Biosynthesis of Phytosterols in the Pea. Mode of Incorporation of Carbon-2 Hydrogen Atoms of Mevalonic Acid into Sitosterol

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Abstract: The biosynthesis of sitosterol from (3*RS*,2*R*)- and (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]mevalonic acid in the pea was investigated. It was determined that the incorporation and the stereochemistry of the tritium atoms at C-1, -7, and -15 of the (*R*)- and (*S*)-sitosterols was the same as in cholesterol biosynthesized from the same precursors by rat liver enzymes. It is inferred that irrespective of whether cycloartenol or lanosterol is the precursor in the pea and in rat liver tissues, the patterns of transformations of the tetracyclic moieties are essentially similar.

In contrast to the biosynthesis of cholesterol in rat liver preparations, the biosynthesis of plant sterols has attracted much less attention.[1,2] Based largely on indirect evidence, it was accepted that cycloartenol and not lanosterol is the primary cyclization product of 2,3(*S*)-oxidosqualene leading to plant sterols.[3,4] The mechanism of the transformation of cycloartenol to phytosterols has not been investigated in detail.[1,2]

Several years ago, evidence consistent with the possible retention of two hydrogen atoms derived from C-2 of mevalonic at C-15 of sitosterol biosynthesized in *Calendula officinalis* was reported.[5] This was in sharp contrast to the biosynthesis of cholesterol by rat liver enzymes, which proceeds with the loss of a hydrogen derived from 2-pro-*S* of MVA from C-15.[6-9] In the broader sense, the observation in *C. officinalis* could be viewed as an indication that the sequence of anabolic transformations leading from cycloartenol to phytosterols differs from that leading from lanosterol to cholesterol. For example, the possibility had to be considered that the sequence from cycloartenol to sitosterol does not involve an intermediate having a 14(15) double bond such as a 8(9),14(15)-diene. In rat livers the formation of this diene proceeds with the loss of a hydrogen derived from the 2-pro-*S* position of MVA from C-15.

The feasibility of a process not involving a 14(15) double-bond intermediate in plant sterol biosynthesis was enhanced by the isolation of 5α-stigmasta-8(14),22-diene-3β-ol from rayless goldenrod (*Aplopappus heterophyllus*).[10] In essence, this brought to mind the possibility that the formation of plant sterols might involve an intermediate having an 8(14) double bond. It may be recalled that based on the concepts of Gautschi and Bloch,[11] Fried et al.[12] and Schroepfer et al.[13] have suggested that the biosynthesis of cholesterol proceeds via an 8(14)-olefinic intermediate obtained in the course of the removal of the 14α-methyl group.

For a critical evaluation of the suggested alternative pathway, it was necessary first to provide firm evidence linking the biosynthesis of phytosterols directly with cycloartenol. Indeed, we have proven that in germinating peas, cycloartenol, or a precursor having an anionic terminus at C-19, is an obligatory intermediate in the formation of sitosterol.[14,15] Very likely, the intermediate with an anionic terminus located at C-19 could be formed in the course of the enzymatic opening of the cyclopropane ring. The evidence supporting the intermediacy of cycloartenol consists of the fact that sitosterol isolated from peas germinated in deuterium oxide (99.8% $^{2}H_2O$) contained ca. 0.4–0.45 atoms of deuterium at C-19. The detection of the relatively large amount of deuterium (0.4–0.45 atoms) at C-19 is most significant since, mechanistically, a maximum of one atom of deuterium could be incorporated into the 10β-methyl moiety of sitosterol. In contrast, no deuterium was incorporated at C-18 of the sitosterol. It might be recalled that the biosynthesis of cholesterol involves the intermediacy of lanosterol in which the three hydrogen atoms of the 10β-methyl are derived from the 3^{1}-methyl of MVA.[1] Accordingly, when cholesterol was biosynthesized from mevalonic acid by rat liver enzymes in a deuterium oxide medium, deuterium was not incorporated into the 10β- (and 13β-) methyl groups.[16-18]

Having established a direct biosynthetic link between cycloartenol (or an intermediate with an anionic terminus at C-19) and phytosterols in the pea, we turned our attention to the evaluation of the transformations occurring at carbons 1, 7, and 15 in the course of the elaboration of sitosterol in this plant. A portion of the results reported in this paper was the subject of preliminary communications.[19-21]

Experimental Section

Materials. Peas (Blue Bantam variety) obtained from W. Atlee Burpee Company were used. (3*RS*)-[2-^{14}C]Mevalonic acid lactone (6.40 mCi/mM), (3*RS*,2*S*)-[2-^{3}H]mevalonic acid lactone (250 mCi/mM), and (3*RS*,2*R*)-[2-^{3}H]mevalonic acid lactone (180 mCi/mM) were obtained from Amersham/Searle Corp. Aliquots of [2-^{14}C]mevalonic lactone (10 μCi of ^{14}C) and (*R*)-[2-^{3}H]mevalonic lactone were mixed to yield (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]mevalonic acid lactone (^{3}H:^{14}C ratio 10.0). Similarly, [2-^{14}C]mevalonic lactone (10 μCi of ^{14}C) and (*S*)-[2-^{3}H]mevalonic lactone were mixed to yield (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]mevalonic acid lactone (^{3}H:^{14}C ratio 3.14).

Sitosteryl acetate, free of campesterol, was prepared from stigmasteryl acetate (purchased from Upjohn Co.). Stigmasteryl acetate was selectively hydrogenated in batches of 2 g in ethyl acetate (100 mL) over Raney nickel (W. R. Grace Co., No. 28). The course of the hydrogenation was followed by argentation TLC (chloroform freed of alcohol) and/or GLC (see below). Usually the hydrogenation was terminated after 18 h, at which time the GLC indicated the presence of stigmasteryl acetate (ca. 10%), sitosteryl acetate (ca. 80%), and stigmastanyl acetate (ca. 10%). The recovered products were fractionated by argentation TLC to yield homogeneous sitosteryl acetate (1.5 g). The sitosteryl acetate was crystallized from MeOH–$CHCl_3$.

Physical Measurements. Melting points were taken on a hot-stage apparatus and are corrected. Ultraviolet spectra were recorded on a Perkin-Elmer 202 spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer 237 spectrophotometer as KBr disks.[22] Proton magnetic resonance spectra were recorded on Varian HA-100 D-15 spectrometer equipped with a Varian C1024 computer or on a Varian M360 spectrometer. Peaks are quoted in parts per million downfield from the tetramethylsilane internal standard. The assignment of the chemical shifts of the methyl groups is discussed elsewhere.[23] Mass spectra were recorded on a Nuclide 12-90-G mass spectrometer equipped with a Nuclide DA/CS I.2 data acquisition system and a Hewlett-Packard 7620A gas chromatography system or on a Du Pont 21-491 instrument.

