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(54) Title: SECO-STEROL COMPOUNDS EFFECTIVE IN INDUCING THE DIFFERENTIATION OF MALIGNANT CELLS

(57) Abstract

Seco-sterol compounds which are effective in inducing the differentiation of malignant cells and are therefore useful for the treatment of malignancies such as leukemia.

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SECO-STEROL COMPOUNDS EFFECTIVE IN INDUCING THE DIFFERENTIATION OF MALIGNANT CELLS

This invention relates to a group of seco-sterol compounds that are effective in inducing the differentiation of malignant cells, such as the differentiation of leukemic cells to normal macrophages.

A useful therapeutic method for the treatment of malignancies is the administration of compounds that stimulate the differentiation of malignant cells to normal cells, thereby inhibiting and/or reversing the malignant transformation. Thus, it has been shown by Suda et al. (U.S. Patent 4,391,802) that 1α-hydroxyvitamin D compounds (e.g. specifically $1\alpha,25$-dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_3) possess, for example, potent antileukemic activity by virtue of inducing the differentiation of malignant cells (specifically leukemia cells) to non-malignant macrophages (monocytes). Hence, these compounds are useful for the treatment of certain malignancies, specifically for the treatment of leukemia. When used for such treatment, however, these known 1α - hydroxyvitamin D compounds have the disadvantage that they are also very potent calcemic agents, i.e. they cause elevated blood calcium levels by stimulating intestinal calcium

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absorption and bone calcium resorption. This calcemic
activity represents, indeed, the well-known, classical
function of these compounds. Furthermore, the cell
differentiation activity (and, hence, antileukemic activity)
of these compounds correlates with their calcemic activity.
For example, 1,25-dihydroxyvitamin D_3, the most potent
compound in inducing the differentiation of malignant cells to
macrophages, is also the most potent vitamin D metabolite in
stimulating calcium transport or raising serum calcium levels.
For practical use as cell-differentiating agents, this potent
calcemic activity is, of course, an undesired side effect,
since the doses required for efficacy in differentiating
malignant cells can lead to excessively high and
non-physiological serum calcium levels in the treated
subjects.

It has now been found that effective cell differentiation
(i.e. reversal of malignant transformation) can be achieved with a
novel class of seco-sterols, that do not have the undesired
side-effects (potent calcemic action) mentioned above. This
selectivity and specificity of action makes the secosterols of
this invention useful and preferred agents for achieving
malignant cell differentiation.

Purely structurally, this class of secosterols has
similarity with some of the known vitamin D compounds. Unlike
the known vitamin D compounds, however, the secosterols of the
present invention do not express the classic vitamin D
activities _in vivo_, i.e. stimulation of intestinal calcium
transport, or the mobilization of bone calcium, and hence they
cannot be classified as vitamin D derivatives from the
functional point of view. In light of the prior art, it was
all the more surprising and unexpected to find that
these secosterols are remarkably effective in inducing the
differentiation of leukemia cells to normal (non-malignant)
macrophages, since, as mentioned above, potent cell

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differentiation activity among the known vitamin D-related
compounds was always closely correlated with potent calcemic
activity. Thus, the secosterols of the present invention
overcome the shortcomings of the known vitamin D-related
antileukemic agents mentioned above, and can be considered
preferred agents for the control and treatment of malignant
diseases such as leukemia. This finding provides an effective
method for the treatment of malignancies, since these
secosterols can be administered to subjects in doses
sufficient to cause differentiation of malignant cells to
normal cells, without producing simultaneously
unphysiologically high and deleterious blood calcium levels.

The secosterols of the present invention exhibiting
this unique and heretofore unrecognized activity pattern
are characterized by the general structures I and II shown below:
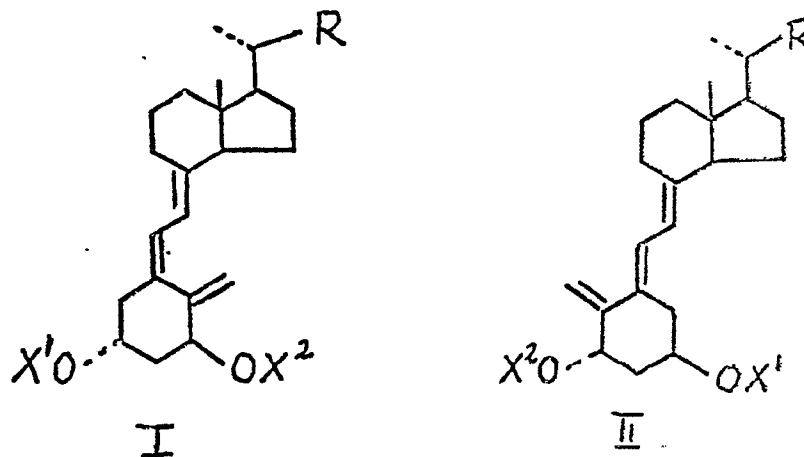
where R is hydrogen, methyl, ethyl or propyl and where
each of X^1 and X^2 represent, independently, hydrogen or an
acyl group.

As used herein, an acyl group
is an alkanoyl group of 1 to 6 carbons in all its isomeric
forms, or an aroyl group, such as benzoyl, or halo-, nitro- or
alkyl-substituted benzoyl groups, or a dicarboxylic acyl group
such as oxalyl, malonyl, succinoyl, glutaroyl, or adipoyl. An
alkyl group is a hydrocarbon radical of 1 to 6 carbons in all
isomeric forms.

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Biological Properties of Secosterol Compounds

Biological activity of compounds of structure I in the differentiation of human leukemia cells.

Chemicals and reagents required for the assays below were obtained from commercial sources as follows: 4β-phorbol 12-myristate-13-acetate (PMA), Nitro blue tetrazolium (NBT) and reagents for α-napthyl acetate esterase assay (α-naphthyl acetate, TRIZMAL^{TM} 7.6 buffer concentrate, Mayer's hematoxylin solution, Fast Blue RR salt, citrate concentrate, ethylene glycol monomethyl ether) were obtained from Sigma Chemical Company, St. Louis, MO; sheep erythrocytes (50% cells in Alsevers solution), fetal calf serum and RPMl-1640 medium were from Gibco Laboratories, Grand Island, NY, and rabbit antiserum to sheep erythrocytes (rabbit hemolysin) was from Flow Laboratories, McLean, VA.

Cell culture: 10 ml aliquots of human leukemia cells (HL-60 cells) (~ 2x10^5 cells/ml in RPMl-1640 media with 10% heat-inactivated fetal calf serum) were plated out on Petri dishes and cultured at 37°C in a 5% CO$_2$ atmosphere. After 16 hours, duplicate dishes were dosed with various amounts of test compounds (as listed in Table 1, below) dissolved in 10-20 μl of ethanol. Control cultures received ethanol alone. The cells were harvested 4 days after dosing and an aliquot of each was counted under a hemocytometer. To assess the degree of differentiation induced by the test compounds, the harvested cultures were resuspended in phosphate-buffered saline (PBS) (~10^6 cells/ml) and each of the following three assays were then performed on aliquots of the cell cultures.

(a) Nitro blue tetrazolium (NBT) reduction assay for differentiation.

This assay is based on the fact that monocytes can be induced by phorbol esters to produce superoxide. The superoxide can be detected by its capability to reduce soluble (nitro-blue tetrazolium (NBT) to a black:blue precipitate, formazan. The NBT-reduction activity exhibited by the HL-60

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cells thus provides a measure of their differentiation to
non-malignant monocytes. The assay was performed according to
the procedure given by Yen et al., J. Cellular Physiol. 118,
277 (1984). The NBT reagent was prepared by dissolving 50 mg
NBT and 5 μg of 4β-phorbol 12 myristate-13-acetate in 50 ml of
phosphate-buffered saline. This reagent (200 μl) was added to
200 μl of the harvested cells (ca. 10^6 cells/ml
phosphate-buffered saline). The mixture was incubated in a
water bath at 37°C for 30 min. The cells were then counted
and the percentage of cells that reduced NBT to formazan blue
was recorded. Results are given in Table 1, below.

(b) Assay for rosette formation.

    This assay is based on rosette formation between
differentiated monocytes and sheep erythrocytes coated with
rabbit antibody and mouse complement. The assay was done
according to the procedure of Lotem and Sachs, Internat. J.
Cancer 15, 731 (1975). Sheep erythrocytes were washed three
times with phosphate-buffered saline and resuspended to a 0.5%
(v/v) suspension. Equal volumes of erythrocytes and a 1:1500
dilution of rabbit antiserum to sheep erythrocytes were mixed
and incubated at 37°C for 30 min. The antibody-coated
erythrocytes (EA) were washed three times with
phosphate-buffered saline, pH 7.0 and resuspended at 0.5%
(v/v). Fresh mouse blood was spun down and the serum was
collected. Equal volumes of EA and a 1:10 dilution of the
mouse serum were mixed and incubated at 37°C for 30 min, then
washed three times with phosphate-buffered saline and
resuspended in RPMI medium, 1% (v/v) to give the erythrocytes
coated with antibody and complement (EAC), 100 μl of EAC was
mixed with an aliquot of HL-60 cells (about 10^6 cells in 100
μl RPMI), incubated for 30 min at 37°C, and then centrifuged
for 3 min at 500xg. The pellet was dispersed and the cells
with attached EAC (i.e. the number of rosettes) were
determined, and expressed as a percent of the total cells
present. The "% rosette formation", indicative of the

differentiation of HL-60 cells to monocytes, is listed in
Table 1, below.

(c)   Assay for α-naphthyl acetate esterase activity.

