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Biological Properties of 20-Isocholesterol

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(20*S*)-Cholest-5-en-3β-ol(20-isocholesterol) was chemically prepared from bisnorcholenic acid. Incubation of cytochrome P-450$_{scc}$ with 20-isocholesterol produced no pregnenolone. 20-Isocholesterol did not satisfy the silkworm sterol requirement. Liposomes containing cholesterol or 20-isocholesterol behaved similarly when examined either by differential scanning calorimetry, or for permeation of carboxyfluorescein from the liposomes.

Keywords——20-isocholesterol; cytochrome P-450$_{scc}$; insect sterol; liposome; differential scanning calorimetry

Cholesterol is an ubiquitous sterol in nature. In living systems, cholesterol functions as a biogenetic precursor of steroid hormones and bile acids. Another important role of cholesterol is as an architectural component of cell membranes, endowing them with appropriate structure and functions.[1] The chemical structure of cholesterol should be intimately related to these biological functions. As part of our continuing studies on sterol structure–biological function correlation,[2-4] we have now examined the influence of alteration in the stereochemistry at C-20. For this purpose, 20-isocholesterol, which has the opposite configuration to cholesterol at C-20, was chemically prepared and some of its biological properties were compared with those of cholesterol.

Synthesis of 20-isocholesterol (**7**) was carried out according to the following Chart 1.

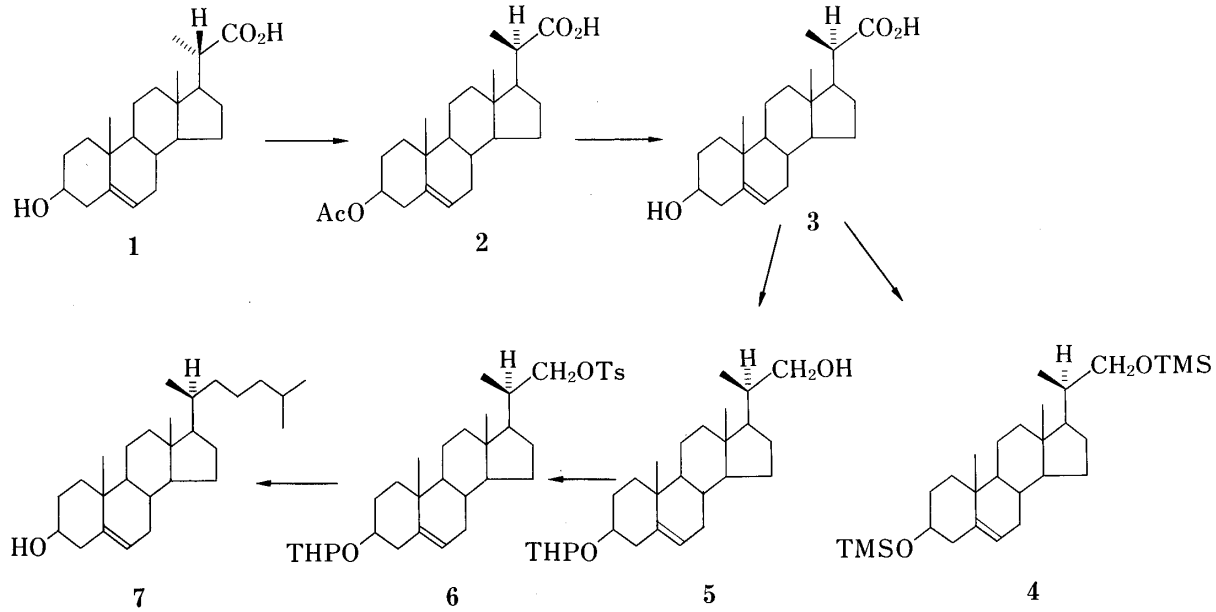


Chart 1

The starting compound (20*R*)-acid **2** has recently been prepared[5] from the commercially available (20*S*)-bisnorcholenic acid (**1**) through base-catalyzed isomerization of the cor-

responding aldehyde derivative followed by chromatographic resolution on Sephadex LH-20. The acetate **2** was saponified with methanolic KOH to give the alcohol **3**. The absence of epimerization during this process was indicated by gas chromatography-mass spectrometry (GC-MS) analysis of the bistrimethylsilyl ether **4** prepared from **3** by $LiAlH_4$-reduction followed by treatment with trimethylsilylimidazole. Thus, GC showed a single peak at 4.2 min, without any peak at 4.9 min corresponding to the (20*S*)-isomer which was derived from the (20*S*)-acid **1** in the same manner. Their MS, including *m/z* 476 (M^+), were indistinguishable from each other. The tetrahydropyranyl ether of the acid **3** was reduced with $LiAlH_4$ to yield the 22-alcohol **5**, which was then converted into the corresponding *p*-toluenesulfonate **6**. According to the previously described method,[6] this tosylate **6** was coupled with isoamylmagnesium bromide in the presence of Li_2CuCl_4 to afford, after acidic treatment, 20-isocholesterol (**7**). The synthetic 20-isocholesterol has physical properties identical with the published data.[7] Its trimethylsilyl ether and the benzoate appeared at slightly shorter retention times on GC[8] and high performance liquid chromatography (HPLC) (reversed phase column)[9] respectively, compared to those of cholesterol derivatives.