Table I. Distribution and Stereochemistry of ^3H at C-1 and C-7 of (*R*)- and (*S*)-Sitosterol Biosynthesized in the Pea from (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]- and (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA, Respectively

		Odd numbered compounds, (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA			Even numbered compounds, (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA		
			^{3}H:^{14}C ratio			^{3}H:^{14}C ratio	
Entry	Compound	^{14}C specific act.	Isotopic	Atomic	^{14}C specific act.	Isotopic	Atomic
1	MVA amide		3.14	1:1		9.97	1.00:1
2	Squalene 6HCl		2.01	6.00:6		5.16	6.00:6
3	Sitosteryl acetate	2.98	1.29	3.21:5	3.26	5.23	5.07:5
4	5α-Stigmastanone	2.94	1.29	3.21:5	3.20	5.15	4.99:5
5	5α-Stigmastanone equilibrated with base	3.04	1.28	3.19:5	3.19	5.21	5.05:5
6	5α-Stigmast-1-en-3-one	3.01	1.02	2.54:5	3.26	5.02	4.89:5
7	1,3-Seco-2-nor-5α-stigmasta-1,3-dioic acid	2.17[a]	0.925[a]	2.30:5[a]	2.34	4.26	4.13:5
8	Sitosterol	3.17	1.29	3.20:5	3.25	5.15	4.99:5
9	5α-Hydroperoxystigmast-6-en-3β-ol	3.20	1.23	3.06:5	3.20	5.17	5.01:5
10	3β-Hydroxystigmast-5-en-7-one	3.23	1.21	3.00:5	3.21	4.34	4.21:5

[a] The error of this ^3H and ^{14}C determination is considerably larger (see counting in the Experimental Section).

Chromatography. Silica gel (Merck HF 254 + 366) and ready-made silica gel sheets (JB-F, J. T. Baker Chemical Co.) were used for thin-layer chromatography in the indicated solvent systems. The compounds were visualized under ultraviolet light, by spraying the plates with an acetone solution of rhodamine or with 50% aqueous sulfuric acid and heating. Radiochromatograms were analyzed on a Packard Scanner (Model 7200).

Glass plates coated with silica gel containing 10% $AgNO_3$ or silica gel sheets (JB-F) impregnated with an $AgNO_3$[24] solution were used for thin-layer argentation chromatography.

GLC was carried out on a Hewlett-Packard Model 7620A instrument equipped with a flame ionization detector and a 6 ft by 2 mm column. The column was packed with 1% SE-30 on Chromosorb Q (100 mesh) support, and the chromatographic separations were carried out isothermally at 260 °C. The temperature of the injection port was 275 °C and of the flame ionization block 280 °C. The column was eluted with He 30 mL/min.

Counting. The samples were crystallized several times from the indicated solvents until they showed a constant specific activity of ^{14}C and constant ^3H:^{14}C ratio. Samples were counted on a Nuclear Chicago Mark II automatic liquid scintillation counter. The samples were dissolved in 10 mL of a scintillation solution of toluene containing 160 mL of Liquifluor (New England Nuclear; PPO–POPOP toluene concentrate) per 1 gal of toluene (spectral grade). The samples were counted until a minimum of 5000 counts of ^{14}C was accumulated. This assured a statistical error of less than 3% [except (*S*)-diacid **9**]. Quenching was determined with the use of ^{14}C and ^3H standards with known dpm.

Because only a small amount of the purified (*S*)-diacid **9** was available, two aliquots (1.030 mg and 1.125 mg) were twice counted for 100 min each on three different occasions. The ^{14}C and ^3H counts were scattered in the range of 10%. Therefore the determination of the ^3H:^{14}C ratio of the (*S*)-diacid **9** (Table I, entry 7) is less precise than the results for the other compounds.

(3*RS*,2*S*)-[2-^{14}C,2-^{3}H]- and (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]Mevalonic Acid Benzhydrylamides. A mixture of (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA (ca. 1.28×10^5 dpm of ^{14}C), cold MVA (90 mg), and benzhydrylamine (diphenylmethylamine) (0.2 mL) in benzene (5 mL) was stirred for 34 h at room temperature in a CO_2-free atmosphere. Ethyl ether (60 mL) was then added and the solution was washed with 1 N HCl (3 × 25 mL) and water, dried over $MgSO_4$, and evaporated. The obtained residue was purified by preparative TLC [silica gel; acetone–hexane (3:7)] to yield the (*S*)-amide (59 mg), which was crystallized (ethyl ether) to a constant specific activity and constant ^3H:^{14}C ratio.

A mixture of (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA (ca. 3.7×10^5 dpm), cold MVA lactone (259 mg), and benzhydrylamine (0.5 mL) in benzene (20 mL) was stirred (12 h) and processed as above to yield the (*R*)-amide (125 mg) which was counted as above. The results are given in Table I (entry 1).

Germination of Peas in the Presence of (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA and (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA. A solution of (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA (10 μCi of ^{14}C) in benzene (0.35 mL) was placed in a Petri dish (100 × 12 mm) and the solvent was removed in a gentle stream of N_2. Then, 45 peas were placed in the dish and water (5 mL) was added. The assembly was placed in a crystallizing dish (190 × 100 mm), covered with a glass plate, and stored at room temperature. When the MVA solution was absorbed (ca. 10 h), water was added, as needed. The peas started to germinate after 2 days and the experiment was terminated after 6 days, at which time the shoots were about 1.2–1.5 cm long.

An analogous experiment was carried out with (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA (10 μCi of ^{14}C) and peas (45) (6 days).

Isolation of Metabolites. The peas from both experiments were processed in an identical manner.

The seedlings (45-g wet weight) were ground with Na_2SO_4 (100 g), then methanolic KOH (30%, 600 mL) was added, and the mixture was stirred and refluxed (3 h) under N_2. After cooling, the solution was decanted and the residue was washed extensively with MeOH. The combined extract was diluted with water (1.3 L) and distilled until ca. 400 mL of distillate was collected.

The aqueous phase was extracted with ether (6 × 150 mL); the extract was washed, dried (Na_2SO_4), and concentrated to yield an unsaponifiable residue. The residue from the 2*R* experiment contained 7.33×10^6 dpm of ^{14}C. The residue from the 2*S* experiment contained 1.07×10^7 dpm of ^{14}C.

The unsaponifiable residues were resolved by TLC [silica gel; petroleum ether (bp 30–60 °C)] into three zones with mobilities similar to (a) squalene, (b) β-amyrin, and (c) sitosterol.

The products of the sitosterol zone were further purified by preparative TLC [silica gel; hexane–$CHCl_3$–MeOH (20:10:1)] into fractions with mobilities similar to (1) sterols, (2) pentacyclic triterpene monoalcohols, and (3) tetracyclic triterpene monoalcohols.

Processing of (*R*)- and (*S*)-Squalene Zones. The (*S*)-squalene fraction (2.75×10^5 dpm of ^{14}C) was diluted with cold squalene (5 mg) and rechromatographed (silica gel, petroleum ether). The recovered (*S*)-squalene was mixed with cold squalene (53.5 mg). Then *n*-butyl alcohol (1 mL) and a saturated methanolic solution of thiourea (3 mL) were added sequentially. The mixture was stored at 0–5 °C for 48 h and the squalene–thiourea adduct was recovered with hexane (30 mL). The hexane extract was washed with water (3 × 10 mL), dried (Na_2SO_4), and evaporated to yield (*S*)-squalene (47 mg). The (*S*)-squalene (43.5 mg) was dissolved in cold (0 °C) dry acetone saturated with HCl (4 mL), and dry HCl was bubbled through the cooled (0 °C) solution for 60 min. The acetone and HCl were removed in a stream of N_2 and the dark-brown residue was washed with cold ethanol (0.5 mL). The resulting solid was crystallized (three times, acetone) to yield (*S*)-squalene hexahydrochloride (23.7 mg) (Table I, entry 2).

