LITERATURE CITED

1. USSR Invertor's Certificate No. 618113, Otkrytiya, No. 29 (1978).
2. State Pharmacopoeia of the USSR [in Russian], Moscow (1968).
3. Z. L. Meneneva and A. G. Khomusaridze, Probl. Éndokrinol., No. 1, 102-109 (1972).
4. Z. L. Lemeneva, V. S. Zelenetskaya, and L. A. Zheldak, Probl. Éndokrinol., No. 4, 23-26 (1980).
5. L. D. Mashkovskii, Medicinal Agents [in Russian], Moscow (1984), pp. 537-538, Chap. 1.
6. Patent 3674865, 1972 (USA).
7. S. L. Steelman and F. M. Pehley, Endocrinology, 53, 604-616 (1953).
8. S. Stevens, in: Recent Research in Gonadotropic Hormones, Edinburgh (1966), pp. 140-142.

INVESTIGATION OF THE DEPENDENCE OF THE DIRECTION OF MICROBIOLOGICAL HYDROXYLATION ON THE STRUCTURE OF SUBSTITUTED PREGNANES

V. M. Ryzhkova, T. M. Bykova,
G. A. Klubnichkina, M. L. Zvereva,
T. I. Gusarova, L. M. Alekseeva,
and G. S. Grinenko

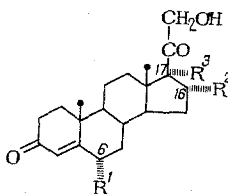
UDC 615.31:547.689.6'116

In previous studies the microbiological process of 11-hydroxylation of the steroid molecule in the synthesis of the highly active drug sinaflan was discussed [8, 9]. It was shown that the introduction of isopropylidine protection into the 16α,17α-dihydroxy group leads to a substantial inhibition of the formation of the 11α-epimer in cultures of *Tieghemella orchidis* and *Cunninghamella blakesleeana* and the consequent production of a high yield of the 11β-epimer.

The present work is devoted to a further study of the stereospecificity of the reactions of microbiological hydroxylation in order to select the optimum variant of the synthesis of steroid drug preparations.

The control of the microbiological hydroxylation of steroids by the introduction of substituents into various positions of the molecule is rather well-known [1]. However, these data are frequently uncoordinated. To obtain comparable results under comparable conditions we studied the influence of substituents at C(6), C(16), C(17) of the steroid molecule on the transforming activity of well-known hydroxylating cultures, *T. orchidis*, *T. hyalospora*, *C blakesleeana*, *Curvularia lunata*, and *Trichothecium roseum*.

Pregnanes containing various substituents at C(6), C(16), C(17), such as hydroxyl, fluorine, methyl, the isopropylidenedihydroxy group, and the acetoxy group, were used as substrates.



I: $R^1 = F$; $R^2 = R^3 = H$; II: $R^1 = R^2 = H$, $R^3 = OH$; III: $R^1 = H$, $R^2 = CH_3$, $R^3 = OH$; IV: $R^1 = H$, R^2 and $R^3 = OC(CH_3)_2O-$; V: $R^1 = F$, R^2 and $R^3 = -OC(CH_3)_2O-$; VI: $R^1 = R^2 = H$, $R^3 = OAc$.

S. Ordzhonikidze All-Union Chemicopharmaceutical Scientific-Research Institute, Moscow. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 20, No. 7, pp. 871-876, July, 1986. Original article submitted February 4, 1985.

0091-150X/86/2007-0511$12.50

© 1987 Plenum Publishing Corporation

TABLE 1. Dependence of the Direction of Microbiological Hydroxylation on the Structure of Substituted Pregnanes

Contnet of steroids after 24h of recording, %

Substrate	F. orchidis				C. blakesleeana				C. lunata			
	11β-OH	11α-OH	6α, 14α	uniden-tified products	11β-OH	14α-OH	6α, 14α	uniden-tified products	11β-OH	14α-OH	uniden-tified products	initial steroid
I	30	45	—	—	27	—	—	~5	36	—	—	—
II	58	26		~5	31	16	—	—	67	15	~8	—
III	59	28		—	30	20	—	~15	79	6	—	—
IV	73	6	—	—	65	6	—	—	21	—	—	51
V	77	7	—	—	63	9	—	—	30	—	—	25
VI	53	7	20	—	52	3	20	—	90	—	—	—