α-Naphthyl acetate esterase is an enzyme charcteristic of
monocytes. The presence of the enzyme thus indicates
differentiation of HL-60 cells to monocytes. The assay is
based on the enzymatic hydrolysis of the α-naphthyl acetate to
liberate free naphthol which couples with a diazonium salt to
form highly colored deposits at the sites of enzyme activity.
The assay was carried out as described in Technical Bulletin
No. 90 (Sigma Chemical Co., St. Louis, MO 63178). Cells were
fixed on slides in a citrate-acetone-methanol fixative for 30
sec at room temperature. The slides were then washed with
deionized water and air-dried at least 20 min. The slides
were then stained in a staining solution prepared by
dissolving 25 mg Fast Blue RR salt in 50 ml of a 1:10 dilution
of TRIZMAL^{TM} buffer concentrate pH 7.6, followed by the
addition of 20 mg of α-naphthyl acetate (in 2 ml ethylene
glycol monomethyl ether). The slides were incubated at 37°C
for 30 min (protected from light). They were then washed,
counter-stained for 5-10 min in Mayer's hematoxylin solution,
washed and then air-dried. The percentage of cells with black
granulation, indicative of α-naphthyl esterase activity, was
determined. Results are listed in Table 1, below.

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Table 1

Percent differentiation of HL-60 cells induced by seco-sterols
or by known 1α-hydroxyvitamin D compounds administered at
various concentrations as measured by NBT-reduction, rosette
formation and esterase activity assays

Compound Administered	Concentration (M)	NBT Reduction (%)	Rosette Formation (%)	Esterase Activity (%)
EtOH	Control	4.5	9	3.5
Secosterol I ($R=CH_3$, $X^1=X^2=H$)	1×10^{-7}	9	9	2
	1×10^{-6}	14	23	11
	5×10^{-6}	59	68	69
Secosterol I ($R=CH_2CH_3$ $X^1=X^2=H$)	1×10^{-7}	15	23	30
	1×10^{-6}	28	30	77
	1×10^{-5}	69	70	91
1α-OH-D_3	1×10^{-7}	10	44	12
	1×10^{-6}	39	61	90
	1×10^{-5}	85	79	100
$1\alpha,25$-(OH)$_2D_3$	1×10^{-8}	39	44	65
	1×10^{-7}	83	76	90

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The above results illustrate the efficacy of the seco-sterols
of general structure I as agents for the differentiation of
human leukemia cells to macrophages (monocytes). The
compounds show highly significant activity in all three of the
differentiation assays used; 50% differentiation is achieved
at concentrations of about 10^{-6} M. For comparative purposes,
the table above also includes the cell differentiation
activity exhibited by 1α-hydroxyvitamin D_3 (1α-OH-D_3) and
$1\alpha,25$-dihydroxyvitamin D_3 ($1,25$-$(OH)_2 D_3$), two known vitamin D
derivatives with potent antileukemic action. The tabulated
data show that the level of activity of the seco sterols (I)
is lower than that shown by $1,25$-$(OH)_2 D_3$ (the most potent
vitamin D-derived agent for differentiation of leukemia
cells), but is approximately equivalent to that shown by
1α-hydroxyvitamin D_3, a compound known to be effective in the
treatment of human leukemoid diseases (Suda et al., U.S.
Patent 4,391,802).

Assay of secosterols of structure I for bone calcium mobiliza-
tion and calcium transport.

Male weanling rats, purchased from the Holtzman Co.,
Madison, WI, were fed the low calcium, vitamin D-deficient
diet described by Suda et al. [J. Nutr. 100, 1049 (1970)] ad
libitum for 3 weeks. The rats were then divided into 4 groups
of 6 animals each. The first group (control group) received
0.05 ml of 95% EtOH by intrajugular injection. The second and
third groups were dosed by the same route with 625 picomoles
and 6250 picomoles, respectively, of secosterol I (R=CH_3,
X^1=X^2=H) dissolved in 0.05 ml of EtOH, and the fourth group
received an intrajugular injection of 625 picomole of
$1\alpha,25$-dihydroxyvitamin D_3 (in 0.05 ml of EtOH). Seven hours
after dosing, the rats were killed by decapitation and their
blood was collected and centrifuged to obtain serum. Serum
calcium concentration was determined with an atomic absorption
spectrometer according to the conventional protocol. Results
are listed in Table 2 below.

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The small intestines of these same rats were removed,
rinsed and everted for measurement of calcium transport
activity according to the technique of Martin and DeLuca [Am.
J. Physiol. 216, 1351 (1969)]. The measured intestinal
calcium transport activity data, expressed as the ratio of
serosal/mucosal calcium concentration, are also listed in
Table 2.

Table 2

Compound Administered	Amount (pmole)	Serum Calcium Concentration (mg/100 ml) mean ± S.D.	Intestinal Ca-transport [Ca-serosal]/ [Ca-mucosal] mean ± S.D.
EtOH (control)	—	2.6 ± 0.1	3.6 ± 0.1
Secosterol I (R=CH$_3$, X^1=X^2=H)	625	2.9 ± 0.1	3.4 ± 0.1
Secosterol I (R=CH$_3$, X^1=X^2=H)	6250	3.0 ± 0.1	3.4 ± 0.1
1,25-(OH)$_2$D$_3$	625	3.8 ± 0.2	6.7 ± 0.8

The above results show that secosterol I (R=CH$_3$, X^1=X^2=H)
expresses no significant calcemic activity even at high doses.
The compound does not elevate serum calcium levels and thus is
devoid of significant bone calcium mobilization activity.
Further, the compound does not stimulate calcium transport in
the intestine at a dose level of 6250 picomole per animal.
Under the same conditions, the known active vitamin D
metabolite, 1,25-(OH)$_2$D$_3$, is, as expected, fully active at 10
times lower dose levels.

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Assay of the seco-sterol homologue of structure I (where R=H, and $X^1=X^2=H$) under conditions analogous to those described above (except that response was measured 12 hr after injection of test compound) gave very similar results, as shown in Table 3, below.

Table 3

Compound Administered	Amount (pmole)	Serum Calcium Concentration (mg/100 ml) mean ± S.D.	Intestinal Ca-transport [Ca-serosal]/ [Ca-mucosal] mean ± S.D.
EtOH (control)	—	4.4 ± 0.3	1.7 ± 0.2
Secosterol I (R=H, $X^1=X^2=H$)	6250	4.4 ± 0.1	1.8 ± 0.1

Again the data in Table 3 demonstrate that the seco-sterol of structure I(R=H; $X^1=X^2=H$) elicits no response _in vivo_ with respect to intestinal calcium transport or calcium mobilization from bone, even when administered at high doses.

It can be concluded, therefore, that these seco steroids of general structure I (where R is hydrogen, methyl, ethyl, propyl) do not carry out the classical vitamin D functions _in vivo_, since they elicit no significant _in vivo_ biological response with respect to bone mineral mobilization, and intestinal calcium transport activation.

The above data establish that the seco-sterols of this invention possess an unusual and unexpected spectrum of activities. They exhibit highly significant cell differentiation activity, like some of the known vitamin D-related compounds, but do not express the calcemic activity typical of vitamin D-derviatives. Thus, in being devoid of

the undesired calcemic action of the known antileukemic
vitamin D-compounds, the seco-steroids of this invention
provide a novel and preferred method for the treatment of
malignancies, such as leukemia.

The compounds may be formulated as pills, capsules or
tablets, or in liquid form as solutions, emulsions,
dispersions or suspensions in pharmaceutically innocuous and
acceptable solvent or oils, and such prepratlons may contain
in addition other pharmaceutically innocuous or beneficial
components, such as antioxidants, emulsifiers, coloring
agents, binders or coating materials.

The compounds may be administered as oral doses, or by
injection or infusion of suitable sterile solutions. The
compounds are advantageously administered in amounts
sufficient to effect the differentiation of malignant cells to
normal macrophages. Dosages from 2 μg to 1000 μg per day are
suitable, it being understood that the amounts given are to be
adjusted in accordance with the severity of the disease, and
the condition and response of the subject as is well
understood in the art.

Preparation of seco-sterol compounds.

Secosterol I (where R=H) can be prepared according to the
method of Lam et al. [Steroids 26, 422 (1975)]. The secosterols
of structuresI and II where R is methyl, ethyl or
propyl, are new compounds which can be prepared according to
the general process illustrated in Process Scheme I. Suitable starting
materials are the i-ether steroids of general structure 1,
where, depending on the final product desired, R may be
methyl, ethyl or propyl. Conversion of a compounds of
structure 1 to the 5-ene steroid of structure 2 is
accomplished by solvolysis in glacial acetic acid, according
to known procedures. The 5-ene steroid is then dehydrogenated
to the 5,7-diene steroid (3) using the sequence of allylic
bromination at C-7 followed by dehydrobromination, and the
acetate group of compound 3 is saponified to obtain the

corresponding alcohol of structure 4. Irradiation of a
solution of alcohol 4 with ultraviolet light open ring B of
the steroid to provide the initial secosterol derivative (the
5(10),6,8-triene), which can be purified and isolated if
desired by standard chromatographic methods, but can also, and
generally most advantageously, be directly thermally
isomerized to the 5,7,10(19)-triene compound of structure 5.

The further conversion of this intermediate to the
desired 1α-hydroxylated analog can be accomplished according
to the general procedures given in U.S. Patents 4,195,027 and
4,260,549. Intermediate 5 (Process Scheme I) is first
tosylated to obtain the 3β-tosylate (6), and this tosylate is
then solvolyzed in buffered methanol to produce the
6-methoxy-3,5-cyclo-derivative represented by structure 7.
Solvolysis of the tosylate in other alcoholic solvents (e.g.
ethanol, propanol, butanol produces the analogous —
6-0-alkyl-3,5-cyclo intermediates, where the alkyl group
derives from the alkyl portion (e.g. ethyl, propyl, butyl,
etc.) of the alcohol used. Any of these 6-0-alkyl-homologs of
compound 7 can be used for the subsequent reactions of this
process. Oxidation of intermediate 7 with selenium dioxide in
the presence of a hydroperoxide introduces the desired
1α-hydroxy function and provides compound 8. This
intermediate is then solvolyzed in a medium containing a
low-molecular weight organic acid to yield the 3-acylate,
having structure 9 (X^1=acyl, X^2=H) and the corresponding
5,6-trans-isomer of structure 10 (X^1=acyl, X^2=H) (where the
acylate groups in each case derive from the organic acid used
in the solvolysis reaction). These 5,6-cis and 5,6-trans
3β-acylates (compounds 9 and 10) are advantageously separated
at this stage so as to obtain each compound in pure form.
They can then be subjected to saponification (e.g. base in
methanol) to obtain the corresponding free hydroxy compounds 9
(X^1=X^2=H) and 10 (X^1=X^2=H). Alternatively, the monoacylates
of 9 and 10, or the free hydroxy compounds can be subjected to

acylation under conventional and known conditions (e.g. acid anhydride or acyl halide in a nitrogenous base solvent) to produce any desired C-1-mono acyl, C-3-mono acyl or C-1,3-di-acyl derivative, e.g. the compounds of structure 9 or 10, where X^1=acyl and X^2=H, or where X^1=H and X^2=acyl, or where X^1=acyl and X^2=acyl, where the acyl groups may be the same or different.