The (*R*)-squalene fraction (4.74×10^5 dpm of ^{14}C) was processed

as described above to yield (*R*)-squalene hexahydrochloride (25 mg) (Table I, entry 2).

Sitosteryl Acetates [(*S*)-1 and (*R*)-2]. The (*S*)-sterols (4.56×10^6 cpm of ^{14}C) were acetylated (pyridine, 2 mL; Ac_2O, 2 mL; 18 h, room temperature) and the recovered steryl acetates were purified by TLC [silica gel; hexane–$CHCl_3$–MeOH (40:20:1)]. The obtained steryl acetates were resolved by argentation TLC [silica gel–10% $AgNO_3$; chloroform (freed of alcohol)] into three radioactive zones with mobilities similar to stigmastanyl acetate, sitosteryl acetate, and stigmasteryl acetate.

The (*S*)-sitosteryl acetate fraction was diluted (2 mg) with authentic compound and purified by argentation TLC in the same system. An aliquot of the recovered sitosteryl acetate (4%) (6.18×10^5 cpm of ^{14}C) was further diluted with sitosteryl acetate (48 mg) and crystallized (four times, MeOH–$CHCl_3$) to constant ^{14}C-specific radioactivities. The four times crystallized (*S*)-sitosteryl acetate was further purified via the 5,6-dibromide, but the ^{14}C-specific activity remained unchanged.

It is worthy of note that a significant drop of ^{14}C-specific activity was noted in first and second crystallizations; however, after the third and fourth crystallizations, the ^{14}C-specific activity remained constant and did not change following additional purification through the 5,6-dibromide.

Consequently, the bulk of (*S*)-sitosteryl acetate (1.61×10^5 dpm of ^{14}C) was diluted with cold sitosteryl acetate (2.002 g) and crystallized twice to yield 1.907 g of radiochemically homogeneous (*S*)-sitosteryl acetate (**1**).

The (*R*)-sterol fraction (4.6×10^6 cpm of ^{14}C) was processed in a similar manner to yield (*R*)-sitosteryl acetate (**2**) (797 mg). The results are given in Table I (entry 3).

Determination of the Presence and the Stereochemistry of Tritium Atoms at C-1 in (*R*)- and (*S*)-Sitosterols. 5α-Stigmast-3β-ol Acetates (3 and 4). The (*S*)-sitosteryl acetate (**1**) was hydrogenated (EtOAc; PtO_2–$HClO_4$) in two batches (550 and 930 mg) to yield (*S*)-5α-stigmastanol acetate (**3**). The conditions of the hydrogenations were as follows. For 1 g of sitosteryl acetate, 10 mL of EtOAc, 135 mg of PtO_2, and 27 μL of 70% $HClO_4$ were used.

The (*R*)-sitosteryl acetate (**2**) (792 mg) was hydrogenated in the same manner to yield (*R*)-5α-stigmastanol acetate (**4**). The samples were recrystallized and were processed in an identical manner: mp 136.5 °C; *m/e* 458 (M^+, 86), 398 (M − 60, 50), 383 [M − (60 + 15), 19], 276 (M − 182, 41), 275 (M − 183, 38), 230 (M − 228, 47), 216 (60), 215 (100); NMR spectrum 0.660 (s, 3 H, 18-CH_3, calcd 0.667), 0.824 (s, 3 H, 19-CH_3, calcd 0.825), 0.910 (d, J = 6.0 Hz, 3 H, 21-CH_3), 0.810 (d, J = 6.0 Hz, 3 H, 26-CH_3), 0.836 (d, J = 6.8 Hz, 3 H, 27-CH_3), 0.920 (d, J = 6.0 Hz, 3 H, 29-CH_3), 2.010 (s, 3 H, 3β-OAc), 4.68 (m, 1 H, 3α-H).

The (*S*)-5α-stigmast-3β-ol acetate (**3**) (300 mg) was treated with $LiAlH_4$ in ether to yield (*S*)-5α-stigmast-3β-ol (**3a**). Similarly, **4** (97 mg) gave the (*R*)-5α-stigmast-3β-ol (**4a**).

5α-Stigmast-3-ones [(*S*)-5 and (*R*)-6]. Jones reagent was added dropwise to a stirred solution of the (*S*)-5α-stigmast-3β-ol (**3a**) (185 mg) in acetone–ether (1:1; 20 mL), at room temperature, until the color persisted for 10 min. TLC [silica, methanol–benzene (1:4)] showed that the oxidation was completed. The reaction was terminated with methanol (5 drops). Water (40 mL) was added and the mixture was extracted with ether. The ether extract was washed with water, dried (Na_2SO_4), and concentrated to yield crude 5α-stigmast-3-one (**5**) (177 mg). The product was crystallized from ethanol.

An analogous treatment of **4a** (86 mg) gave (*R*)-5α-stigmast-3-one (**6**) (83 mg) which was crystallized. The results are summarized in Table I (entry 4).

The crystallized samples of **5** and **6** showed mp 158–160 °C; MS *m/e* 414 (M^+, 23), 399 (M − 15, 9), 317 (4), 273 (4), 247 (M − 167, 5.5), 246 (M − 168, 9), 233 (13), 232 (54), 231 (100), 217 (36); IR ν (KBr) 1715 cm^{-1} (3-ketone).

Base Equilibration of 5α-Stigmast-3-ones (5 and 6). The (*S*)-5α-stigmast-3-one (**5**) (20 mg) was dissolved in ether (2 mL), then methanolic KOH (5%, 20 mL) was added, and the mixture was refluxed (4 h) under N_2. Water (100 mL) was added and the sterol (**5**) was recovered (ether) and processed in the usual manner. The obtained residue was crystallized (EtOH, twice).

The ketone **6** (30 mg) was equilibrated and processed in a similar manner. The results are given in Table I (entry 5).

5α-Stigmast-1-en-3-ones (7 and 8). A mixture of 5α-stigmast-3-one (**5**) (120 mg), dichlorodicyanobenzoquinone (DDQ) (120 mg), and freshly purified dioxane (15 mL) was refluxed for 16 h under N_2.[25] The reaction was monitored by argentation TLC (chloroform; freed of alcohol) and it was terminated when, in addition to the required 5α-stigmast-1-en-3-one, a diene appeared. The mixture was diluted with ether (100 mL), washed with 1 N NaOH and water, and dried and the solvent evaporated. The residue was fractionated by preparative argentation TLC (chloroform; freed of alcohol) and crystallized (MeOH–$CHCl_3$) to yield homogeneous 5α-stigmast-1-en-3-one (**7**) (14 mg).

The recovered 5α-stigmast-3-one (**5**) (67 mg) was reprocessed as described above to yield an additional 19 mg of **7**.

From **6** (80 mg), following a similar treatment, 5α-stigmast-1-en-3-one (**8**) (31 mg) was obtained. The results are given in Table I (entry 6).

