BUTTERWORTH HEINEMANN

The scarlet letter: Reichstein's Substance S. A comparison of the angiostatic properties of 5α-tetrahydro S and 5β-tetrahydro S

Harry E. Hadd,* A. Prztjazny,† and John M. Kokosa†

**Holling's Cancer Center, Charleston, South Carolina; †GMI Institute, Flint, Michigan, USA*

5β-Tetrahydro-Reichstein's Substance S (3α, 5β-THS) from different sources yielded variable bioassay activity in the chick chorio-allantoic membrane assay system. Physical characterization showed impure products. Synthesis of this compound by two different routes yielded active and inactive 3α,5β-THS. Of the other two epimers, 3β,5β-THS (epi-THS) and 3α,5α-THS (allo-THS), only the latter was active. These results suggest that the impurities present in 3α,5β-THS synthesized by reduction of the α,β-unsaturated ketone of Substance S might be either or both the epi-/allo-epimers (3β,5β-THS and 3α,5α-THS, respectively), with only the latter contributing the positive angiostatic activity to the mixture. Of the two synthetically derived compounds, only the latter was shown to maintain the activity, whereas 3α,5β-THS was not antiangiogenic. (*Steroids* **60**:650–655, 1995)

Keywords: chick chorioallantoic membrane (CAM) assay; angiostatic steroids; 5α-tetrahydro-Reichstein's Substance S; 5β-tetrahydro-Reichstein's Substance S.

Introduction

Folkman and colleagues identified a series of steroids that inhibited the new growth of blood capillaries in the chick chorioallantoic membrane assay (CAM) and defined them as angiostatic steroids.[1,2] One of the most potent was 5β-tetrahydro-Reichstein's Substance S (5β-pregnane-3α,17α,21-triol-20-one; 3α,5β-THS), and it appeared to hold considerable promise as a therapeutic agent with great angiostatic properties.[1] 3α,5β-THS is a steroid with no known biological activities; it has no glucocorticoid activity, such as that of cortisol. In contrast, cortisol, the main glucocorticoid, also possesses a high angiostatic index in the CAM assay, although not to the degree of 3α,5β-THS. Thus, the angiostatic function of 3α,3β-THS is not a minor characteristic of glucocorticoids.

However, 3α,5β-THS posed a problem. Only one lot of this compound obtained from Sigma Chemical Co. was active (J. Folkman, Personal Communication, 1989). The steroid obtained from other suppliers yielded variable activities in the CAM assay (Table 1). None of the steroid from other suppliers succeeded in equalling the activity from a specific lot of steroid from Sigma; for example, 3α,5β-THS obtained from Berlichem Corporation was devoid of angiostatic activity.

In general, 3α,5β-THS has been synthesized principally by two routes: if the precursor is 5β-pregnan-3α-ol-20-one, as shown in Figure 1, and the two hydroxyl functions are inserted at C-17 and C-21, then the 3α,5β-THS shows no activity in the CAM assay; this was the synthetic route employed by Berlichem Corporation and Steraloids.

If Substance S is the starting material, the reduction of the α,β-unsaturated ketone yields the 3α-ol,5β-H configuration, first by reduction of the 4,5-ene double bond by Pd/C, and then by reduction of the 3-ketone by Li-Selectride treatment (Figure 2). The 3α-hydroxyl function may also be generated by inversion of the 3β-hydroxyl group if the 3-ketone is reduced by H_2/Raney Ni. 3α,5β-THS from various suppliers derived by this synthetic route yielded a steroid with variable and inconsistent activity in the CAM assay. It should be noted that the melting point of all commercial preparations was uniformly low when compared to reference values (Table 1). Upon recrystallization of the compound obtained from Berlichem Corp., the melting point was 215°C, in accord with literature values,[3] but the angiostatic activity was still absent. Sodium borohy-

Address reprint requests to Harry E. Hadd, 1051 B North Jamestown Road, Decatur, GA 30033, USA.
Received January 20, 1995; accepted May 4, 1995.