It is evident from the above description and the Process Scheme that the nature of the side-chain in the starting material determines the side-chain structure in the final products. Thus, using as starting material the sterol of structure 1, where R is methyl, provides the products of structures 9a and 10a, where R is methyl. Compound 1, where R is ethyl, as starting material, gives products 9a and 10a, where R is ethyl, and upon processing of steroid 1, where R is propyl, through the above described process, there is obtained the product 9c and 10c, where R represents propyl.

The 5,6-_trans_-compounds of structure 10 in Process Scheme I have utility as biologically active analogs of the corresponding 5,6-_cis_-compounds of structure 9, or they may be converted to the 5,6-_cis_ products of structure 9, by the _trans_ to _cis_ isomerization processes well-known in the art.

Preparation of starting materials. The starting materials of structure 1 (where R=CH_3, CH_2CH_3 or $CH_2CH_3CH_3$) can be prepared by conventional methods from stigmasterol. Thus, by conversion of stigmasterol to its i-ether derivative, followed by ozonolytic cleavage of the side-chain double bond, and subsequent hydride reduction, there is obtained the known 22-alcohol, having the structure:

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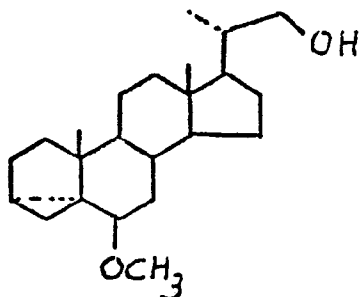
Tosylation of this alcohol, followed by hydride reduction of
this 22-tosylate provides directly the compound of structure
$\underset{\sim}{1}$, where R=CH$_3$.

Treatment of the above 22-tosyl intermediate with sodium
cyanide gives the corresponding 23-nitrile derivative. By
two-step hydride reduction of this nitrile, there is obtained
the 23-alcohol which, after tosylation, and another hydride
reduction of this 23-tosyloxy intermediate, provides the
starting compound of structure $\underset{\sim}{1}$, where R=ethyl.

Similarly, the above 23-tosyloxy intermediate, by
treatment with sodium cyanide, gives the corresponding
24-nitrile derivative, which after hydride reduction (to
obtain the 24-alcohol) and another tosylation and hydride
reduction sequence, provides the compound of structure $\underset{\sim}{1}$,
where R represent n-propyl.

The preparation of the novel compounds of this invention
is more specifically described by the following Examples. In
these Examples, the identification of products by Arabic
numerals (e.g. compounds $\underset{\sim}{1}$, $\underset{\sim}{2}$, $\underset{\sim}{3}$, etc.) refers to the
structures so numbered in the Process Scheme.

Example 1

Preparation of Compounds $\underset{\sim}{9a}$ and $\underset{\sim}{10a}$ (X^1=X^2=H)

3β-acetoxy-23,24-dinorchol-5-ene ($\underset{\sim}{2}$)

A solution of compound $\underset{\sim}{1}$ (R=CH$_3$) (1.26 g, 3.8 mmol) in
glacial acetic acid (35 mL) was heated at 70°C for 4.5 h. The
reaction mixture was cooled and poured into ice water,
neutralized with 10% aqueous sodium hydroxide and extracted
with chloroform (3 x 100 mL); the chloroform extracts were
washed with water (2 x 50 mL), saturated sodium chloride
solution (2 x 50 mL), dried over anhydrous magnesium sulfate,
filtered and concentrated to dryness to give 0.86 g (63%
yield) of compound $\underset{\sim}{2}$ (R=CH$_3$).

Mass spectrum: $\underline{m/e}$ (relative intensity), 358 (M$^+$, 2), 298
(100), 283 (15), 255 (6), 190 (9), 177 (23); ^{1}H-NMR (CDCl$_3$) δ
0.66 (s, 18-H), 0.81 (d, J=7 Hz, 22-H), 0.95 (d, J=7.0 Hz,

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21-H), 1.02 (s, 19-H), 2.06 (s, 3-OCOCH$_3$), 4.59 (m, 3-H), 5.38
(m, 6-H).

3β-acetoxy-23,24-dinorchola-5,7-diene (3) (R=CH$_3$)

A stirred solution of 2 (R=CH$_3$) (0.6 g, 8 mmol) in dry
hexane (50 mL) containing finely divided sodium bicarbonate
(0.7 g, 8 mmol) was heated to 80°C at reflux under nitrogen
before 1,3-dibromo-5,5-dimethylhydantoin (0.24 g, 0.85 mmol)
was added. The reaction was allowed to proceed for 20 min.
The mixture was cooled, then filtered and concentrated under
reduced pressure.

The residue was immediately dissolved in 15 mL dry xylene
and added dropwise to a mixture of xylene (25 mL) and
s-collidine (0.4 g, 3.3 mmol). The mixture was flushed with
nitrogen and was then cooled, diluted with benzene (50 mL),
washed with 3% aqueous hydrochloric acid (3 x 20 mL),
saturated sodium bicarbonate solution (1 x 25 mL), water (1 x
25 mL), saturated aqueous sodium chloride (2 x 25 mL), dried
over anhydrous magnesium sulfate and filtered. The solvent
was evaporated under reduced pressure.

The oily residue was dissolved in dry dioxane (40 mL) and
p-toluenesulfonic acid (0.076 g, 0.4 mmol) was added. The
mixture was flushed with nitrogen and refluxed at 70°C for 0.5
h. After cooling, it was diluted with water (100 mL) and
extracted with ethyl acetate (1 x 70 mL, 2 x 60 mL), the
organic extract was washed with saturated sodium bicarbonate
solution (1 x 30 mL), saturated sodium chloride (2 x 30 mL),
dried over anhydrous magnesium sulfate, filtered and the
solvent was evaporated under reduced pressure to give an oil
containing the desired the 5,7-diene (3) (Rf 0.29 in 10% ethyl
acetate-hexane) and the 2,4,6-triene (Rf 0.5 in 10% ethyl
acetate:hexane). Preparative TLC using 10% ethyl
acetate-hexane gave 0.26 g of 3 (R=CH$_3$) in ca. 43% yield.
UV (C$_2$H$_5$OH) λ_{max} 282, 293, 272, 262 nm; mass spectrum: m/e
(relative intensity), 356 (M$^+$, 5), 296 (100), 281 (59), 253
(56), 211 (29), 158 (75), 143 (74); ^{1}H-NMR (CDCl$_3$): 0.62 (s,

18-H), 0.88 (d, J=7.0 Hz, 22-H), 0.95 (s, 19-H), 0.96 (d,
J=7.0 Hz, 21-H), 2.04 (s, 3-OCOCH$_3$), 4.7 (m, 3-H), 5.4 (m,
7-H), 5.58 (m, 6-H).

3β-Hydroxy-23,24-dinorchola-5,7-diene (4) (R=CH$_3$)

A 10% sodium hydroxide in methanol solution was added
dropwise to a stirred solution of 3 (R=CH$_3$) 259 mg; Rf 0.56 on
25% ethyl acetate-hexane) in ether (15 mL) over a 5 min period
under nitrogen. The reaction proceeded at 23°C and was
monitored on TLC. It was completed within 35 min. The
mixture was diluted with ether (100 mL) and water (30 mL) was
added. The layers were separated and the aqueous layer was
extracted with ether (2 x 50 mL). The combined ether
fractions were washed with water (2 x 30 mL), saturated sodium
chloride solution (2 x 30 mL), dried over anhydrous magnesium
sulfate, filtered and evaporated to dryness under reduced
pressure to give 200 mg of 4 (77% yield) after preparative TLC
using 25% ethyl acetate/hexane.

UV (EtOH): λ_{max} 282, 293, 272, 262 nm; mass spectrum: m/e
(relative intensity), 314 (M$^+$, 85), 296 (12), 281 (100), 261
(6), 255 (60), 211 (22), 171 (30), 143 (78); ^{1}H-NMR (CDCl$_3$): δ
0.62 (s, 18-H), 0.87 (d, J=7.0 Hz, 22-H), 0.95 (s, 19-H), 0.97
(d, J=7.0 Hz, 21-H), 3.63 (sh, 3-H), 5.38 (m, 7-H), 5.58 (m,
6-H).

Seco-sterol 5 (R=CH$_3$)

The 5,7-diene (4) (36 mg) dissolved in a mixture (1:4) of
benzene-ether (100 mL) was placed in a jacket around a
double-walled, water-cooled quartz immersion well equipped
with a Hanovia 608A quartz-medium pressure mercury vapor
ultraviolet lamp with a vycor filter. The mixture was
irradiated for 4.5 min. The system was purged continuously
with nitrogen throughout irradiation. The solvent was then
removed under reduced pressure and the residue redissolved in
dryethanol. It was flushed with nitrogen and then heated to
70°C at reflux under nitrogen for 3 h. It was then cooled and
concentrated under reduced pressure. Purification by TLC

using 20% ethyl acetate-hexane afforded 5 (R=CH$_3$) in 31.7%
yield (11.4 mg).