The crystallized samples of **7** and **8** showed mp 140–141 °C; MS *m/e* 412 (M^+, 100), 372 (M − 40, 22), 371 (M − 41, 61), 328 (M − 84, 55), 315 (M − 97, 30), 292 (32), 271 (34), 245 (25), 230 (33), 229 (53); ν (KBr) 1680 cm^{-1}; λ (MeOH) 233 nm (ε 9850); NMR spectrum 0.705 (s, 3 H, 18-CH_3), 1.010 (s, 3 H, 19-CH_3), 0.928 (d, J = 6.0 Hz, 3 H, 21-CH_3), 0.823 (d, J = 6.5 Hz, 3 H, 26-CH_3), 0.844 (d, J = 6.8 Hz, 3 H, 27-CH_3), 0.920 (d, J = 6.0 Hz, 3 H, 29-CH_3), 5.83 (10.0, 2-H), 7.12 (10.0, 1-H).

1,3-Seco-2-nor-5α-stigmasta-1,3-dioic Acids (9 and 10). 5α-Stigmast-1-en-3-one (**7**) (21 mg) was diluted with cold 5α-stigmast-1-en-3-one (14 mg).

Ruthenium tetraoxide was generated by stirring a suspension of ruthenium dioxide (6.6 mg) in acetone with an aqueous solution of metaperiodic acid (53 mg in 4–5 drops of water). To the stirred yellow-brown solution of ruthenium tetraoxide, a solution of 5α-stigmast-1-en-3-one (**7**) (35 mg) in acetone (2 mL) was added dropwise. The yellow-brown color of the solution was maintained by the dropwise addition of a 12.5% solution of metaperiodic acid in acetone–water (1:1) as needed.[26] After 5 h, the reaction was terminated with 2-propanol (0.5 mL) and the stirring was continued for 2 h. The mixture was filtered (Celite) and the filtrate was concentrated in a stream of N_2. The residue was fractionated by TLC (in the above indicated system; developed twice). The seco-acid **9** (18 mg) was recovered with ether. Similar treatment of **8** (35 mg) gave the seco-acid **10** (18 mg) (Table I, entry 7).

The samples were crystallized (twice, hexane–EtOAc) and showed mp 219–222 °C; MS *m/e* 430 (M − 18, 7), 404 (M − 44, 7), 403 (M − 45, 17), 402 (M − 46, 46), 388 (M − 60, 11), 359 (M − 89, 11), 261 (25), 235 (29), 234 (55), 221 (83), 220 (100), 219 (55); NMR spectrum 0.665 (s, 3 H, 18-CH_3), 1.273 (s, 3 H, 19-CH_3), 0.970 (d, J = 7.5 Hz, 3 H, 21-CH_3), 0.828 (d, J = 6.5 Hz, 3 H, 26-CH_3), 0.845 (d, J = 7.0 Hz, 3 H, 27-CH_3).

Determination of the Presence and the Stereochemistry of Tritium at C-7 in Sitosterols (1a and 2a). A mixture of sitosteryl acetate (**1**) (300 mg), $LiAlH_4$ (300 mg), and ether (10 mL) was stored (2 h) at room temperature. The mixture was worked up in the conventional manner and crystallized to constant specific radioactivity to yield (*S*)-sitosterol (**1a**) (257 mg).

Similarly, (*R*)-sitosteryl acetate (**2**) (20 mg) was diluted with an authentic sample (180 mg) and treated with $LiAlH_4$, as described above, to yield (*R*)-sitosterol (**2a**) (174 mg) (Table I, entry 8).

5α-Hydroperoxystigmast-6-en-3β-ols (11 and 12). A mixture of **1a** (257 mg), pyridine (26 mL), and hematoporphyrin (16 mg) was irradiated with a Hanovia mercury (500) lamp placed 2–3 cm from the wall of a Pyrex reaction vessel.[9,27] During the irradiation, a slow stream of oxygen was bubbled through the solution. The course of the reaction was monitored by TLC [silica; methanol–benzene (1:4)] on aliquots removed at 1-h intervals. When the sitosterol was consumed (ca. 3 h), the reaction was terminated, ether (100 mL) and Norit (300 mg) were added, and the mixture was stirred (30 min). The solid was removed by filtration and the filtrate was first distilled on a rotatory evaporator. The remaining pyridine was removed in a stream of N_2. The residue was dried under reduced pressure and the crude brown (*S*)-5α-hydroperoxide (**11**) (240 mg) was crystallized from methanol (**11**). An aliquot (87 mg) was recrystallized (three times, MeOH) and counted.

The (*R*)-**2a** (174 mg) was treated in a similar manner to yield (*R*)-hydroperoxide **12** (168 mg). An aliquot (64 mg) was crystallized (three times, MeOH) (Table I, entry 9).

The recrystallized samples of **11** and **12** showed mp 119–121 °C; MS *m/e* 446 (M^+, 12), 431 (M − 15, 19), 430 (M − 16, 39), 429 (M − 17, 91), 416 (M − 30, 27), 415 (M − 31, 59), 414 (M − 32, 55),

Table II. Specific Activities of ^{14}C and ^{3}H:^{14}C Ratios of Metabolites and Their Transformation Products (See Text)[a]

Entry	Compound	Odd numbered compounds, (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA			Even numbered compounds, (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA		
		^{14}C specific act.	^{3}H:^{14}C ratio		^{14}C specific act.	^{3}H:^{14}C ratio	
			Isotopic	Atomic		Isotopic	Atomic
1	MVA amide		3.14	1.00:1		9.97	1.00:1
2	Squalene 6HCl		2.01	6.00:6		5.16	6.00:6
3	Sitosteryl acetate	2.94	1.26	3.13:5	3.26	5.15	4.99:5
4	5α-Stigmastanyl acetate	2.90	1.22	3.03:5	3.22	5.18	5.03:5
5	5α-Stigmast-14-en-3β-ol acetate	2.89	1.17	2.91:5	3.22	4.54	4.40:5
6	5α-Stigmasta-3β,14α,15β-triol 3-acetate	2.92	1.19	2.96:5	3.25	4.53	4.39:5
7	3β,14α-Dihydroxy-5α-stigmast-15-one 3-acetate	2.90	1.16	2.89:5	3.23	4.49	4.35:5

[a] The results in Tables I and II are the average of at least three crystallizations in which the ^{14}C specific activity and ^{3}H:^{14}C remained constant (±3%). ^{14}C specific activity × 10^4 dpm per mmol. The MVA and squalene were counted as benzhydrylamide and hexahydrochloride, respectively. The results are significant to ±3%. The atomic ratios are calculated on the basis of the atomic ratio of squalene (6-^{3}H:6-^{14}C).

413 (M − 33, 100), 400 (M − 46, 27), 399 (M − 47, 71), 398 (M − 48, 23), 397 (M − 49, 22), 396 (M − 50, 22), 386 (M − 60, 26); ν (KBr) 3420 (3β-OH), 3220 cm^{-1} (5α-OOH).