655 Avenue of the Americas, New York, NY 10010

0039-128X/95/$10.00
SSDI 0039-128X(95)00080-A

Table 1 Physical constants and biological activity of 5β-THS and 5α-THS

Source	Melting point (°C)	CAM assay percent activity	
		Heparin	CD14S
3α,5β-THS			
Synthesized via reduction of Substance S			
Sigma			
lot #49F4025	202–204	66	
lot #49F4024		23	
lot #38F384-4046		20	
lot #4024		57	66
lot #4025		20	63
Steraloids	204–206	40	12
Rsch Plus	204–210	33	0
Mattox	208–210; 204–211	10	28
This synthesis	214	0	0
Synthesized from pregnanolone			
Berlichem	202–204; 204–206	0	0
Crystallized EtOAc	215	0	0
Steraloids		0	0
3α,5α-THS			
This synthesis	223–225	40%	40%
ALCO	223–225	40%	

dride reduction of the 20-carbonyl of the Diosyn compound yielded 5β-pregnane-3α,17α,20β,21-tetrol, which also showed no activity in the CAM assay.

Research plan

It appeared that the stereochemistry about C-5 and C-3 might hold the answer to the problem: Were 3α,5α-THS (allo-THS) and/or 3β,5β-THS (epi-THS) present as impurities in the 3α,5β-THS synthesized by the reduction of Substance S? If so, it might account for the observed angiostatic activity of the 3α,5β-THS obtained from Sigma. Figure 3 shows the four epimers of THS.

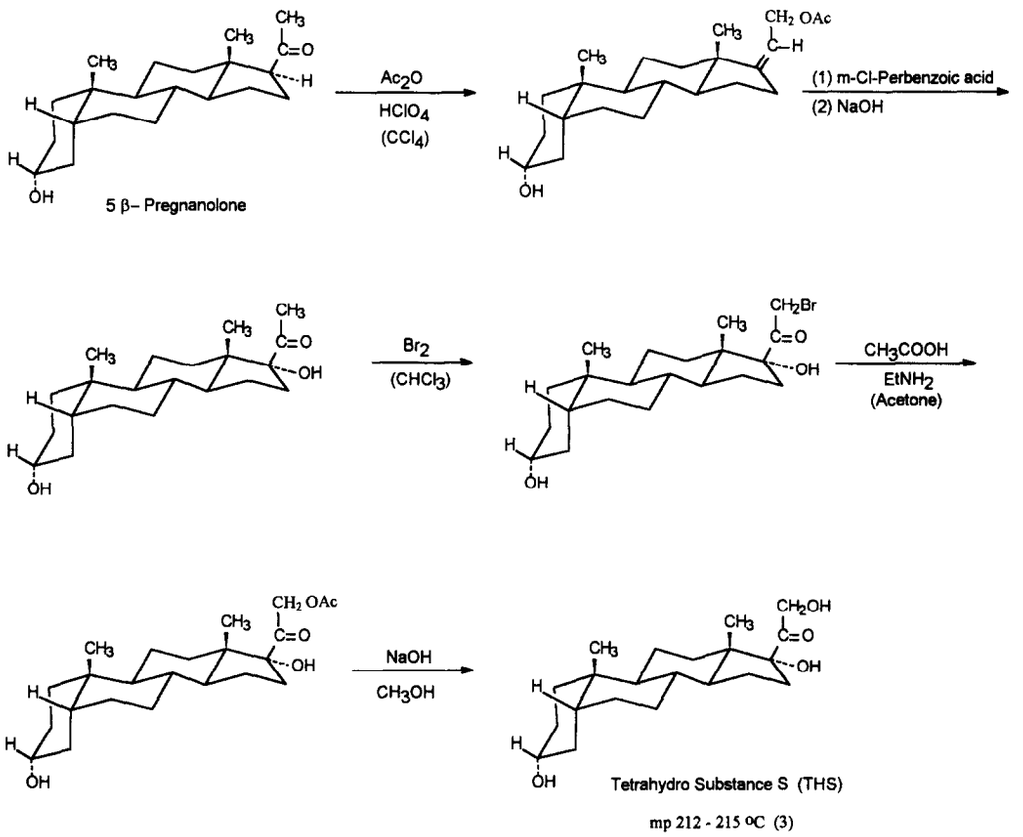
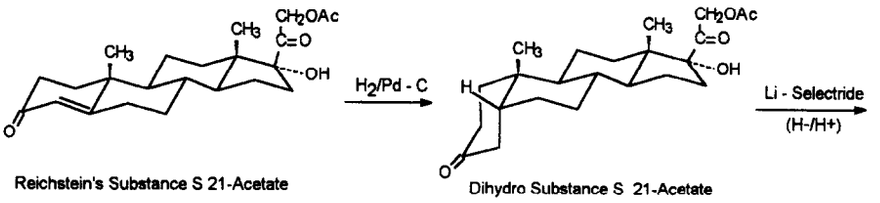