UV (EtOH) λ_{max} 264 nm; mass spectrum: m/e (relative intensity)
314 (M$^+$, 14), 296 (21), 281 (4), 271 (2), 253 (3), 136 (82),
118 (100); ^{1}H-NMR (CDCl$_3$): δ 0.54 (s, 18-H), 0.85 (d, J=7.0
Hz, 22-H), 0.94 (d, J=7.0 Hz, 21-H), 3.93 (m, 3-H), 4.82 (m
(sharp), 19(Z)-H), 5.04 (m (sharp), 19(E)-H), 6.02 (d, J=12.0
Hz, 7-H), 6.22 (d, J=12.0 Hz, 6-H).

Tosylate 6 (R=CH$_3$)

A solution of 5 (15 mg, 47 mmol, Rf 0.28 in 30% ethyl
acetate-hexane) in dry pyridine (0.5 mL) was treated with
p-toluenesulfonyl chloride (22 mg, 117 mmol) at 5°C under
nitrogen for 24 h. The reaction was quenched with ice water
and the mixture extracted with ether (3 x 30 mL). The
combined extracts were washed with 3% aqueous hydrochloric
acid (2 x 30 mL), saturated aqueous sodium bicarbonate (1 x 50
mL), saturated sodium chloride solution (1 x 50 mL), dried
over anhydrous magnesium sulfate and concentrated under
reduced pressure to give 20 mg of tosylate 6 (R=CH$_3$) (Rf 0.6
in 30% ethyl acetate-hexane).

3,5-Cyclo-derivative 7 (R=CH$_3$)

The crude tosylate 6 (R=CH$_3$) (20 mg, 0.41 mmol, Rf 0.5 in
25% ethyl acetate-hexane) was added to a stirred solution of
finely divided sodium bicarbonate (200 mg, 2.4 mmol) in
anhydrous methanol (20 mL). The mixture was heated to 55°C at
reflux under nitrogen for 8 h, cooled, diluted with ether (100
mL) and washed with water (3 x 30 mL), saturated aqueous
sodium chloride solution (1 x 30 mL), dried over anhydrous
magnesium sulfate, filtered and the solvent was evaporated
under reduced pressure. The crude mixture was chromatographed
on preparative TLC using 10% ethyl acetate-hexane to obtain 7
(R=CH$_3$) (7 mg, Rf 0.66 in 10% ethyl acetate-hexane) in 50%
yield.

Mass spectrum: m/e (relative intensity), 328 (M$^+$, 14), 296
(15), 281 (7), 253 (37), 159 (28), 135 (34), 145 (30); ^{1}H-NMR

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(CDCl$_3$): δ 0.55 (s, 18-H), 0.85 (d, J=7.0 Hz, 22-H), 0.93 (d,
J=7.0 Hz, 21-H), 3.26 (s, 6(R)-OCH$_3$), 4.18 (d, J=10 Hz, 6-H),
4.89 (m (sharp), 19(Z)-H), 5.0 (d, J=10 Hz, 7-H), 5.06 (m
sharp)

<u>1α-Hydroxy-compound 8</u> (R=CH$_3$)

t-Butyl hydroperoxide (7 μl, 0.05 mmol) was added to a
suspension of selenium dioxide (SeO$_2$; 1.1 mg, 10 μmole) in 1%
dry pyridine-methylene chloride (5 mL) under nitrogen. The
mixture was stirred at 23°C for 0.5 h, then diluted with
another 10 mL of 1% pyridine-methylene chloride solution. The
mixture was cooled on an ice bath and intermediate 7 (R=CH$_3$)
(7 mg, 21 μmole; Rf 0.62 in 25% ethyl acetate-hexane) in dry
methylene chloride was introduced. The reaction was monitored
on TLC. It proceeded at 23°C for 16 min before 10% sodium
hydroxide solution (20 mL) was added to quench the reaction.
The mixture was diluted with ether (100 mL), phases were
separated and the ether phase was washed with 10% aqueous
sodium hydroxide (2 x 25 mL), water (2 x 20 mL), saturated
aqueous sodium chloride (2 x 20 mL) and dried over anhydrous
magnesium sulfate. It was filtered and the solvent was
evaporated under reduced pressure. Preparative TLC of the
residue using 25% ethyl acetate-hexane gave the
1α-hydroxy-derivative 8 (R=CH$_3$) (3 mg, Rf 0.15 in 25% ethyl
acetate-hexane) in 41% yield.

Mass spectrum: m/e (relative intensity): 344 (M$^+$, 34), 312
(77), 271 (42), 177 (567), 135 (100), ^{1}H-NMR (CDCl$_3$): δ 0.55
(s, 18-H), 0.85 (d, J=7.0 Hz, 22-H), 0.94 (d, J=7.0 Hz, 21-H),
3.26 (s, 6(R)-OCH$_3$), 4.17 (m(sharp), 6-H), 4.22 (m (sharp),
1-H), 4.94 (d, J=9 Hz, 7-H), 5.16 (d, J=2.2 Hz, 19(Z)-H), 5.24
(d, J=2.2 Hz, 19(E)-H).

<u>5,6-cis and trans secosterol 3β-acetates</u> 9a and 10a
(X^1=Ac, X^2=H)

A solution of 8 (R=CH$_3$) (3 mg) in glacial acetic acid
(0.5 mL) was heated to 55°C under nitrogen for 20 min, cooled
and poured over ice cold sodium bicarbonate solution (15 mL)

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and extracted with ether (3 x 30 mL). The combined ether
extracts were washed with saturated aqueous sodium bicarbonate
(1 x 20 mL), water (2 x 20 mL), saturated sodium chloride (1 x
20 mL) and dried over anhydrous magnesium sulfate, filtered
and concentrated under reduced pressure. The residue was
chromatographed in 35% ethyl acetate-hexane (multiple
elutions, 4 times) to yield 0.8 mg of the 5,6-cis-3β-acetate
(9a) and .45 mg of the 5,6-trans-3β-acetate (10b),
respectively.

Acetate 9a (X^1=Ac, X^2=H): UV (EtOH): λ_{max} 264 nm, λ_{min}
228 nm.; mass spectrum: m/e (relative intensity) 372 (M^+, 7),
312 (40), 269 (10), 189 (15), 134 (100); ^{1}H-NMR (CDCl$_3$): δ
0.54 (s, 18-H), 0.85 (d, J=7.0 Hz, 22-H), 0.92 (d, J=7.0 Hz,
21-H), 2.02 (s, 3-OCOCH$_3$), 4.4 (broad, 1-H), 5.01 (m(sharp),
19(Z)-H), 5.21 (m, 3-H), 5.33 (m (sharp), 19(E)-H), 6.0 (d,
J=12.0 Hz, 7-H), 6.34 (d, J=12.0 Hz, 6-H).

Acetate 10a (X^1=Ac, X^2=H): UV (EtOH): λ_{max} 273 nm, λ_{min}
228 nm; mass spectrum: m/e (relative intensity) 372 (M^+, 3),
328 (4), 312 (14), 269 (6), 177 (37), 149 (58), 135 (100);
^{1}H-NMR (CDCl$_3$): δ 0.54 (s, 18-H), 0.85 (d, J=7.0 Hz, 22-H),
0.92 (d, J=7.0 Hz, 21-H), 2.02 (s, 3-OCOCH$_3$), 4.4 (broad,
1-H), 4.99 (m(sharp), 19(Z)-H), 5.13 (m (sharp), 19(E)-H), 5.8
(d, J=12.0 Hz, 7-H), 6.58 (d, J=12.0 Hz, 6-H).

1α-Hydroxy-secosterol 9a (X^1=X^2=H).

An ether solution (10 ml) of the 3β-acetate 9a as
obtained in the preceding experiment was hydrolyzed using 10%
sodium hydroxide in methanol at 23°C under nitrogen for 0.5 h.
The mixture was diluted with water (10 mL) and extracted with
ether (3 x 50 mL). The combined ether extracts were washed
with water (2 x 10 mL), saturated sodium chloride solution (2
x 10 mL), dried over anhydrous magnesium sulfate, filtered and
evaporated to dryness under reduced pressure.

The residue was purified by HPLC on a Zorbax-Sil
analytical column (4.6 mm x 25 cm) using 5% isopropanol-hexane
at 265 nm to give 9a (X^1=X^2=H). High resolution mass

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spectral analysis: calc. for $C_{22}H_{34}O_2$, 330.2559; found
330.2541; UV (EtOH): λ_{max} 264 nm λ_{min} 227 nm; mass spectrum,:
m/e (relative intensity) 330 (M^+, 55), 312 (71), 287 (7), 269
(7), 251 (5), 189 (21), 152 (73), 134 (100); ^{1}H-NMR (CDCl$_3$): δ
0.54 (s, 18-H), 0.85 (d, J=7.0 Hz, 22-H), 0.93 (d, J=7.0 Hz,
21-H), 4.23 (m, 3-H), 4.42 (m, 1-H), 5.0 (s, 19(Z)-H), 5.34
(s, 19(E)-H), 6.02 (d, J=12.0 Hz, 7-H), 6.39 (d, J=12.0 Hz,
6-H).

5,6-trans-analog 10a (X^1=X^2=H)

The 3β-acetate (10a) was hydrolyzed in a similar manner
and purified on HPLC using 5% isopropanol-hexane at 273 nm to
give 10a (X^1=X^2=H). High resolution mass spectral analysis:
calc. for $C_{22}H_{34}O_2$, 330.2559; found, 330.2532; UV (EtOH): λ_{max}
273 nm, λ_{min} 227 nm; mass spectrum: m/e (relative intensity),
330 (M^+, 69), 312 (48), 287 (21), 269 (18), 251 (15), 189
(31), 152 (76), 134 (100).