3β-Hydroxystigmast-5-en-7-ones (13 and 14). The (*S*)-5α-hydroperoxystigmast-6-en-3β-ol (**11**) (40 mg) was dissolved in pyridine (1 mL) containing 350 μg of $CuCl_2 \cdot 2H_2O$, and the mixture was stored at ambient temperature. The reaction was monitored by TLC [silica, benzene–methanol (4:1)] and was terminated after 24 h. The pyridine was removed in a stream of N_2; the residue was dried in vacuo, dissolved in ether (5 mL), and filtered through Celite. The crude 3β-hydroxy-(*S*)-stigmast-5-en-7-one (39 mg) was crystallized (three times, methanol) to yield homogeneous **13** (25 mg).

From (*R*)-hydroperoxide **12** (50 mg), 3β-hydroxy-(*R*)-stigmast-5-en-7-one (**14**) (25 mg) was obtained (Table I, entry 10).

The ketones **13** and **14** showed mp 136 °C; MS *m/e* 428 (M^+, 100), 413 (M − 15, 12), 396 (M − 32, 13), 395 [M − (15 + 18), 28], 287 (M − 141, 33), 247 (M − 181, 24), 245 (21), 205 (64); ν (KBr) 3410 (3β-OH), 1660 cm^{-1} (7-ketone); λ (MeOH) 239 nm (ε 13 040); NMR 0.685 (s, 3 H, 18-CH_3), 1.198 (s, 3 H, 19-CH_3), 0.923 (d, *J* = 6.5 Hz, 3 H, 21-CH_3), 0.814 (d, *J* = 6.8 Hz, 3 H, 26-CH_3), 0.835 (d, *J* = 6.5 Hz, 3 H, 27-CH_3), 0.915 (d, *J* = 6.0 Hz, 3 H, 29-CH_3), 3.62 (m, 1 H, 3α-H), 4.64 (6-H).

Determination of the Presence and the Stereochemistry of Tritium at C-15. 5α-Stigmast-14-en-3β-ol Acetates (15 and 16). Stigmastanyl acetates **3** and **4** (Table II, entry 4) were used. Benzene was purified by stirring with concentrated sulfuric acid for 48 h and decanted. Prior to use the benzene was distilled from P_2O_5 through a 60-cm long fractionation column filled with glass turnings. The stigmastanyl acetate **3** (870 mg) was dissolved in freshly purified benzene (203 mL) and flushed with N_2 for 10 min. Then, freshly crystallized $C_6H_5ICl_2$ (523 mg) was added and the bubbling of dry nitrogen was continued (15 min). The mixture was irradiated with two sun lamps (Sylvania, 275 W) while nitrogen was passed.[28,29] When the color of the solution turned from yellow to pinkish (ca. 15 min), the reaction was terminated and the benzene solution was concentrated to ca. 10 mL. Acetone (100 mL) and powdered $AgClO_4$ (ca. 400 mg) were added and the mixture was stirred (15 min). The solid was removed by filtration (Celite) and the treatment with $AgClO_4$ was repeated twice more. Subsequently, a saturated aqueous solution of Na_2CO_3 (15 mL) was added to the acetone solution and the brown solid was removed by filtration (Celite). The filter cake was extensively washed with acetone and then with ether. This treatment was also repeated twice more.

The filtrate was concentrated by distillation and the aqueous–oily residue was extracted with ether. The combined ether extract was washed, dried (Na_2SO_4), and concentrated. The residue was fractionated by argentation TLC [silica gel–10% $AgNO_3$; chloroform (freed of alcohol)], and the 5α-stigmast-14-en-3β-ol acetate was separated from 5α-stigmast-9(11)-en-3β-ol acetate, sitosteryl acetate, and stigmastanyl acetate. The recovered "14-ene" was further purified by sequential argentation TLC in two solvent systems: (1) hexane–ethyl acetate (85:15); (2) chloroform (freed of alcohol).

From the (*S*)-**3** (870 mg), 5α-stigmast-14-en-3β-ol acetate (**15**) (138.5 mg), 5α-stigmast-9(11)-en-3β-ol acetate (113 mg), and starting material **3** (435 mg) were obtained. The recovered **3** was reprocessed in the same manner to yield an additional amount of the "14-ene" **15** (51 mg). The obtained (*S*)-14-ene **15** (189 mg) was further purified by argentation TLC to yield homogeneous **15** (134 mg).

The (*R*)-**4** (792 mg) was processed in a similar manner to yield the (*R*)-14-ene **16** (160 mg) and 5α-stigmast-9(11)-en-3β-ol (105 mg). The 14-enes **15** and **16** were crystallized and counted (Table II, entry 5).

The 14-ene acetates **15** and **16** were difficult to crystallize. The product obtained after several crystallizations (three times, MeOH) showed mp 88–89 °C; MS *m/e* 456 (M^+, 16), 441 (M − 15, 10), 394 (7), 381 (6), 344 (7), 317 (8), 316 (M − 140, 48), 315 (M − 141, 100), 314 (M − 142, 15), 256 [M − (140 + 60), 20], 255 [M − (141 + 60), 93]; NMR 0.890 (s, 3 H, 18-CH_3, calcd 0.917), 0.838 (s, 3 H, 19-CH_3, calcd 0.833), 0.860 (d, *J* = 6.5 Hz, 3 H, 21-CH_3), 0.804 (d, *J* = 6.5 Hz, 3 H, 26-CH_3), 0.826 (d, *J* = 6.8 Hz, 3 H, 27-CH_3), 2.000 (s, 3 H, 3β-OAc), 4.69 (m, 1 H, 3α-H).

5α-Stigmast-14α,15α-epoxy-3β-ol Acetates (17 and 18). A solution of the (*S*)-5α-stigmast-14-en-3β-ol acetate (**15**) (126 mg) in $CHCl_3$ (1.95 mL) was added to a solution of *m*-chloroperbenzoic acid (89 mg) in $CHCl_3$ (1.80 mL). The mixture was stirred at 22 °C in the dark and the reaction was followed by TLC [hexane–EtOAc (19:1)]. When the epoxidation was nearly completed (30 min) ether (50 mL) was added and the mixture was worked up in the conventional manner to yield the crude 5α-cholest-14α,15α-epoxy-3β-ol acetate (**17**). The product was purified by TLC [silica gel; hexane–EtOAc (23:2)] to yield the homogeneous epoxide **17** (98 mg).

From the (*R*)-5α-stigmast-14-en-3β-ol acetate (**16**) (141 mg) following a similar treatment, (*R*)-5α-stigmast-14α,15α-epoxy-3β-ol acetate (**18**) (103 mg) was obtained: mp 150–152 °C; *m/e* 472 (M^+, 21), 457 (M − 15, 10), 454 (M − 18, 18), 412 (M − 60, 7), 379 [M − (60 + 18 + 15), 13], 341 (M − 131, 10), 332 (M − 140, 36), 331 (M − 141, 100), 317 [M − (140 + 15), 14], 313 [M − (141 + 18), 18], 293 (M − 179, 19), 292 (M − 180, 64); NMR 0.855 (s, 3 H, 18-CH_3, calcd 0.850), 0.855 (s, 3 H, 19-CH_3, calcd 0.850), 0.813 (d, *J* = 6.5 Hz, 3 H, 26-CH_3), 0.833 (d, *J* = 6.5 Hz, 3 H, 27-CH_3), 1.990 (s, 3 H, 3β-OAc), 4.66 (m, 1 H, 3α-H), 3.30 (15β-H).