Figure 1 Synthesis of 5β-tetrahydro Substance S from pregnanolone



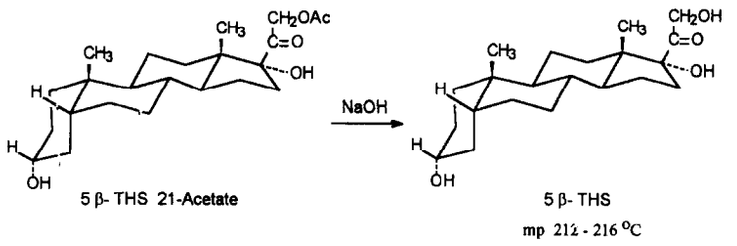
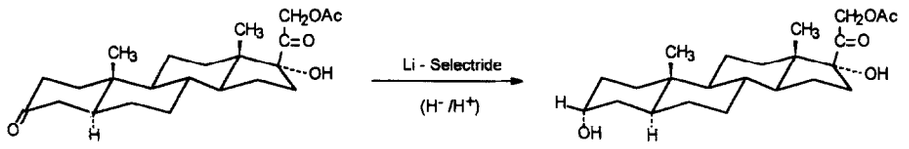
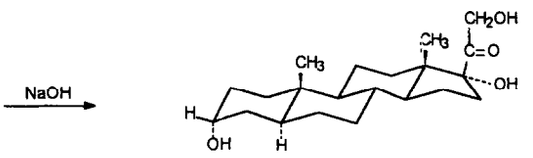


Figure 2 Synthesis of 5α-/β-tetrahydro Substance S





Therefore, the reduction of Substance S was carried out, essentially by the procedure described by Harnik,[3] Hosoda et al.,[4] van Euw et al.,[5] and Templeton et al.[6] Two compounds resulted and were identified as 3α,5β-THS[3,4,5] and 3α,5α-THS.[5] The ^{1}H NMR of each was in accord with the values of Hosoda et al.[4] Additionally, 3α,5β-THS from various sources was characterized by FTIR, mass spectra, and high-performance liquid chromatography (HPLC). The CAM assay of all compounds was carried out in Dr. Folkman's laboratory, in the presence of heparin or β-cyclodextrin tetradecasulfate. Only 3α,5α-THS was anti-angiogenic in the CAM assay. Two samples of 3α,5α-THS, prepared by two different routes, were tested (Table 1). Additionally, 3β,5β-THS obtained from Steraloids was tested in the CAM assay and shown to be inactive.