Example 2

Preparation of Compounds 9b and 10b (X^1=X^2=H)

3β-Acetoxy-24-norchol-5-ene (2, R=ethyl)

A solution of 1 (R=Et) (0.5 g; Rf 0.8 in 25% ethyl
acetate-hexane) in glacial acetic acid (7 mL) was heated at
70°C for 4.5 h. The reaction mixture was cooled, poured into
ice water, neutralized with 10% aqueous sodium hydroxide and
extracted with chloroform (3 x 70 mL). The organic extract
was washed with water (2 x 25 mL) and saturated sodium
chloride (2 x 25 mL) and then dried over anhydrous magnesium
sulfate. It was filtered and concentrated to dryness under
reduced pressure to give 0.5 g of residue containing 2 (R=Et)
(Rf 0.18 in 25% ethyl acetate-hexane) in ca. 90% yield. This
material was used without further purification.
Mass spectrum: m/e (relative intensity) 372 (M^+, 1), 312
(100), 298 (38), 283 (7), 255 (12), 191 (30); ^{1}H-NMR (CDCl$_3$):
δ 0.69 (s, 18-H), 0.82 (t, J=7.5 Hz, 23-H), 0.9 (d, J=7.0 Hz,
21-H), 1.05 (s, 19-H), 2.04 (s, 3-OCOCH$_3$), 4.58 (m, 3-H), 5.38
(d, J=4 Hz, 6-H).

3β-Acetoxy-24-norchola-5,7-diene (3) (R=Et)

A stirred solution of 2 (R=Et) (270 mg, 0.72 mmol) in dry
hexane (50 mL) containing finely divided sodium bicarbonate
(600 mg, 7.14 mmol) was heated to 80°C at reflux under
nitrogen and 1,3-dibromo-5,5-dimethylhydantoin (125 mg, 0.43
mmol) was then added. The reaction was allowed to proceed for
20 min at 80°C, then cooled and filtered. The filtrate was
evaporated to dryness in vacuo.   The residue was immediately
dissolved in dry xylene (5 mL) and added dropwise to a mixture
of xylene (30 mL) and s-collidine (174 mg, 1.44 mmol). The
mixture was then flushed with nitrogen and refluxed at 145°C
for 1.5 h. It was then cooled, diluted with benzene (100 mL),
washed with 3% aqueous hydrochloric acid (3 x 20 mL),
saturated aqueous sodium bicarbonate (1 x 20 mL), saturated
sodium chloride solution (2 x 20 mL), dried over anhydrous
magnesium sulfate, filtered and evaporated to dryness under
reduced pressure.

The residue was dissolved in dioxane (20 mL) and
p-toluenesulfonic acid (60 mg, 0.32 mmol) was added. . The
mixture was flushed with nitrogen and refluxed at 70°C for 30
min, cooled, diluted with water (10 mL) and then extracted
with ethyl acetate (1 x 100 mL, 2 x 50 mL). The organic phase
was washed with saturated aqueous sodium bicarbonate (1 x 25
mL), water (1 x 25 mL), saturated aqueous sodium chloride (2 x
30 mL) and then dried over anhydrous magnesium sulfate. It
was filtered and evaporated under reduced pressure to dryness.
The residue was chromatographed on preparative TLC using 10%
ethyl acetate-hexane to yield 148 mg (54%) of 3 (R=Et).
UV (EtOH): λ_{max} 282 nm, 293, 272, 262, mass spectrum: m/e
(relative intensity) 370 (M$^+$, 4), 328 (3), 310 (100), 296
(22), 253 (14), 158 (45), H-NMR (CDCl$_3$): δ 0.63 (s, 18-H),
0.84 (t, J=7.5 Hz, 23-H), 0.95 (d, J=7.0 Hz, 21-H), 0.96 (s,
19-H), 2.04 (s, 3-OCOCH$_3$), 4.71 (m, 3-H), 5.4 (m, 7-H), 5.58
(m, 6-H).

3β-Hydroxy-24-norchola-5,7-diene (4) (R=Et)

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Sodium hydroxide in methanol (10% solution) was added dropwise to a stirred solution of 3 (130 mg, 0.35 mmol, Rf 0.4 in 15% ethyl acetate-hexane) in ether (20 mL) under nitrogen. The reaction was allowed to proceed at 23°C for 40 min. It was then diluted with ether (100 mL), water (20 mL) was added and the phases were separated. The aqueous layer was further extracted with ether (2 x 60 mL) and the ether extracts were combined, washed with water (2 x 30 mL), saturated aqueous sodium chloride (2 x 30 mL), dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure. The residue was chromatographed on preparative TLC in 30% ethyl acetate-hexane to yield 103 mg (89%) of 4 (R=Et) (Rf 0.09 in 15% ethyl acetate-hexane). UV (EtOH): λ_{max} 282, 293, 272, 262 nm; mass spectrum: $\underline{m/e}$ (relative intensity), 328 (M^+, 100), 314 (16), 310 (8), 295 (86), 281 (15), 269 (45), 255 (13), ^{1}H-NMR (CDCl$_3$): δ 0.63 (s, 18-H), 0.84 (t, J=7.5 Hz, 23-H), 0.94 (d, J=7.0 Hz, 21-H), 0.96 (s, 19-H), 3.64 (m, 3-H), 5.38 (m, 7-H), 5.57 (m, 6-H).

Secosterol analog 5 (R=Et)

The 5,7-diene 4 (R=Et) (125 mg) in 1:4 dry benzene-ether (150 mL) was irradiated in a manner similar to that described in Example 1, above, for 25 min. The solvent was evaporated under reduced pressure. The crude residue was immediately dissolved in dry ethanol (30 mL) saturated with nitrogen. The solution was refluxed at 70°C under nitrogen for 3 h, then cooled and concentrated under reduced pressure. Purification by preparative TLC using silica gel plates in 30% ethyl acetate-hexane afforded 5 (R=Et) (30 mg) in ca. 24% yield. UV (EtOH): λ_{max} 264 nm, λ_{min} 227 nm; mass spectrum: $\underline{m/e}$ (relative intensity) 328 (M^+, 23), 310 (3), 295 (10), 271 (9), 253 (11), 136 (81), 118 (100); ^{1}H-NMR (CDCl$_3$): δ 0.55 (s, 18-H), 0.83 (t, J=7.5 Hz, 23-H), 0.92 (d, J=7.0 Hz, 21-H), 3.93 (broad, 3-H), 4.81 (m (sharp), 19(Z)-H), 5.01 (m (sharp), 19(E)-H), 6.02 (d, J=12.5 Hz, 7-H), 6.22 (d, J=12.5 Hz, 6-H).

Tosylate 6 (R=Et)

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A solution of 5 (R=Et) (15 mg, 0.045 mmol; Rf 0.23 in 25%
ethyl acetate-hexane) in dry pyridine (1 mL) was treated with
p-toluenesulfonyl-chloride (20 mg, 0.105 mmol) at 5°C under
nitrogen for 24 h. The reaction was quenched with ice water
and the mixture extracted with ether (3 x 50 mL). The organic
phases were combined and washed with water (2 x 20 mL), 3%
aqueous hydrochloric acid (2 x 20 mL), saturated sodium
bicarbonate solution (2 x 20 mL) and saturated sodium chloride
solution (1 x 20 mL), then dried over anhydrous magnesium
sulfate and concentrated under reduced pressure to yield 22 mg
of the tosylate 6 (R=Et) in greater than 95% purity.
Mass spectrum: $\underline{m/e}$ (relative intensity) 484 (M^+, 8), 310 (72),
296 (18), 295 (20), 281 (10), 253 (39) 158 (65), 143 (47), 118
(100).

3,5-Cyclo-derivative 7 (R=Et)

The tosylate 6 (R=Et) (22 mg, 0.045 mmol; Rf 0.54 in 25%
ethyl acetate-hexane) was added to a stirred suspension of
finely divided sodium bicarbonate (60 mg, 0.71 mmol) in
anhydrous methanol (20 mL). The mixture was heated at 55°C
under nitrogen for 8 h, cooled, diluted with ether (150 mL)
and washed with water (2 x 30 mL) and saturated aqueous sodium
chloride (2 x 30 mL). It was dried over anhydrous magnesium
sulfate, filtered and concentrated under reduced pressure to
yield a crude product 7 (R=Et) (Rf 0.6 in 25% ethyl
acetate-hexane) in ca. 68% yield.
Mass spectrum: $\underline{m/e}$ (relative intensity) 342 (M^+, 10), 328
(12), 310 (11), 295 (10), 253 (8), 149 (41), 136 (67), 118
(100).

1α-Hydroxy-3,5-cyclo-derivative 8 (R=Et)

t-Butyl hydroperoxide (14.0 μl, 0.1 mmol) was added to a
suspension of selenium dioxide (2.4 mg, 0.022 mmol) in dry 1%
pyridine-methylene chloride (10 mL) and cooled on an ice bath.
The cyclovitamin 7 (R=Et) (15 mg, 0.044 mmol; Rf 0.6 in 25%
ethyl acetate-hexane) in dry methylene chloride (5 mL) was
added. The reaction was monitored on TLC and proceeded for 40

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min before 10% sodium hydroxide (10 mL) was added to quench
the reaction. The mixture was diluted with ether (150 mL),
phases were separated and the ether phase was washed with 10%
sodium hydroxide solution (2 x 30 mL), water (2 x 30 mL),
sodium chloride (2 x 30 mL) and dried over anhydrous magnesium
sulfate. It was then filtered and concentrated under reduced
pressure. Preparative TLC using 25% ethyl acetate-hexane gave
8 (R=Et) (8 mg, Rf 0.18 in 25% ethyl acetate-hexane) in ca.
50% yield.

Mass spectrum: m/e (relative intensity) 358 (M^+, 21), 326
(48), 285 (35), 269 (15), 191 (50), 135 (100); ^{1}H-NMR ($CDCl_3$):
δ 0.55 (s, 18-H), 0.85 (t, J=7.5 Hz, 23-H), 0.92 (d, J=7.0 Hz,
21-H), 3.27 (s, 6R-OCH_3), 4.18 (d, J=10 Hz, 6-H), 4.22 (m,
1-H), 4.95 (d, J=10 Hz, 7-H), 5.16 (d, J=2.0 Hz, 19(Z)-H),
5.26 (d, J=2.2 Hz, 19(E)-H).