5α-Stigmasta-3β,14α,15β-triol 3-Acetates (19 and 20). A mixture of (*S*)-5α-stigmast-14α,15α-epoxy-3β-ol acetate (**17**) (98 mg), acetone (3.1 mL), water (1.65 mL), and periodic acid (50 mg) was stirred at room temperature. The reaction was monitored by TLC [hexane–EtOAc (4:1)] and when the bulk of the starting material was consumed, the reaction was terminated (2 h). The acetone was removed in a stream of N_2 and ether (1 mL) was then added. The obtained solution was fractionated by preparative TLC [hexane–EtOAc (4:1)] to yield (*S*)-5α-stigmasta-3β,14α,15β-triol 3-acetate (**19**) (53 mg). The product was crystallized from hexane–benzene and counted (Table II, entry 6).

From the (*R*)-5α-stigmast-14α,15α-epoxy-3β-ol acetate (**18**) (92 mg) following a similar treatment, (*R*)-5α-stigmasta-3β,14α,15β-triol 3-acetate (**20**) (58 mg) was obtained (Table II, entry 6).

The recrystallized **9** and **10** showed mp 160–162 °C; *m/e* 472 (M − 18, 3), 359 (M − 131, 5.5), 332 [M − (140 + 18), 8], 331 [M − (141 + 18), 18], 306 (6.5), 305 (M − 185, 31), 293 (23), 292 (100),

253 (20), 237 (9), 233 (17), 217 (9.5), 216 (31), 215 (25); NMR 0.783 (s, 3 H, 18-CH_3, calcd 1.111), 0.995 (s, 3 H, 19-CH_3, calcd 0.858), 0.925 (d, J = 6.0 Hz, 3 H, 21-CH_3), 0.818 (d, J = 6.5 Hz, 3 H, 26-CH_3), 0.828 (d, J = 6.5 Hz, 3 H, 27-CH_3), 0.905 (d, J = 6.5 Hz, 3 H, 29-CH_3), 2.000 (s, 3 H, 3β-OAc), 4.68 (m, 1 H, 3α-H), 4.04 (15α-H).

3β,14α-Dihydroxy-5α-stigmast-15-one 3-Acetates (21 and 22). A solution of 5α-stigmasta-3β,14α,15β-triol 3-acetate (**19**) (40 mg) in pyridine (0.4 mL) was added to a complex prepared from CrO_3 (40 mg) and pyridine (0.4 mL). The mixture was stored at room temperature and the progress of the reaction was followed by TLC [hexane–EtOAc, (17:5)]. When the reaction was completed (2 h), ether (1 mL) was added and the mixture was fractionated by preparative TLC [silica; hexane–EtOAc (4:1)]. The recovered (*S*)-3β,14α-dihydroxy-5α-stigmast-15-one 3-acetate (**21**) (32 mg) was crystallized from MeOH–$CHCl_3$.

The (*R*)-5α-stigmasta-3β,14α,15β-triol 3-acetate (**20**) (45 mg) was oxidized in the same manner to yield 3β,14α-dihydroxy-(*R*)-5α-stigmast-15-one 3-acetate (**22**) (40 mg) (Table II, entry 7).

The recrystallized **11** and **12** showed mp 146–148 °C; MS *m/e* 488 (M^+, 14), 470 (M − 18, 5), 445 (M − 43, 14), 347 (M − 141, 6), 329 [M − (141 + 18), 10], 294 (M − 194, 22), 293 (M − 195, 100), 292 (M − 196, 100), 253 (M − 235, 100); NMR 0.788 (s, 3 H, 18-CH_3, calcd 0.859), 1.020 (s, 3 H, 19-CH_3, calcd 0.833), 0.938 (d, J = 6.5 Hz, 3 H, 21-CH_3), 0.810 (d, J = 6.5 Hz, 3 H, 26-CH_3), 0.825 (d, J = 6.5 Hz, 3 H, 27-CH_3), 0.920 (d, J = 6.0 Hz, 3 H, 29-CH_3), 2.000 (s, 3 H, 3β-OAc), 4.68 (m, 1 H, 3α-H).

5α-Stigmast-9(11)-en-3β-ol Acetate. The *S* and *R* samples of 5α-stigmast-9(11)-en-3β-ol acetate were isolated in the course of chromatographic separation of the *S* and *R* samples of 5α-stigmast-14-en-3β-ol acetates. The recovered "9(11)-ene's" were further purified by argentation TLC in chloroform (freed of alcohol).

The samples were crystallized from methanol–chloroform and showed mp 118–119 °C; MS *m/e* 456 (M^+, 61), 441 (M − 15, 16), 382 (M − 74, 24), 381 [M − (60 + 15), 59], 315 (M − 141, 17), 288 (M − 168, 23), 255 [M − (141 + 60), 43], 229 (40), 214 (30), 213 (100); NMR 0.590 (s, 3 H, 18-CH_3, calcd 0.600), 0.960 (s, 3 H, 19-CH_3, calcd 0.967), 0.908 (d, J = 6.5 Hz, 3 H, 21-CH_3), 0.815 (d, J = 6.5 Hz, 3 H, 26-CH_3), 0.835 (d, J = 6.5 Hz, 3 H, 27-CH_3), 0.913 (d, J = 6.0 Hz, 3 H, 29-CH_3), 2.010 (s, 3 H, 3β-OAc), 4.68 (m, 1 H, 3α-H), 5.24 (11-H).

Results and Discussion

We have indicated in the introductory section that the purpose of the present study was to compare certain mechanistic aspects of the transformations of lanosterol to cholesterol in rat livers with those of cycloartenol to sitosterol in the germinating pea. Specifically, we wished to define the incorporation and the stereochemistry of tritium atoms at C-1, -7, and -15 of sitosterol biosynthesized in the germinating pea from samples of mevalonic acid labeled stereospecifically with tritium at C-2.

With this in mind, (3*RS*,2*S*)-[2-^{14}C,2-^{3}H]-MVA (10 μCi of ^{14}C; ^{3}H:^{14}C ratio 3.14) and (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]-MVA (10 μCi of ^{14}C; ^{3}H:^{14}C ratio 10.0) were incubated with germinating peas. After 6 days, the peas were processed and the recovered *S* and *R* metabolites were purified to yield homogeneous (*S*)- and (*R*)-squalenes (6HCl) and (*S*)- (**1**) and (*R*)- (**2**) sitosteryl acetates (Table I, entries 2 and 3). A very large drop of the ^{3}H:^{14}C ratio between the (*S*)- and (*R*)-MVA amides and the (*S*)- and (*R*)-squalenes (6HCl), respectively, may be noticed (Table I, entries 1 and 2).

The observed large loss of tritium is in sharp contrast to our results on the biosynthesis of yeast sterols[29,30] and the in vivo biosynthesis of sterols in the brains of rats raised and maintained on isocaloric diets containing 8 and 25% casein.[31] It should be pointed out that all these studies (yeast homogenates, rat brains, germinating peas, petals of *C. officinalis*, etc.) were carried out with aliquots of (3*RS*,2*R*)-[2-^{3}H]-MVA from the same batch (pool).