Substrate	T. roseum					T. hyalospora					
	11β-OH	11α-OH	6α, 14α	initial steroid	uniden-tified products	14α-OH	11β-OH	11α-OH	6α, 14α	uniden-tified products	starting materials
I	20	15	—	—	—	—	—	—	—	—	—
II	27	17	—	10	—	10	—	80	—	~5	—
III	35	21	—	15	—	—	—	80	—	~5	—
IV	15	62	—	—	—	—	—	—	—	~10	80
V	13	65	—	—	—	—	—	—	—	~10	80
VI	27	6	21	—	~10	—	15	32	22	—	—

The most indicative results obtained from the work are presented in Table 1.

A distinct relationship between the chemical structure of the substrate and the direction of hydroxylation was exhibited by a culture of T. orchidis. In the hydroxylation of 6α-fluorodeoxycorticosterone, containing no substituents at C(16) and C(17), the 11α-epimer predominates; its content relative to the 11β-hydroxy derivative is 1.5:1. Fermentation of cortexolone (II), containing a hydroxyl at C(17), leads to a shift of the reaction in the direction of the β-isomer; in this case twice as much hydrocortisone is formed in comparison with epicortisol. The data obtained on the influence of the hydroxyl C(17) on the ratio of the transformation products agree with the information cited earlier with regard to a culture of Absidia orchidis, which gave a ratio of 11α- and 11β-hydroxy compounds of 7:1 on progesterone and 4:5 on II, respectively [11].

The presence of a methyl group in the 16α position in the molecule (compound III) had no effect on the ratio of the indicated epimeric alcohols.

The introduction of such a voluminous substituent as the isopropylidene group into the molecule at the hydroxyls 16α,17α (IV) permitted us to obtain a high yield of the 11β-hydroxy isomer, with a low degree of formation of the 11α-epimer: The ratio of the epimers was 11:1. Additional introduction of 6α-fluorine into the molecule in compound V practically does not change the direction of hydroxylation.

Extremely interesting results with a culture of T. orchidis were obtained when the 17α-acetate of VI was used: In this case, just as in the example with IV or V, there is a shielding of the α-region, but transformation occurs less directedly, and no increase in the yield of the 11β-hydroxylated product is observed on account of the formation of an appreciable amount of the 17α-acetate of 6α,14α-dihydroxycortexolone. The change in the direction of hydroxylation and the appearance of OH at C(6) can be explained according to the Brennon hypothesis by nonspecificity of the enzymatic hydroxylation, due to the equivalence of positions 11 and 6 in two different orientations of the steroid molecule at the moment of its fixation on the surface of the enzyme [10].

For a culture of C. blakesleeana, results similar to the preceding were obtained on the influence of the steroid structure on the process of hydroxylation: The voluminous substituent of substrates IV and V directed the process toward the predominant formation of the 11β-hydroxysteroid on account of shielding of the α-region; in the case of a less voluminous substituent of the substrate VI, instead of an 11α-hydroxysteroid a 6α,14α-dihydroxysteroid is formed.

We should mention that when a culture of C. blakesleeana, possessing a wide spectrum of transforming activity (introduction of the hydroxyl in positions 11, 9, 14, and 7), substituents at C(16), C(17) of the steroid substrates III-VI inhibit the process of decomposition of steroids observed with substrates I and II. This fact may be associated with shielding of the α-region, including position 9α, hydroxylation at which is one of the first steps of the oxidative degradation of the steroid molecule [14].

In addition, the presence of a 6α-fluorine in substrate I may promote a polarization of the latter with the fluorine of the position, which leads to a change in the direction of hydroxylation into position 9α instead of 7α, followed by destruction of the molecules, as has been noted for C. lunata [13]. In experiments that we conducted with C. lunata with substrate I, a low yield of the hydroxylated product was also noted according to the more profound conversion of the steroid molecule. From II, C. lunata forms 11β- and 14α-hydroxylated products in a 3.4:1 ratio, respectively. The use of 16α-methylcortexolone is extremely promising for this culture; in this case the amount of the side product, 14α-hydroxysteroid, decreases from 15 to 6% in comparison with the hydroxylaton of II, and the yield of the 11β-hydroxylated product correspondingly increases. The influence of the 16α-methyl is discussed in a work with the fungus Mucor griseocyanus; moreover, it has been suggested that the 14α-hydroxylation of 16α-methyldeoxycorticosterone is sterically inhibited, since the distance in the molecule between the 16α-methyl and the hydrogen atom in position 14α is only about 0.3 nm [15].