    5,6-cis and trans secosterol 3β-acetates 9b and 10b
(X^1=Ac, X^2=H)

    A solution of 8 (R=Et) (8 mg) in glacial acetic acid (0.5
mL) was heated to 55°C under nitrogen for 15 min, cooled and
poured over ice-cold sodium bicarbonate solution (15 mL). The
mixture was extracted with ether (3 x 30 mL), and the ether
extract was washed with saturated sodium bicarbonate solution
(1 x 20 mL), water (2 x 20 mL), saturated aqueous sodium
chloride (1 x 20 mL), dried over anhydrous magnesium sulfate
and filtered. The filtrate was concentrated under reduced
pressure and chromatographed by preparative TLC on 30% ethyl
acetate-hexane (x 2) to give the 5,6-cis-3β-acetate 9b (X^1=Ac,
X^2=H) (Rf 0.13 on 25% ethyl acetate-hexane) and the
5,6-trans-3β-acetate 10b (X^1=Ac, X^2=H) (Rf 0.11 on 25% ethyl
acetate-hexane).

    The products were further purified on HPLC on a
Zorbax-Sil column (4.6 mm x 25 cm) in 1% isopropanol-hexane to
give the acetates of 9b and 10b in 28.9% and 10.4% yields,
respectively (retention volumes of 39 mL and 46.5 mL)

    3β-Acetate 9b (X^1=Ac, X^2=H)

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UV (EtOH) λ_{max} 264 nm, λ_{min} 226 nm; mass spectrum: $\underline{m}/\underline{e}$
(relative intensity) 386 (M^+, 17), 326 (45), 308 (8), 269
(16), 203 (17), 134 (100), ^{1}H-NMR (CDCl$_3$): δ 0.55 (s, 18-H),
0.84 (t, J=7.5 Hz, 23-H), 0.92 (d, J=7.0 Hz, 21-H), 2.04 (s,
3-OCOCH$_3$), 4.41 (m, 1-H), 5.02 (m (sharp), 19(Z)-H), 5.22 (m,
3-H), 5.35 (m (sharp), 19(E)-H) 6.03 (d, J=12.5 Hz, 7-H), 6.35
(d, J=12.5 Hz, 6-H).

3β-Acetate 10b (X^1=Ac, X^2=H)

UV (EtOH) λ_{max} 273 nm, λ_{min} 226 nm; mass spectrum: $\underline{m}/\underline{e}$
(relative intensity): 386 (M^+, 12), 326 (64), 312 (8), 297
(9), 279 (4), 269 (21), 203 (28), 134 (100); ^{1}H-NMR (CDCl$_3$): δ
0.56 (s, 18-H), 0.85 (t, J=7.5 Hz, 23-H), 0.93 (d, J=7.0 Hz,
21-H), 2.30 (s, 3-OCOCH$_3$), 4.49 (m, 1-H), 5.0 (m (sharp),
19(Z)-H), 5.14 (m (sharp), 19 (E)-H), 5.26 (m, 3-H), 5.82 (d,
J=12.5 Hz, 7-H), 6.58 (d, J=12.5 Hz, 6-H).

1α-Hydroxy-secosterols 9b and 10b (X^1=X^2=H)

The 3β-acetate 9b (X^1=Ac, X^2=H) (1.5 mg, Rf 0.31 in 20%
ethyl acetate-hexane) was hydrolyzed using 10% sodium
hydroxide in methanol (2 mL) at 23°C under nitrogen for 0.5 h.
The mixture was diluted with ether (50 mL) and water (5 mL)
was added. The phases were separated and the aqueous layer
was extracted with ether (2 x 30 mL). The ether extracts were
combined, washed with water (2 x 10 mL), saturated sodium
chloride solution (2 x 10 mL), dried over anhydrous magnesium
sulfate, filtered and concentrated under reduced pressure to
give 9b (X^1=X^2=H) (Rf 0.06 in 20% ethyl acetate-hexane).

Similarly, acetate 10b (X^1=Ac, X^2=H) (0.75 mg, Rf 0.26 in
40% ethyl acetate-hexane) was hydrolyzed to give
5,6-trans-isomer 10b (R=Et, X^1=X^2=H) (Rf 0.06 in 40% ethyl
acetate-hexane).

Each of the products was chromatographed on preparative
TLC in 60% ethyl acetate-hexane, followed by HPLC on a
Zorbax-Sil analytical column (4.6 mm x 25 cm) in 7%
isopropanol-hexane and then reverse phase HPLC (RPHPLC) on a

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Zorbax-ODS analytical column (4.6 mm x 25 cm) in 90%
methanol-water.

Compound 9b (X^1=X^2=H)

UV (EtOH): λ_{max} 264 nm, λ_{min} 227 nm; high resolution mass
analysis: calc. for $C_{23}H_{36}O_2$, 344.2715; found, 344.2707;
mass spectrum: m/e (relative intensity) 344 (M^+, 23), 326
(12), 287 (7), 269 (6), 251 (5), 203 (9), 152 (7), 134 (100);
^{1}H-NMR (CDCl$_3$): δ 0.52 (s, 18-H), 0.82 (t, J=7.5 Hz, 23-H),
0.90 (d, J=7.0 Hz, 21-H), 4.29 (m, 3-H), 4.42 (m, 1-H), 4.99
(m (sharp), 19(Z)-H), 5.31 (m (sharp), 19(E)-H), 6.0 (d,
J=12.5 Hz, 7-H), 6.37 (d, J=12.5 Hz, 6-H).

5,6-trans-compound 10b (X^1=X^2=H)

UV (EtOH): λ_{max} 273 nm, λ_{min} 226 nm; high resolution mass
analysis: calc. for $C_{23}H_{36}O_2$, 344.2715; found, 344.2705; mass
spectrum: m/e (relative intensity) 344 (M^+, 12), 326 (6), 287
(4), 269 (4), 251 (3), 203 (6), 152 (30), 134 (100); ^{1}H-NMR
(CDCl$_3$): δ 0.56 (s, 18-H), 0.84 (t, J=7.5 Hz, 23-H), 0.91 (d,
J=7.0 Hz, 21-H), 4.22 (m (sharp), 3-H), 4.48 (m (sharp), 1-H),
4.96 (s, 19(Z)-H), 5.12 (m (sharp), 19(E)-H), 5.88 (d, J=12.5
Hz, 7-H), 6.57 (d, J=12.5 Hz, 6-H).

Example 3

Preparation of compounds 9c and 10c

From 6β-methoxy-3α,5-cyclo-5α-cholane (compound 1, where
R=propyl), processed through all the reaction steps given in
Example 2 above under analogous experimental conditions, there
is obtained the 1α-hydroxy-secosterol analog of structure 9c
(X^1=X^2=H) and the corresponding 5,6-trans-compound of
structure 10c (X^1=X^2=H).

Example 4

Synthesis of Starting Material, Compound 1 (R=Me)

(a) (22E)-6β-Methoxy-3α,5-cyclo-5α-stigmast-22-ene
(Stigmasteryl i-methylether)

Freshly crystallized p-toluenesulfonyl chloride (20 g,
0.10 mole) was added to a solution of stigmasterol (25 g, 0.06

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mole; Rf 0.26 in 30% ethyl acetate-hexane) in dry pyridine
(250 mL). The reaction mixture was stirred at 23°C for 24 h,
after which it was slowly poured into saturated aqueous sodium
bicarbonate solution. The precipitate was collected by
filtration, washed with water several times until neutral and
dried under reduced pressure overnight to yield
stigmasteryl-3β-tosylate (32.06 g) in 93.2% yield.

The tosylate was converted to the i-ether without further
purification. A solution of the tosylate (32 g, 0.06 mole) in
chloroform (100 mL) was added slowly to a refluxed solution of
finely divided sodium bicarbonate (30 g, 0.36 mole) in
methanol (400 mL). The mixture was stirred at reflux for 14
h, cooled and concentrated to ca. 100 mL. Hexane was added
(300 mL) and the resulting mixture was washed with water (100
mL). The phases were separated and the aqueous layer was back
extracted with hexane (2 x 200 mL, 1 x 100 mL). The organic
layers were combined and washed with water (2 x 100 mL),
saturated aqueous sodium chloride (2 x 100 mL), dried over
anhydrous magnesium sulfate, filtered and concentrated under
reduced pressure to give 24 g of a crude oil containing the
desired i-methyl ether (Rf. 0.52 in 10% ethyl acetate-hexane).
Mass spectrum: $\underline{m/e}$ (relative intensity) 426 (M^+, 87), 411
(34), 394 (54), 371 (59), 368 (19), 351 (21), 255 (61), 83
(100).

(b) 6β-Methoxy-3α,5-cyclo-23,24-dinor-5α-cholan-22-ol.

A stirred solution of stigmasteryl i-ether as obtained
above (5.0 g, 11.7 mmol, Rf 0.64 in 30% ethyl acetate-hexane)
in 1% pyridine-methylene chloride (100 mL) was cooled to -69°C
on a dry ice-acetone bath and treated with ozone generated by
a Welsbach model T816 ozonator, until a pale blue color
persisted due to excess ozone. The mixture was purged with
oxygen for 5 min and allowed to warm to 23°C. Sodium
borohydride (0.7 g, 18.4 mmol) in ethanol was added. After 2
h, the reaction mixture was diluted with ether (200 mL).

Water (100 mL) was added, the phases were separated and the
aqueous layer was extracted with ether (2 x 200 mL). The
combined organic fractions were washed with saturated aqueous
sodium chloride (2 x 50 mL) and dried over anhydrous magnesium
sulfate, filtered and evaporated to dryness under reduced
pressure. The residue was applied on a silica gel column and
eluted using 20% ethyl acetate-hexane to afford 2.6 g of the
desired 22-alcohol derivative in 65% yield. Mass spectrum:
$\underline{m}/\underline{e}$ (relative intensity) 346 (M^+, 75), 331 (52), 314 (89), 291
(100), 288 (30); ^{1}H-NMR ($CDCl_3$): δ 0.44 (m, 3-H), 0.65 (m,
4-H), 0.75 (s, 18-H), 1.04 (s, 19-H), 1.08 (d, J=7.0 Hz,
21-H), 2.77 (m, sharp, 6-H), 3.32 (s, 6-OCH_3).