Experimental

^{1}H NMR values were recorded in $CDCl_3$ with a 400 mHz Varian instrument, Hollings Cancer Center, MUSC, Charleston, SC, or a 300 mHz GE instrument Chemistry Department, Emory University. FTIR spectra were recorded on samples in KBr with a Perkin Elmer. Mass spectroscopy by EI, and performed by Dr. Adams, Department of Chemistry, Emory University. HPLC was performed with Waters instrumentation by Mike Woodman in Oak Brook, IL, USA. Melting point determinations were observed with the Leitz melting point hot stage and are uncorrected. Chemicals and solvents were from Aldrich Chemical Co. Steroid sample sources were from Sigma Chemical Co., St. Louis, MO; Research Plus Laboratories Inc. (Bayonne, NJ, USA); Dr. V.R. Mattox, Mayo Clinic (Rochester, MN, USA), Steraloids (Wilton, NH, USA), ALCO (Lugano, Switzerland), and Berlichem Corp. (Wayne, NJ, USA).

Synthesis

Reduction of 4,5-double bond. Under N_2 Substance S-21-acetate was introduced to the hydrogenator bottle, and dried ethyl acetate was added, followed by 5% Pd/C. Under H_2 at 30 lbs/in^2, reduction was carried out for 18 h. The 5α- and 5β-dihydro-Substance S-21-acetates were easily separable by the technique described by Harnik, i.e., following removal of the Pd, the ethyl acetate solution was reduced to approximately 40%, and the 5α-DHS-21-acetate separated easily. Following filtration, further reduction in

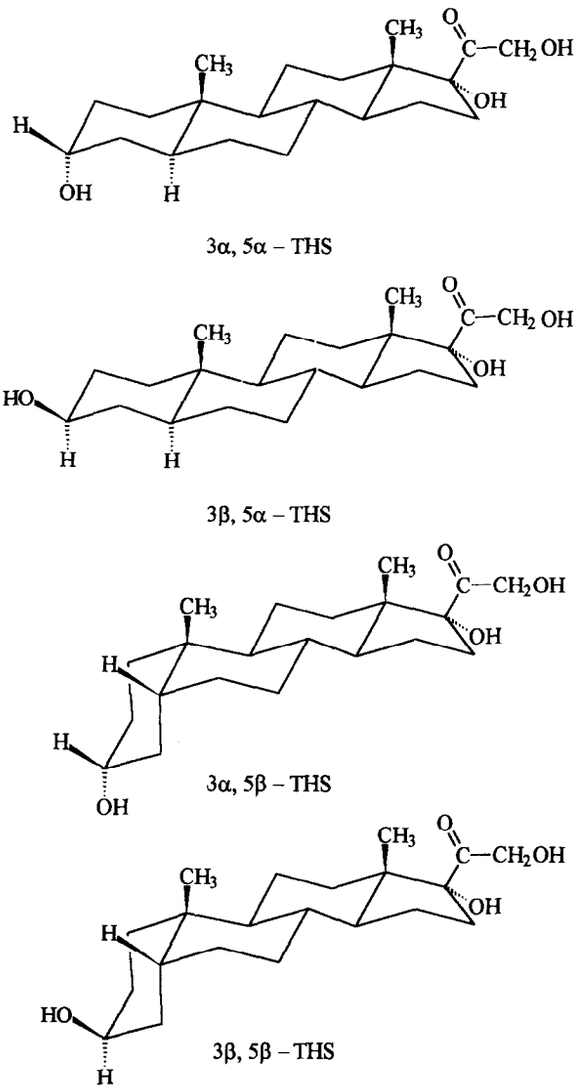


Figure 3 The four epimers of tetrahydro-11-desoxycortisol (THS)

volume of the ethyl acetate solution was reflected in the crystallization of the 5β-DHS-21-acetate.

Reduction of the 3-ketone of 5α-DHS-21-acetate. The Li-Selectride procedure according to Templeton et al.[6] was followed.