The introduction of a 16α,17α-acetonide group into the molecule hindered the transformation of the steroid by the fungus C.lunata. Fermentation on substrates IV and V proceeded to an extent of only 20-30%. The greatest effect for C. lunata was obtained with the 17α-acetate of cortexolone (VI), where the reaction occurs virtually quantitatively with the formation of only 1 11β-hyroxylated transformation product, which agrees greatly with the available literature data [4].

Both 11α- and 11β-hydroxylation are known for strains of T. roseum. The culture of T. roseum on substrates I, II used in the experiments presented showed some advantage in the direction of 11β-hydroxylation at a low general yield of the transformation products on account of a more profound digestion of the steroid molecule. When 16α-methylcortexolone (III) is used, a decrease in the percentage of destruction and inhibition of 14α-hydroxylation are observed. On account of the introduction of the acetonide group in to the molecule, with IV and V a high total yield of the hydroxylated products can be obtained with an absolute predominance of 11α-hydroxylation. In the transformation of the 17α-acetate VI, the main reaction products are 16β- and 6α,14α-dihydroxysteroids, formed in approximately equal quantities.

A culture of T. hyalospora, transforming II and III to the corresponding 11α-hydroxyderivatives with a yield of up to 80% [3], gave a mixture of products consisting of 11β-, 11α-, and 6α,14α-dihydroxysteroids in the ratio of 1:2:1.5, respectively, when the acetate VI was used. The presence of an acetonide group in the substrates IV and V virtually entirely suppressed the possibility of transformation of these compounds by a culture of T. hyalospora. Thus, in the transformation of steroid substrates by representatives of the phycomycetes, T. orchidis, C. blakesleeana, and T. hyalospora, a pronounced dependence of the 11β-hydroxylation on the presence of a voluminous 16α-17α-substituent, shielding the α-region, is observed.

When a representative of the fungi imperfecta, T. roseum, was used on substrates IV and V, an inverse directing action of the acetonide group was observed, i.e., predominant formation of an 11α-hydroxy compound, which may be associated with differences in the orientation of the enzyme complexes relative to the steroid molecule in taxonomically different groups of microorganisms.

The presence of a 17α-acetate group leads to an inhibition of the formation of an 11α-hydroxysteroid and a substantial shift of the reaction toward the formation of a 6α,14α-dihydroxysteroid for all the cultures considered, with the exception of a culture of C. lunata, which is characterized by the formation only of an 11β-hydroxycompound on account of shielding of the 14α-position.

A definite decrease in the degree of formation of the 14α-hydroxycompound is observed when a 16α-methyl group is introduced into the molecule. It should be noted that the presence of substituents (especially voluminous ones) at C(16), C(17) protects the steroid molecule from more profound transformation.

Since the mechanism of microbiological hydroxylation, predetermining the direction of transformation of steroids, is still being studied [12], the detection of the peculiarities of the transformation by representatives of different groups of microorganisms may promote a more substantiated selection of the microorganism in one reaction sequence or another in the general scheme of corticosteroid synthesis.

The structure of the compounds obtained in microbiological transformation was established with the aid of the mass spectra and ^{1}H-NMR spectra. In the case of inoculation of the substrate I with a culture of T. orchidis, a mixture of 11α- and 11β-hydroxylated products was obtained, and they were isolated and characterized in the form of 21-acetates. Under conditions of mass spectrometric fragmentation, both compounds give a molecular ion, which corresponds to the molecular weight of these isomers (m/e = 406). The main direction of fragmentation is stripping of an $AcOCH_2$ group from the molecular ion, with the formation of a fragment from which a water molecule is ejected (m/e = 315); however, the intensity of this peak in the spectra of the stereoisomers differs. In the case of the 11β-compound the intensity of the peak with m/e = 315 is basic, which is evidence of an axial arrangement of the 11β-hydroxyl group. In the spectrum of the 11α-isomer the intensity of the peak of stripping of water is low, which is evidently caused by an equatorial orientation of the hydroxyl group.