The biosynthesis of (*R*)-squalene in yeast homogenates proceeded with the loss of less than 5% tritium. The losses of tritium in the formation of (*R*)-squalenes in the brains of rats derived from the 8 and 25% casein diets were 5 and 2%, respectively.

Similar aliquots of (3*RS*,2*S*)-[2-^{3}H]-MVA from the same batch (pool) were used in the studies of the in vivo biosynthesis of fusidic acid in *F. coccineum*[32] and in germinating peas.[33] While the in vivo biosynthesis of (*S*)-fusidic acid by *F. coccineum* involved the loss of 12% tritium, the biosynthesis of (*S*)-squalene in germinating peas proceeded with the loss of 36% tritium.

The *large* losses of tritium noted in the germinating peas and in the petals of *C. officinalis*[33] are difficult to explain by the proposed mechanism.[34]

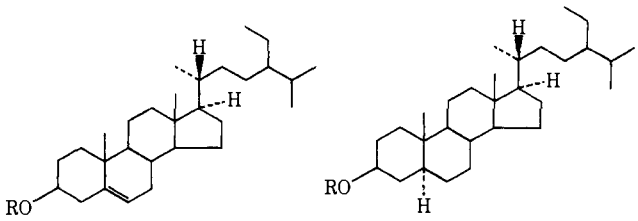
For the determination of the tritium content at C-1 of the (*S*)- (**1**) and (*R*)- (**2**) sitosteryl acetates, the samples were hydrogenated and the resulting acetates **3** and **4** were saponified ($LiAlH_4$) to yield after oxidation the 5α-stigmastan-3-ones. The outlined sequence of transformations from sitosteryl acetates to the 5α-stigmastanones **5** and **6** proceeded without loss of tritium. Similarly, no loss of tritium was observed when the ketones were equilibrated with base, indicating the absence of tritium at C-2 (and -4) of the (*S*)- and (*R*)-sitosterols, as expected.

The stigmastanones **5** and **6** were dehydrogenated with DDQ to yield the 5α-stigmast-1-en-3-ones **7** and **8**, respectively. It was shown that this dehydrogenation involves the removal mainly of the axial 1α- and 2β-hydrogen atoms.[25,35,36] For the determination of the tritium remaining at C-1, the conjugated ketones **7** and **8** were oxidized[26] with RuO_4 and the resulting 1,3-seco-2-nor acids **9** and **10** were isolated. The results of the transformations are summarized in Table I (entries 4–7). It can be noticed that introduction of the C-1 double bond in the (*S*)-3-ketone (**5**) and the subsequent cleavage of the resulting conjugated (*S*)-1-en-3-one (**7**) to the (*S*)-1,3-seco-2-nor-1,3-dioic acid (**9**) involved the loss of 0.67 and 0.24 atoms of tritium, respectively. However, it should be recalled that the determination of the ^{3}H:^{14}C ratio of the (*S*)-diacid (**9**) was considerably less precise (see the Experimental Section). Therefore the evaluation of the amount of tritium (if any) at the 1β position requires confirmation. A similar sequence of transformations in the *R* series **6** to **8** and then to **10** involved the loss of 0.1 and 0.76 atoms of tritium, respectively.

For the determination of the tritium content at C-7, the (*S*)- (**1a**) and (*R*)- (**2a**) sitosterols were converted photochemically to the 5α-hydroperoxides **11** and **12**. This reaction involves the stereospecific abstraction of the 7α-hydrogen atom.[9,27] When the (*S*)-sitosterol (**1a**) was converted first to the 5α-hydroperoxide **11** and this, in turn, was rearranged to the (*S*)-7-ketone **13**, the ^{3}H:^{14}C ratio remained essentially unchanged (Table I, entries 8–10). The transformation of the (*R*)-(**2a**) to the 5α-hydroperoxide **12** likewise proceeded without loss of tritium (Table I, entries 8 and 9). However, rearrangement of **12** to the (*R*)-7-ketone **14** involved the loss of 0.8 atom of tritium (Table I, entries 9 and 10).

The results show that, in the course of the elaboration of sitosterol from cycloartenol, a hydrogen derived from 2-pro-*S* of MVA is lost from C-7. Also, the hydrogen atom retained at C-7 and derived from 2-pro-*R* of MVA, which in protosterols[37] and cycloartenol has the 7α configuration, was inverted and has the 7β configuration in sitosterol. These observations are consistent with the view that the biosynthesis of sitosterol proceeds via an intermediate having a C-7 double bond.[1,2] The loss of a hydrogen atom derived from 2-pro-*S* of MVA from C-7 of a precursor most likely occurs during the formation of the double bond. The C-7 double bond is subsequently reduced via the acquisition of 7α- and 8β-hydrogen[9,38] atoms and this accounts for the inversion of the configuration of the hydrogen atom retained at C-7, derived from 2-pro-*R* of MVA.

We now focused our attention on the transformations at



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3, R = Ac
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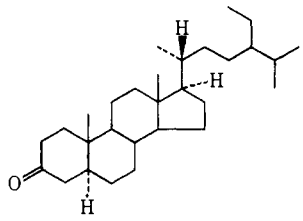

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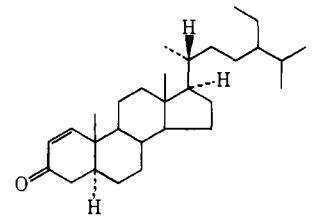

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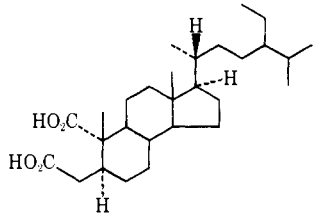

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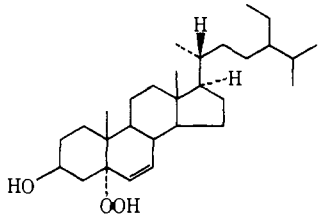

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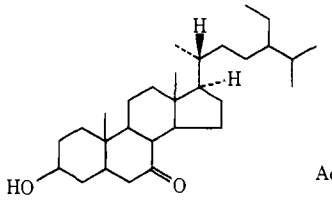

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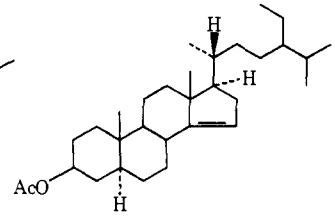

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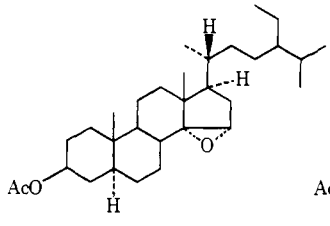

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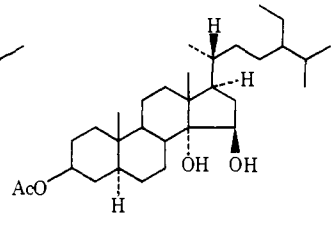

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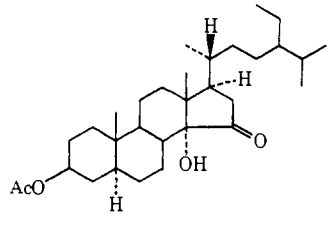

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C-15. We have proven that the overall process of the photochemical dehydrogenation[28] of 5α-cholestanol acetate to 5α-cholest-14-en-3β-ol acetate in the presence of $C_6H_5ICl_2$ is equivalent to the stereospecific abstraction of the cis-14α- and 15α-hydrogen atoms.[29,39] It may be assumed with certainty that the conversions of 5α-stigmastanol acetates **3** and **4** to 5α-stigmast-14-en-3β-ol acetates **15** and **16** will also involve the removal of the 14α- and 15α-hydrogen atoms.

