16 acetate and formate cardenolides indicates that, in contrast to the C-16-hydroxy compounds, C-16-esters have a preferred D-ring and side-chain conformation. The ester group may serve to lock the side-chain in the putative active conformation. In addition, we speculate that the 16β-formate group (or acetate) forms another and separate interaction with the receptor site which accounts for its increased activity. Further studies will be needed to verify the cause of the increased activity of 16β-formate and acetate substitution on compound **II**. If all of the compounds in a series with variation in the C-17 side-group show an equal increase in activity with 16β-acetate or formate substitution, the concept that there is a second binding site for the 16-substituent would be supported. There is preliminary evidence for this in the equivalent effect on activity in the series of monodigitoxosides, 3β-formates, and 3β-acetates reported in Table 1.

Acknowledgments

Mr. Michael Barone and Miss Dani Vinh provided excellent assistance in carrying out the biochemical studies.

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