(c) 6β-Methoxy-3α,5-cyclo-23,24-dinor-5α-cholan-22-yl tosylate
        p-Toluenesulfonyl chloride (2.0 g, 11 mmol) was added to
a solution of the 22-alcohol obtained in the previous
experiment (1.9 g; 5.5 mmol) in dry pyridine (35 mL). The
reaction mixture was stirred at 23°C for 18 h, then poured
into ice-cold saturated aqueous sodium bicarbonate (50 mL) and
extracted with ethyl acetate (2 x 150 mL, 1 x 100 mL). The
organic extract was washed with water (3 x 50 mL), saturated
sodium chloride (2 x 50 mL) and dried over anhydrous magnesium
sulfate. It was then filtered and evaporated to dryness under
reduced pressure. The residue was dried under reduced
pressure overnight to give 2.2 g of the 22-tosylate in 91.0%
yield. Mass spectrum: $\underline{m}/\underline{e}$ (relative intensity) 500 (M^+, 69),
485 (49), 468 (100), 445 (79), 442 (20), 296 (20), 273 (24).

(d) 6β-Methoxy-3α,5-cyclo-23,24-dinor-5α-cholane (compound 1,
R=Me)
        To the 22-tosylate (2.15 g, 4 mmol) in anhydrous ether
(100 mL) was added 0.22 g lithium alumnium hydride (liAlH,
0.22 g, 6 mmol). The reaction mixture was refluxed 10 h,
cooled and excess reagent was decomposed by saturated aqueous
sodium chloride. The mixture was filtered and the layers

separated. The aqueous fraction was back-extracted with ether
(2 x 100 mL). Ether fractions were combined, washed with
water (1 x 50 mL), saturated sodium chloride solution (2 x 50
mL), dried over anhydrous magnesium sulfate, evaporated to
dryness and dried under vacuum to give compound $\underline{1}$ (R=Me) in
96.0% yield. Mass spectrum: $\underline{m/e}$ (relative intensity) 330
(M^+, 41), 315 (46), 298 (100), 283 (21), 275 (81), 272 (18),
177 (45); ^{1}H-NMR (CDCl$_3$): δ 0.40 (m, 3-H), 0.64 (m, 4-H), 0.72
(s, 18-H), 0.85 (d, J=7.0 Hz, 22-H), 0.92 (d, J=7.0 Hz, 21-H),
2.77 (m, sharp), 6-H), 3.35 (s, 6-OCH$_3$).

Example 5

Preparation of Starting Material, Compound $\underline{1}$ (R=Et)

(a)   6β-Methoxy-3α,5-cyclo-24-nor-5α-cholane-23-nitrile.

To the 22-tosylate obtained in Example 4(c) above (10 g,
20 mmol, Rf 0.53 in 25% ethyl acetate-hexane) dissolved in
dimethylsulfoxide (200 mL) was added sodium cyanide (1.95 g,
40 mmol). The mixture was stirred at 80°C under nitrogen for
2 h, then cooled and stirred at room temperature for 1 h. It
was then poured over ice-saturated ammonium chloride solution
(250 mL) and extracted with ether (3 x 450 mL). The combined
ether fractions were washed with water (3 x 200 mL), saturated
sodium chloride (2 x 200 mL), dried over anhydrous magnesium
sulfate, filtered and concentrated under reduced pressure to
give the desired 23-nitrile derivative (7.05 g crude, Rf 0.57
in 25% ethyl acetate-hexane). The crude mixture was used in
the next step without further purification. Mass spectrum:
$\underline{m/e}$ (relative intensity) 355 (M^+, 33), 340 (46), 323 (64), 308
(4), 297 (17), 300 (100), 218 (11), 149 (31); ^{1}H-NMR (CDCl$_3$):
δ 0.43 (m, 3-H), 0.65 (m, 4-H), 0.74 (s, 18-H), 1.02 (s,
19-H), 1.15 (d, J=7.0 Hz, 21-H), 2.77 (t, J=2.5 Hz, 6-H).

(b)   6β-Methoxy-3α,5-cyclo-24-nor-5α-cholan-23-ol.

To the nitrile derivative as obtained in (a) above (7.0
g, 19.7 mmol, Rf 0.27 in 10% ethyl acetate-hexane) dissolved
in dry benzene (150 mL) and cooled on ice under nitrogen was

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added slowly diisobutylaluminum hydride [DIBAL-H, 1.5 mole
solution in toluene (20 mL, 30 mmol)]. The ice bath was
removed after addition was complete and the reaction was
allowed to proceed at 23°C for 3 h. Methanol (150 mL) was
added to decompose the aluminum salt complex and the mixture
was poured over ice water (200 mL). The mixture was filtered
and the aqueous layer was extracted with ether (3 x 150 mL).
The organic phases were combined, washed with saturated sodium
chloride (2 x 60 mL), dried over anhydrous magnesium sulfate
and concentrated under reduced pressure to give 6.25 g of
product. This product was a mixture of the original
23-nitrile and the expected 23-aldehyde. This mixture was
again treated with the same reducing agent. Thus, the crude
product was dissolved in dry benzene and treated with DIBAL-H
(17.5 mL, 26.25 mmol). Work-up as before and chromatography
of the residue on silica gel, eluted with 10% ethyl
acetate-hexane yielded 0.85 g of the desired 23-alcohol
derivative (Rf 0.03 in 10% ethyl acetate-hexane). Mass
spectrum: m/e (relative intensity) 360 (M^+, 75), 345 (59), 328
(99), 305 (100), 302 (29), 281 (22), 255 (23); ^{1}H-NMR ($CDCl_3$):
δ 0.44 (m, 3-H), 0.65 (m, 4-H), 0.72 (s, 18-H), 0.93 (d, J=7.0
Hz, 21-H), 1.02 (s, 19-H), 2.77 (m, sharp, 6-H), 3.3 (s,
6-OCH_3).

The remainder of the product (2 g) was the corresponding
23-aldehyde, which, if desired, can be further reduced using
the above conditions to yield additional quantities of the
23-alcohol product.

(c)   23-Hydroxy-6β-methoxy-3α,5-cyclo-24-nor-5α-cholane
23-tosylate.

p-Toluenesulfonyl chloride (0.95 g, 5.0 mmol) was added
to a solution of the 23-alcohol obtained in (b) above (0.85 g,
2.36 mmol) in dry pyridine (10 mL). The reaction mixture was
kept at 0°C for 20 h, then poured into ice-cold saturated
aqueous sodium bicarbonate (25 mL), extracted with ethyl

acetate (3 x 100 mL), washed with water (3 x 40 mL), saturated
sodium chloride (2 x 40 mL), dried over anhydrous magnesium
sulfate, filtered and evaporated to dryness under reduced
pressure to give the 23-tosylate (1.15 g) in ca. 95% yield.
Mass spectrum: $\underline{m/e}$ (relative intensity) 514 (M^+, 42), 499
(26), 482 (100), 468 (14), 459 (42), 456 (12), 361 (13), 255
(29).

(d) 6β-Methoxy-3,5-cyclo-24-nor-5α-cholane (compound $\underline{1}$,
R=Et).

To the above tosylate (1.15 g, 2.08 mmol, Rf 0.7 in 10%
ethyl acetate-hexane) in anhydrous ether (100 mL) was added
LiAlH$_4$ (0.12 g, 3 mmol). The reaction mixture was refluxed 5
h, cooled and excess reagent was decomposed by saturated
sodium chloride solution. The mixture was filtered, the
phases were separated and the aqueous fraction was extracted
with ether (2 x 100 mL). The ether extracts were combined,
washed with water (1 x 50 mL), saturated sodium chloride (2 x
50 mL), dried over anhydrous magnesium sulfate, evaporated to
dryness under reduced pressure and then dried under vacuum to
give the desired 24-nor-cholane derivative (compound $\underline{1}$, R=Et)
(0.59 g, Rf 0.64 in 10% ethyl acetate-hexane) in ca. 82%
yield. Mass spectrum: $\underline{m/e}$ (relative intensity) 344 (M^+, 9),
329 (12), 312 (19), 289 (22), 286 (4), 255 (7), 191 (11), 69
(90); ^{1}H-NMR (CDCl$_3$): δ 0.44 (m, 3-H), 0.65 (m, 4-H), 0.72 (s,
18-H), 0.82 (t, J=7.5 Hz, 23-H), 0.9 (d, J=7.0 Hz, 21-H), 1.02
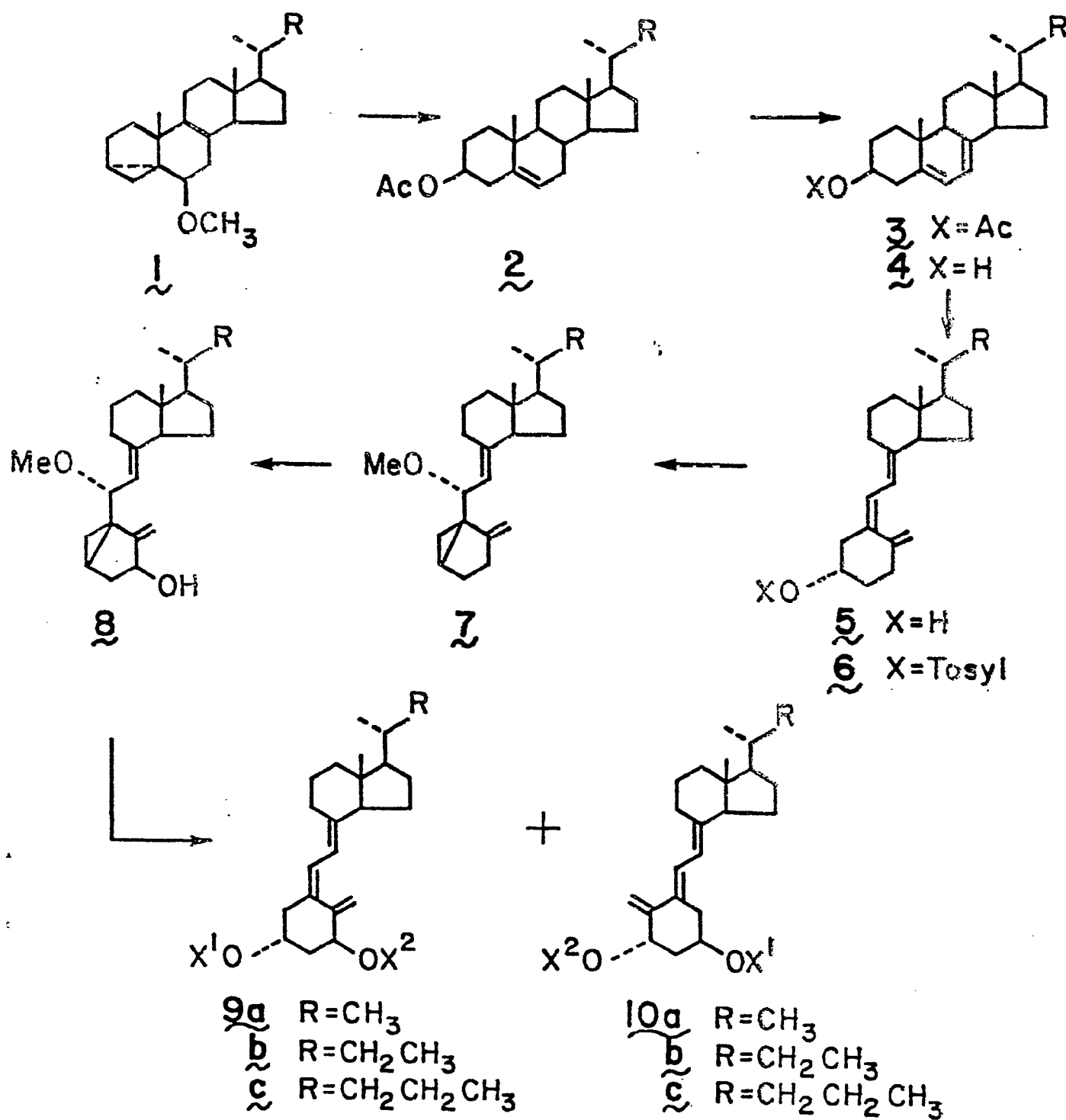(s, 19-H), 2.77 (m, sharp, 6-H), 3.32 (s, 6-OCH$_3$).