The ^{1}H-NMR spectrum of the 11α-compound contains a signal of the axial 11β-proton at 4.0 ppm. On account of the axial-axial interaction with protons at C(9) and C(12) and the axial-equatorial interaction with the proton at C(12), the signal is additionally split and takes the form of sextet, J_{a-a} = 10 Hz, J_{a-e} = 5 Hz.

The signal of the equatorial 11α-proton in the spectrum resonates in a weaker field, the doublet J_{e-e} = 5 Hz at 4.4 ppm.

Under the action of T. orchidis on the 17,21-diacetate of 17α,21-dihydroxypregn-4-ene-3,20-dione, in addition to the 11β- and 11α-hydroxy compounds, about 20% of the 6α,11β,14α,17α,21-pentaol-3,20-dione is formed, the structure of which, in the form of the 21-acetate, was established with the aid of the ^{1}H-NMR spectrum. The presence of hydroxyl in position 6 is confirmed by the presence of a multiplet of the proton at C(6) with δ 4.34 ppm in the ^{1}H-NMR spectrum. The signal of the proton at C(4) with δ 5.74 ppm proves to be shifted in this case into a weaker field region in comparison with the analogous signal in the spectrum of hydrocortisone 17,21-diacetate. A comparison of the chemical shifts of the angular methyl groups in the spectrum of the product isolated and hydrocortisone-diacetate shows that the signal of the CH_3 group at C(19) in the spectrum of this compound is shifted by 0.2 ppm in the strong field direction, whereas the chemical shift of the signal of the CH_3 group at C(18) is virtually unchanged. We estimated the changes in the chemical shifts for the methyl protons at C(19) and C(18) in the transition from hydrocortisonediacetate to the compound isolated. For this purpose we used the increments of the OH group and considered the possible directions of hydroxylaton, namely 11β,6α,6β,14α [2]. The most satisfactory correspondence of the experimental and calculated values of the chemical shifts of the angular methyl groups was obtained for the 17,21-diacetate of pregn-4-ene-6α,11β,14α,-17α,21-pentaol-3,20-dione; on this basis the indicated structure was assigned to the compound isolated.

EXPERIMENTAL

The medium for the culturing of T. orchidis, C. blakesleeana, T. hyalospora, and T. roseum contained 30 g of glucose, 5 g corn extract, 3 g peptone, 3 g yeast autolysate, 5 g KH_2PO_4, and 100 ml of water; pH 6.8-7.2. The inocula were cultured in 0.75-liter flasks on a circular shaker, performing 220 rpm at 26-28°C.

For the culturing of C. lunata we used a medium containing 30 g sucrose, 2.5 g yeast autolysate, 2 g $NaNO_3$, 3.2 g $(NH_4)_2HPO_4$, 1 g K_2HPO_4, 0.5 g KCl, and 0.5 g $MgSO_4$, dissolved in 1,000 ml of water; pH 6.1-6.2. During transformation, mycelium washed free of medium was used. The process was conducted in phosphate buffer at pH 6.0.

The concentration of the initial steroid in the experiments with all the cultures was 0.3-0.5 g/liter; the steroid was introduced in the form of a 2.5% solution in DMFA, and transformation was carried out in flasks under the same conditions as the culturing of the inoculates or (when enlarged loads were delivered) in glass fermenters with a delivery of air of 0.5 liter/(liter·min) and a working mixer performing 400 rpm, for 24-48 h. The process was monitored by the method of thin-layer chromatography of steroids extracted from the sample with methylene chloride on Silufol UV-254 plates in the system methylene chloride—methanol—water (19:1:0.1) and chloroform—acetone (7:3). The chromatograms were examined in UV light and then treated with a 1% solution of vanillin in 10% perchloric acid, followed by heating, identifying the transformation products according to the corresponding standards. For a quantitative determination, the steroids were eluted from the chromatogram by the standard, and the content of steroids was measured spectrophotometrically on an SF-16 instrument.

In experiments with C. blakesleeana, when Reichstein's substance S was used, where the coloration of the transformation product was not distinct, before application on the chromatogram the residue was acetylated at C(21), and the acetylated product was analyzed.

The specific rotation was determined in chloroform. The IR spectra were recorded on Perkin-Elmer (USA) and UR-10 (German Democractic Republic) spectrometers in suspensions of liquid petrolatum. The ^{1}H-NMR spectra were obtained on NM-4H-100 and XL-100-A spectrometers (JEOL, Japan). The chemical shifts are cited in the δ scale, with tetramethylsilane as the internal standard. The mass spectra were obtained on MAT-112 and MX-1303 instruments (direct introduction of the sample into the source). Ionizing voltage 70 eV (MAT-112) and 30 eV (MX-1303).