3α,5α-THS. 5α-DHS-21-acetate (0.996 g) was placed in an oven-dried 100 mL 3-neck flask with a stir-bar and the 3 necks closed with appropriate rubber septa. The system was placed under N_2 (needle in and out), and 50 mL dry tetrahydrofuran (THF) was added. The flask was cooled to −78°C with dry ice/acetone. There was added via syringe 2.8 mL LS-Selectride (10% × 5) over about 1 min. Stirring was continued at −78°C for 15 min, then the flask was warmed to 0°C and stirred for an additional 1 h. The solution was poured into a 1 L separatory funnel containing 300 mL H_2O and 1 mL HOAc. An additional 100 mL of EtOAc was added and the mixture was shaken. Then, 25 mL of a saturated NaCl solution was added to aid in the breaking up of the emulsion, which was allowed to stand for separation of the two phases. The aqueous phase was further extracted with 50 mL of EtOAc. The combined EtOAc phase was washed with 75 mL of saturated NaCl solution and then dried over anhydrous Na_2SO_4. The EtOAc was filtered and evaporated under reduced pressure (rotovap) and yielded 0.9 g of crude 3α,5α-THS-21-acetate. IR spectra indicated one carbonyl absorption peak was gone. One hundred milligrams (2.55×10^{-4} mol) of the latter was treated with 51 mg (5.1×10^{-4} mol) $KHCO_3$ in 10 mL MeOH plus 5 drops H_2O. The mixture under N_2 was stirred at RT overnight. H_2O (15 mL) was added and the precipitate filtered, washed, and dried to yield 38 mg of 3α,5α-THS, m.p. 182–185°C. Recrystallization from EtOAc yielded 25 mg of crystals, m.p. 225–226°C (lit. 224–226°C).[3,4,5] ^{1}H NMR ($CDCl_3$) Δ = 0.65 (3 H,s,18-CH_3). 0.77(3 H, s, 19-CH_3) 4.27, 4.64 (ea 1 H, d, J = 20 Hz, 21-H). 4.02 (1 H, m, 3β-H). IR (KBr; wave no., cm^{-1} −OH str 3426.240, C-H str 2925.425, sh 2856.850, C = 0 1707.775, fingerprint region 1446.847, 1385.927, 1079.799, 1092.208, 1044.534, 1005.904. (Lit.[6].)

3α,5β-THS. The procedure described above for the reduction of the 3-ketone of 5α-DHS-21-acetate was repeated for the preparation of 3α,5β-THS-21-acetate. Li-Selectride reduction of the 3-ketone of 5β-DHS-21-acetate yielded 3α,5β-THS-21-acetate and, following removal of the 21-acetate, 3α,5β-THS, m.p. 215°C after recrystallization from EtOAc (lit. 212–216°C [3]. ^{1}H NMR ($CDCl_3$) 0.66 (3 H, s, 18-CH_3), 0.95 (3 H, s, 19 CH_3), 3.65 (1 H, m, 3β-H), 4.28, 4.64 (ea 1 H, d, J = 19 Hz, 21-H). IR (KBr) wave no., cm^{-1} −OH str 3426, C-H str 2925, sh 2856, C = O 1707, fingerprint region 1446, 1385, 1092, 1079, 1044, 1005. (Lit.[6].)

Results

^{1}H NMR

The spectra were recorded in $CDCl_3$ with a 300 or 400 mHz instrument employing similar settings during one period of observation.

3α,5β-THS

Although the samples of 3α,5β-THS from Sigma showed a high index of bioactivity in the CAM assay, identifiable 3β-H of 3α,5α-THS was absent. However, the ^{1}H NMR of 3β,5β-THS showed the readily identifiable 3α-H at 4.1 ppm. This signal was observed in one sample of 3α,5β-THS (Steraloids). The ^{1}H NMR spectra of all 3α,5β-THS

Table 2 Physical properties of 3α,5β-THS mass spectra electron impact (EI)

Source	Mass spectra
Sigma batch 49F4025	(358), 350 (M^+), 320, 302, 272, 255, 229 (P.I.), 201
batch 49F4024	(358), 350 (M^+), 320, 302, 272, 255, 229 (P.I.), 201
Research plus	(358), 350 (M^+), 320, 302, 272, 255, 229 (P.I.), 201
Diosyn	350 (M^+), 320, 302, 272, 255, 229 (P.I.), 201
Steraloids	350 (M^+), 320, 302, 272, 255, 229 (P.I.), 201