Example 6

Preparation of Starting Material, Compound $\underline{1}$ (R=propyl)

Treatment of the 23-tosylate obtained in Example 5(c)
above, with sodium cyanide under conditions analogous to those
described in Example 5(a), provides
6β-methoxy-3α,5-cyclo-5α-cholan-24-nitrile. By reduction of
this nitrile, using the procedure of Example 5(b) above, there
is obtained 6β-methoxy-3α,5-cyclo-5α-cholan-24-ol. This

alcohol is converted to the corresponding 24-tosylate
derivative by a procedure analogous to that described in
Example 5(c) above, and the 24-tosylate, subjected to hydride
reduction as described in Example 5(d) above, then provides
the desired compound, 6β-methoxy-3α,5-cyclo-5α-cholane
(compound 1, where R=propyl).

Process Scheme I

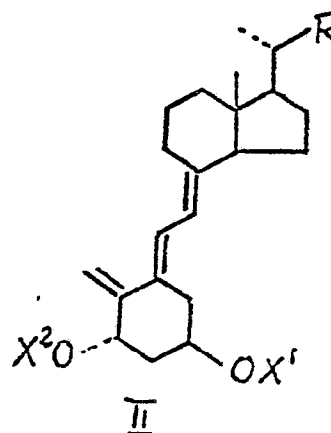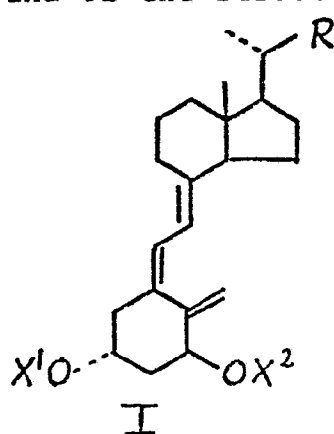9a R=CH₃
b R=CH₂CH₃
c R=CH₂CH₂CH₃

10a R=CH₃
b R=CH₂CH₃
c R=CH₂CH₂CH₃

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CLAIMS

1.   A method for inducting cell differentiation in
a mammal which comprises administering to the mammal a
compound of the structure:

where R is hydrogen, methyl, ethyl or propyl and where X^1
and X^2 independently represent hydrogen or an acyl group.

2.   A method according to Claim 1 wherein the cells
induced to differentiate are leukemic cells.

3.   A method according to Claim 1 or 2 wherein R,
X^1 and X^2 represent hydrogen and the compound is of
structure I.

4.   A method according to Claim 1 or 2 wherein R is
a methyl and X^1 and X^2 represent hydrogen and the compound
is of structure I.

5.   A method according to Claim 1 or 2 wherein R is
ethyl and X^1 and X^2 represent hydrogen and the compound is
of structure I.

6.   A method according to Claim 1 or 2 wherein R is propyl and x^1 and x^2 represent hydrogen and the compound is of structure I.

7.   A method according to Claim 1 substantially as hereinbefore described.

8.   A compound of structure I or II as defined in Claim 1 for use in the treatment of malignant cells.

9.   A compound of the structure
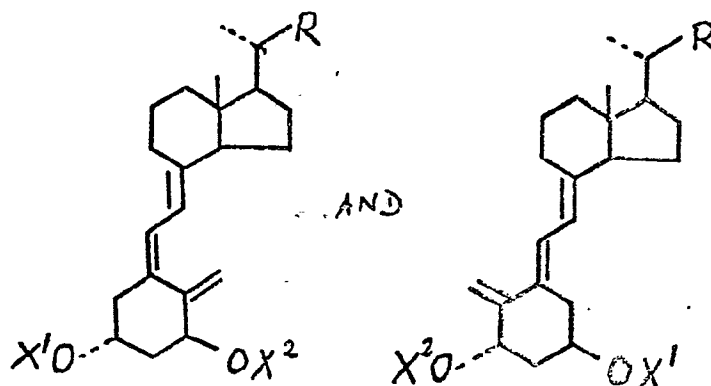
wherein R is methyl, ethyl or propyl and wherein x^1 and x^2 independently represent hydrogen or an acyl group.

10.   A compound as claimed in Claim 9 wherein R is methyl and x^1 and x^2 represent hydrogen and the compound is of structure I.

11.   A compound as claimed in Claim 9 wherein R is ethyl and x^1 and x^2 represent hydrogen and the compound is of structure I.

12.   A compound as claimed in Claim 9 wherein R is propyl and x^1 and x^2 represent hydrogen and the compound is
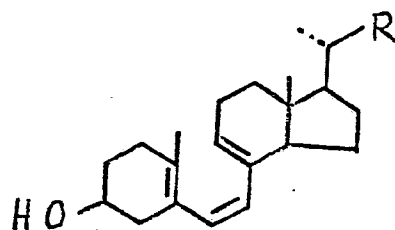
-36-

of structure I.

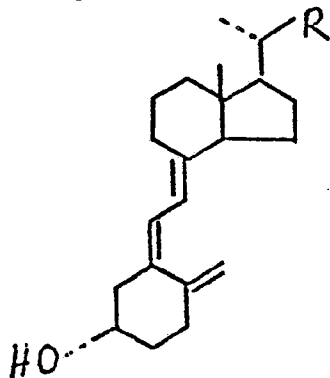13. A compound having the formula
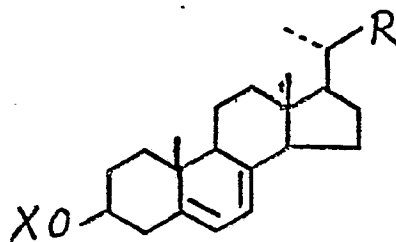
wherein R is methyl, ethyl or propyl and wherein X is
hydrogen or acyl.

14. A compound having the formula
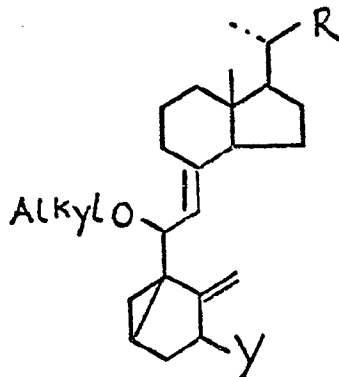

wherein R is methyl, ethyl or propyl.

15. A compound having the formula

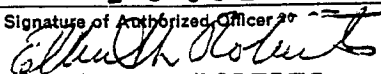wherein R is methyl, ethyl or propyl.

16. A compound having the formula

wherein R is methyl, ethyl or propyl and where Y is hydrogen or hydroxy.

17. A pharmaceutical composition comprising a compound as claimed in any one of claims 9 to 12 together with a pharmaceutically acceptable excipient.

18. A composition according to Claim 17 wherein the compound is one of structure I.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00833

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: A01N 45/00; A61K 31/59
U.S.: 514/167

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	514/167; 260/397.2

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 6

CAS-ON LINE COMPUTERIZE SEARCH.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
A	US,A, 4,391,802 (SUDA ET AL) July 5, 1983	
A	US,A, 4,588,716 (DELUCA ET AL) May 13, 1986	
A	Proc. Natl. Acad. Sci, Vol. 80, Published January 1983, (YOSHIO ET AL) "1ɑ.25-Dihydroxy vitamin D₃ and 1ɑ-hydroxy vitamin D₃ prolong survival time of mice inoculated with myeloid leukemia cells. See pages 201-204	

* Special categories of cited documents: 15

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 3	Date of Mailing of this International Search Report 2
03 July 1986	18 JUL 1986
International Searching Authority 1	Signature of Authorized Officer 20
ISA/US	ELBERT L. ROBERTS

Form PCT/ISA/210 (second sheet) (October 1981)