6α-Fluoropregn-4-en-21-ol-3,20-dione (I). To a solution of 8 g of the 21-acetate of pregn-5-ene-3β,21-diol-20-one in 160 ml of methylene chloride at −60°C we added 5.5 ml of 70% hydrofluoric acid, mixed for 10 min, added 5.7 g of dibromanthin in portions, and neutralized with an ammonia solution after 15 min. The organic layer was removed; the aqueous layer was extracted with methylene chloride, the extracts were combined, washed with water to a neutral pH, dried, the solvent evaporated under vacuum, the residue triturated with ether with an addition of acetone, and the precipitate filtered. We obtained 5.5 g of the

21-acetate of 5α-bromo-6β-fluoropregnane-3β,21-diol-20-one, mp 162.8°C (with dec.), $[\alpha]_2$ + 19.47°. IR spectrum, ν_{max}, cm^{-1}: 3600, 3480 (OH), 1741, 1720 (CO); (CO ester). ^1H-NMR spectrum in $CDCl_3$, δ, ppm: 0.73 ($18\text{-}CH_3$); 1.41 (d., $19\text{-}CH_3$, J 3.5 Hz); 2.18 ($-OCOCH_3$); 3.61 (H-17); 4.65 ($-CH_2-$); 4.59 and 5.67 (H-6); 4.70 (H-3). Found, %: C 58.32; H 7.48; Br 16.68. $C_{23}H_{34}BrFO_4$. Calculated, %: C 58.32; H 7.24; Br 16.88.

From the mother liquor we isolated 0.84 g of the 21-acetate of 5β-fluoro-6α-bromopregnane-3β,21-diol-20-one, mp 176-178°C, $[\alpha]_D$ + 55.34°. IR spectrum, ν, cm^{-1}: 3580 (OH); 1710 (CO); 1350 (CO). Found, %: C 58.04; H 7.0. $C_{23}H_{34}BrFO_4$. Calculated, %: C 58.04; H 7.0.

A 3-g portion of the 21-acetate of 5α-bromo-6β-fluoropregnane-3β,21-diol-20-one was dissolved in 40 ml of acetone, cooled to 0°C, and oxidized with 3 ml of Jones' reagent. The reaction solution was mixed for 5 min, and the excess of Jones' reagent reduced with methanol. The reaction mass was diluted with 20 ml of water, extracted with methylene choride, the extract washed with water, dried with calcium chloride, the solvent evaporated under vacuum to a volume of 20-30 ml, then 200 ml of acetone, and evaporation was continued to a volume of 40-50 ml. To an acetone solution of a steroid we added 1 ml of a 15% solution of hydrogen chloride in methanol and mixed the reaction mass for 2 h at 20°C. In the process of isomerization of the fluorine atom at C(6) there is a partial saponification of the 21-acetoxy group. The mixture of products was isolated by diluting the mass with 300 ml of water. The precipitate was filtered off, washed with water, and dried. To establish the structure of the compounds obtained, the mixture was acetylated at C(21) with acetic anhydride in pyridine solution. Chromatographic monitoring (thin-layer chromatography on Silufol plates, hexane—ethyl acetate, 1:1) shows that one acetylation product is formed in the course of the reaction. The reaction mass was poured out into an ice bath, the precipitate filtered off, washed with a 5% solution of hydrochloric acid and then with water. We obtained 1.9 g of the 21-acetate of 6α-fluoropregn-4-en-21-ol-3,20-dione, mp 170.8°C (with dec.), $[\alpha]_D$ + 177.63°. IR spectrum, ν, cm^{-1}: 1740, 1710, 1670, 1240. ^1H-NMR spectrum ($CDCl_3$), δ, ppm: 0.67 ($18\text{-}CH_3$); 1.15 ($19\text{-}CH_3$); 2.11 ($21\text{-}COOCH_3$); 3.56 (H-17); 4.57 ($-CH_2-$); 4.80 and 5.49 (H-6, J 49 Hz); 6.03 (H-4). Found, %: C 70.48; H 8.18. $C_{23}H_{32}FO_4$. Calculated, %: C 70.56; H 8.24.