Table 3 High-performance liquid chromatography

Source	Retention time (min)	Peak area %
Sigma batch 49F4025	6.47	96.34
Research plus	6.42	89.11
Mattox	6.49	87.89
Berlichem	6.43	90.84
Steraloids	6.43	92.27

Instrument: Waters, 230 nm detector UV absorbance.
Conditions: Column: 3.9 × 75 mm, 4 μM Novapak C18; solvent, CH_3CN/H_2O (30%, 70%) for 3 min; gradient from 3 min to 10 min (30%–90% CH_3CN), hold 2 min, then 100% CH_3CN for 6 min.

samples were identical with regard to the salient features, the proton signals of the CH_3-groups at C-18 and C-19, the 16-CH_2, Hs of the 17α-OH and 21-OH, the 3β-H and the C-21-CH_2. The single exception was the sample from Steraloids that appeared to have a small impurity at 4.1 ppm, identical to the absorption of the 3α-H of 3β,5β-THS; its bioactivity in the CAM assay was insignificant. Commercially obtained 3α,5β-THS compounds that were derived by reduction of the α,β-unsaturated 3-ketone of the A-ring all showed variable and inconsistent activity in the CAM assay.

Compounds derived by introduction of the 17- and 21-hydroxyl groups on 5β-pregnan-3α-ol-20-one had the M^+ ion 350 amu, and none of the ubiquitous 358 amu. This latter ion was observed in those samples derived by reduction of Substance S (Table 2).

High-performance liquid chromatography (HPLC)

HPLC analysis of several of the 3α,5β-THS compounds on an RPC18 column served only to show a single peak with retention time of 6.4 min of area ranging from 87–96% of the total material injected (Table 3). The indicated impurities did not permit isolation and identification.

Three β-cyclodextrins were compared for their relative ability to solubilize 3α,5β-THS and 3α,5α-THS in the CAM assay. Only the β-cyclodextrin tetradecasulfate appeared to function in a manner similar to heparin in this assay system.[1,2] (Table 4).

Chick chorioallantoic membrane assay (CAM)

CAM assay was carried out courtesy of Dr. J. Folkman. The CAM assay expresses the angiostatic activity (the inhibition of new growth of blood capillaries in the chick CAM assay) as a percent positive, (the number of positive units/the number of units × 100 = percent positive). The assay is described in detail in reference 7. In brief, a mixture of 50 μg of steroid and 50 μg of heparin (or β-cyclodextrin tetradecasulfate) in 10 μL of solvent (0.45% methylcellulose), air-dried atop a Teflon rod 3.25 mm in diameter. The disc-film was implanted on the CAM of the 6-day-old chick embryo; 48 h later the presence or absence of avascular zones at the disc-site were evaluated and tabulated as the percent-positive zones.

Table 4 3α,5α-THS and 3α,5β-THS results from the CAM assay using three different β-cyclodextrins: β-cyclodextrin, Molecusol, and β-cyclodextrin tetradecasulfate

	Percent activity	
	3α,5β-THS	3α,5α-THS
β-Cyclodextrin	0	0
Molecusol	0	20
β-cyclodextrin tetradecasulfate	0	40

Discussion

We showed that 3α,5β-THS prepared from Substance S by reducing the α,β-unsaturated ketone, and carefully purified to the correct m.p. purity, yielded an inactive compound in the CAM assay. The 3α,5α-THS that was the second compound resulting from the above synthesis, when carefully purified, yielded 40% activity in the CAM assay. All previous preparations of 3α,5β-THS were impure according to m.p., and upon crystallization yielded a compound with no angiostatic properties. Only one 3α,5β-THS compound obtained from another source showed both the presence of the recognizable impurity and the activity. The impurity was identified via ^1H NMR as the A/B cis epimer, 3β,5β-THS.