When the (*S*)-**3** was converted photochemically to the (*S*)-5α-stigmast-14-en-3β-ol (**15**), no change in the ^3H:^{14}C ratio was observed, indicating the absence of isotopic hydrogen at the 15α position (Table II, entries 4 and 5). Subsequent transformations of the (*S*)-14-stigmastanol (**15**) via the epoxide **17** and the 14α,15β-diol **19** to the (*S*)-3β,14α-dihydroxy-5α-stigmast-15-one 3-acetate (**21**) also proceeded without loss of tritium (Table II, entries 5–7). In contrast, the dehydrogenation of the (*R*)-stigmastanol acetate (**4**) to the (*R*)-14-olefin **16** involved the loss of 0.6 atom of tritium (Table II, entries 4 and 5).[40] No additional loss of tritium was observed in the course of the transformations of the (*R*)-olefin to (*R*)-epoxide **18,** (*R*)-14α,15β-diol **20,** and, finally, to the (*R*)-14α-hydroxy-15-ketone **22.**

It is apparent that, while the (*S*)-sitosterol is devoid of tritium at C-15, the (*R*)-sitosterol has a 15α-tritum atom. It may, therefore, be inferred that the elaboration of sitosterol from cycloartenol involves an intermediate having a 14(15) double bond, for example, a 8(9),14(15)-diene. The formation of the C-14 double bond apparently proceeds via the loss of a hydrogen derived from the 2-pro-*S* of MVA from C-15 of a precursor. It should be noted that the 2-pro-*R* hydrogen (tritium) atom of MVA retained at C-15 also undergoes an inversion of configuration from the 15β configuration in prostosterol[37] and cycloartenol to the 15α configuration in sitosterol. This is consistent with the view that the reduction of the 14(15) double bond involves the acquisition of 14α- and 15β-hydrogen atoms.

Our results indicate that the overall outcome of the biosynthetic transformation at C-1, -7, and -15 in the elaboration of cholesterol from lanosterol in rat liver preparations and of sitosterol from cycloartenol in the germinating peas is the same. A similar pattern of transformation was observed in petals of *C. officinalis.*[33] Therefore, it is likely that the overall sequence of transformations of the tetracyclic moiety in the pea, *C. officinalis,* rat livers, and brains[31] follows similar routes.

Acknowledgment. This work was supported by NIH Grant AM 12156.

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"Inverse Substrates" for Trypsin. Efficient Enzymatic Hydrolysis of Certain Esters with a Cationic Center in the Leaving Group[1]

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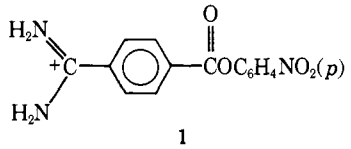
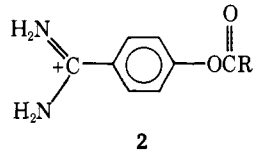
Abstract: The kinetics of the reaction of trypsin with several esters derived from *p*- and *m*-amidinophenol have been studied. These esters are characterized by their linkage, i.e., the specific group for the enzyme (charged amidinium) is not involved in their carbonyl groups but in the leaving portion. Esters of *p*-amidinophenol were demonstrated to undergo efficient and specific tryptic hydrolysis whereas the meta derivatives exhibited only low reactivity. The selectivity and efficiency of the *p*-amidinophenyl esters were compared to those of normal-type substrates, and it was concluded that these esters are almost comparable to normal type substrates both in binding and in acylation. A slow deacylation process was also demonstrated. The process may be governed by the inherent nature of nonspecific acyl residues although it had been introduced specifically. All these enzymatic processes were found identical with those of substrates with normal linkages, together with the effects of competitive inhibitor. These compounds were named "inverse substrates" with respect to these properties. A facile procedure for the preparation of acyl-enzymes carrying nonspecific residue was described and the potential usefulness of the "inverse substrate" concept was discussed.

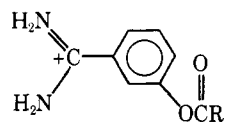
It is generally believed that only esters and amides containing a specificity-determining residue in their carbonyl portions function as good substrates of the endopeptidase, trypsin, and chymotrypsin.[2] Very few attempts have been made to question this empirical rule to which exceptions have not yet been found.[3] Previously, we have reported that the esters of *p*-amidinobenzoic acid (**1**) were efficiently hydrolyzed by trypsin.[4] In an extension of that investigation we have found that several acyl derivatives of *p*-hydroxybenzamidine (**2**), which are related "inverted" types of ester substrates having an amidine function in the leaving group, were hydrolyzable as well by trypsin.[5] At the same time Markwardt et al.[6] reported the inhibitory action of a series of *p*-amidinophenyl benzoates on trypsin and thrombin as well as chymotrypsin. These observations both suggest that the compounds in which the arrangement of the site-specific group is of a type "inverse" to that of the normal substrates may also act as specific substrates if they are properly designed to fit the active site.



1



2



3

a, R = CH_3; **b**, R = $C(CH_3)_3$; **c**, R = C_6H_5

In order to confirm whether these "inverse" esters are "true substrates" or not, a series of aliphatic esters of *p*-amidinophenol (**2a** and **2b**) and *m*-amidinophenol (**3a** and **3b**) has been prepared, and detailed kinetic studies on the individual steps of their tryptic hydrolysis were conducted. Benzoyl esters (**2c** and **3c**) are also included for comparison. In this work we wish to present evidence which indicates that these esters **2** behave like the normal substrates and may be regarded as "inverse substrates".

Results and Discussion

Kinetic parameters for the trypsin-catalyzed hydrolysis of the amidinophenol esters are listed in Table I. The acylation rate constants, k_2, and Michaelis constants, K_s, were obtained by following the formation of the hydrolysis products, the amidinophenols, which absorb at longer wavelength than the substrates. As shown in Table I, all the compounds in the series **2** and **3** were found to have a strong affinity to trypsin, with K_s values ranging around 10^{-5} M. These K_s values are comparable to those for normal-type specific substrates, e.g., *p*-nitrophenyl amidinobenzoate (**1**) and *p*-nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate (ZLysONP)[7] whose parameters are shown in Table II. In contrast, the K_s value for the nonspecific substrate, *p*-nitrophenyl acetate (AcONP),[8] is approximately three orders of magnitude larger than those of **2** and **3**.

The rates of the acylation of trypsin are strongly dependent on the nature of the leaving groups of the substrates. Since the Hammett σ_p values for nitro and amidino substituents are not markedly different, the acylation rates for **2a–c** can be reasonably compared with those for normal substrates which have