A 0.8-g portion of I was inoculated with a culture of T. orchidis; the culture fluid, separated from the mycelium, was extracted twice with equal volumes of ethyl acetate. The solvent was evaporated under vacuum. The residue was dissolved in 14 ml of glacial acetic acid, 0.61 g of barium acetate and 0.52 ml of acetic anhydride were added, the mixture was exposed for 24 h at room temperature, poured out into 50 ml of water, and the precipitate was filtered and washed with water. We obtained 0.32 g of a mixture of two products. Thin-layer chromatography on Silufol plates in the system chloroform—methanol—water (95:5:0.5) showed the presence of two substances with R_f 0.44 (11α-hydroxyisomer) and R_f 0.57 (11β-hydroxyisomer). The mixture was separated as follows: On a Silufol plate we applied 200 μg of a mixture of the substances in a strip and chromatographed in the system indicated above. Then the strips containing the substances with R_f 0.44 and 0.57 were cut out of the plate. The steroid was eluted with a mixture of methylene chloride and methanol in a 4:1 ratio. The operation on separation was multiply repeated, and the combined eluates were evaporated under vacuum, yielding 0.08 g of the 21-acetate of 6α-fluoropregn-4-ene-11β,21-diol-3,20-dione and 0.06 g of the 21-acetate of 6α-fluoropregn-4-ene-11α,21-diol-3,20-dione.

17,21-Diacetate of Pregn-4-ene-6α,, 11β,14α, 17α, 21-pentaol-3,20-dione. A 5 g portion of VI was inoculated with a culture of T. orchidis. After exhaustive extraction of the culture fluid with ethyl acetate and evaporation of the solvent, the residue was acetylated with acetic anhydride in acetic acid. We obtaine 2.83 g of a mixture of three products: the 17,21-diacetate of pregn-4-ene-11β,17α,21-triol-3,20-dione, its 11α-hydroxyisomer, and the 17,21-diacetate of pregn-4-ene-6α,11β,14α,17α,21-pentaol-3,20-dione. The indicated mixture was triturated with 10 ml of methanol, and the precipitate filtered off. The pentaol passes into the mother liquor in this case. The mother liquor was chromatographed on Silufol plates in the system benzene—acetone (4:1). After elution of the steroid and evaporation of the solvent, we obtained 0.2 g of the 17,21-diacetate of the pentaol.

LITERATURE CITED

1. A. A. Akhrem and Yu. A. Titov, Steroids and Microorganisms [in Russian], Moscow (1970), pp. 249-257.

2. N. S. Bhakka and D. G. Williams, The Use of NMR in Organic Chemistry [Russian translation], Moscow (1966), pp. 31-37.
3. L. K. Garsia-Rodriges, K. N. Gabinskaya, V. F. Shner, and O. V. Messinova, Khim.-farm. Zh., No. 4, 62-66 (1977).
4. Great Britain Patent No. 1150955, Chem. Abstr., 69, 77624 p. (1968).
5. Great Britain Patent No. 749414, Chem. Abstr., 51, 1320 (1957).
6. US Patent No. 3079384, Chem. Abstr., 59, 2911 e. (1963).
7. US Patent No. 3549498, Ibid., 72, 44024 b. (1970).
8. V. M. Ryzhkova, T. I. Gusarova, G. A. Klubnichkina, et al., Khim.-farm. Zh., No. 12, 69-71 (1980).
9. V. M. Ryzhkova, T. I. Gusarova, G. S. Grinenko, et al., ibid., No. 3, 61-66 (1982).
10. D. R. Brannon, J. W. Parrich, B. J. Wiley, and L. Long, J. Org. Chem., 32, 1521-1527 (1967).
11. A. Čapek and O. Hanč, Folia Microbiol. (Praha), 6, 237-241 (1961).
12. H. L. Holland, Chem. Soc. Rev., 11, 371-378 (1982).
13. K. Kieslich, K. Petzololt, H. Kosmol, and W. Koch, Justus Liebigs Ann. Chem., 726, 168-174 (1969).
14. C. J. Sih, Biochim. Biophys. Acta, 62, 541-543 (1962).
15. U. Valcavi, B. Cossi, and S. Pedeschi, Farm. Ed. Sci., 28, 873-877 (1973).