The epimeric pair, 3α,5α-THF and 3α,5β-THF, (source: Sigma), showed a similar pattern of activity in the CAM assay, namely that the allo (5α) epimer was more active than the 5β. Additionally, the epi-allo-THS (3β,17α,21-trihydroxy-5α-pregnane-20-one) showed absent activity in the CAM assay (Table 5). These latter three compounds were from Sigma and their structure verified by ^1H NMR (data not shown).

This work compares for the first time the activity in a biological system of the four epimeric pairs (about C-3 and C-5) of C-21 tetrahydrosteroids and showed the trans A/B-ring structure to bear greater activity than the cis A/B-ring structure (see Figure 3). This work also suggests that prior physical characterization (verification) of the purity of compounds from commercial sources might save enormous expenditures of valuable research time and NIH funds.

Conclusion

As shown in Table 1, 3α,5β-THS obtained from different sources and synthesized by two different routes (Figures 1 and 2), yielded different results in the CAM assay system. 3α,5β-THS derived by reduction of the α,β-unsaturated ketone of ring A of Substance S yielded variable results from the CAM assay. Synthesis of this molecule starting

Table 5 Results from CAM assay of 5α-/5β-tetrahydro cortisol, (3α,11β,17α,21-tetra-hydroxy-5α-pregnane-20-one (allo-THF), 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one (THF, Sigma, mixture), 3β,17α,21-trihydroxy-5α-pregnane-20-one (epi-allo-THS), and 3β-17α,21-trihydroxy-5β-pregnane-20-one (epi-THS, Steraloids)

Compound	Percent activity
3α,5α-THF	39% Positive
3α,5β-THF	12% Positive
3β,5α-THS	0%
3β,5β-THS	0%

Compounds were tested at the 50 μg level with 50 μg heparin.

from 3α-hydroxy-5β-pregnan-20-one yielded a compound devoid of angiostatic activity. An in-depth study of the physical constants revealed only that the compounds were uniformly impure compared to literature values. It was observed that allo-THS, the 5α-epimer, was the angiostatic steroid. This compound may have been one of the impurities in the 3α,5β-THS obtained by reduction of the α,β-unsaturated ketone of ring A of Substance S.

Acknowledgments

This work was presented as poster 70, Division of Medicinal Chemistry, 208th American Chemical Society National Meeting, August 21–25, 1994, and was supported in part by the Lake County Medical Center Development Funds, American Cancer Society RP258, NIH HL43035, Institutional Funds to GMI and stipends from Hollings Cancer Center, Charleston, SC, and private sources (HEH). The authors are grateful to Steraloids for supplying some of the steroids. The authors thank Dr. Folkman for the CAM assay results over the past several years.

References

1. Crum R, Szabo S, Folkman J (1985). A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* **230**:1375–1378.
2. Folkman J, Ingber, DE (1987). Angiostatic steroids. *Ann Surg* **206**:375–383.
3. Harnik M (1964). Studies in syntheses of steroid metabolites. Part IV. *Steroids* **3**:359–379.
4. Hosoda H, Takasaki W, Mura H, Tohkin M, Maruyama Y, Nambara T (1985). Chemical conversion of corticosteroids to 3α,5α-tetrahydro derivatives. Synthesis of allotetrahydro-11-deoxycortisol glucuronides. *Chem Pharm Bull (Tokyo)* **33**:4281–4287.
5. van Euw J, Neher R, Reichstein T (1962). 3α,17α,21-Triol-5α-Pregnan-20-on und Subst.TR 1018. *Helv Chim Acta* **45**:224–232.
6. Templeton JF, Kumar VPS, Gupta, RK, Friesen AM (1986). Stereoselective reduction of C-2 substituted steroid C-3 ketones with lithium Tris-(R,S-1,2-dimethylpropyl)-borohydride and sodium borohydride. *Steroids* **48**:339–346.
7. Hadd HE, Folkman J (1995). Angiostatic properties of steroid glucuronides. In: Nixon D (ed), *Chemo Prevention of Cancer*, CRC press, Boca Raton, FL, pp. 99–119.