For examination of its biological properties, 20-isocholesterol was first incubated with cytochrome $P\text{-}450_{scc}$, which is the enzyme catalyzing side chain cleavage of cholesterol.[10] As described previously,[11] incubation was performed with the reconstituted system consisting of cytochrome $P\text{-}450_{scc}$, adrenodoxin and adrenodoxin reductase in the presence of a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system, and the product pregnenolone was quantified as the trimethylsilyl ether by GC-MS in the selected ion monitoring mode. It is evident from Fig. 1 that enzymic formation of pregnenolone from 20-isocholesterol was negligible, whereas cholesterol was effectively converted into pregnenolone. This result is in line with the previous data[12] that (20*S*)-20-hydroxycholesterol (**8**), (20*R*, 22*R*)- and (20*R*, 22*S*)-20,22-dihydroxycholesterol (**9** and **10**), but not (20*R*)-20-hydroxycholesterol (**11**), (20*S*, 22*R*)- and (20*S*, 22*S*)-20,22-dihydroxycholesterol (**12** and **13**) were good substrates for the P-450. The former three compounds may be regarded as C-20 hydroxylation products derived from cholesterol, whereas the latter three could come from 20-isocholesterol. It is concluded therefore, that cytochrome $P\text{-}450_{scc}$ stringently requires a sub-

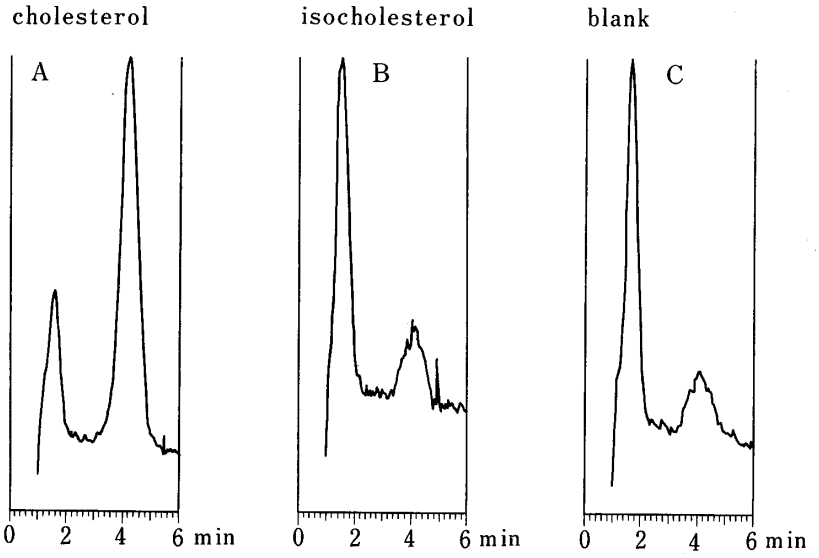
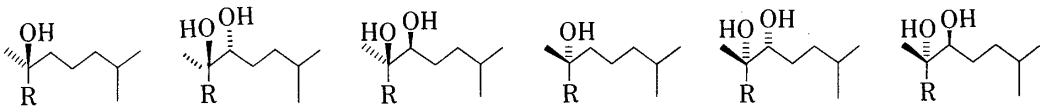


Fig. 1. Selected Ion Monitoring (*m/z* 298) of the Trimethylsilyl Ether of Pregnenolone Formed during Incubation of Cholesterol (A), 20-Isocholesterol (B), and without Addition of Substrate (C)

GC-MS was done on 1% OV-17 (1.5 m) at 248 °C. Standard pregnenolone trimethylsilyl ether appeared at 4.0 min.



8 9 10 11 12 13

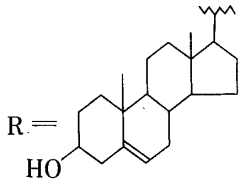


Chart 2

TABLE I. Mean Body Weight of the Silkworm Larvae

Fed sterol	On day 10		On day 14	
	Number of surviving insects	Mean body wt. (mg/head)	Number of surviving insects	Mean body wt. (mg/head)
Cholesterol	37	12.2	37	32.4
20-Isocholesterol	5	2.0	1	3.0

strate with the same configuration at C-20 as that of cholesterol. This report is the first to demonstrate that 20-isocholesterol is not a substrate for cytochrome P-450$_{scc}$, although this sterol has been reported to be an inhibitor of enzymic formation of pregnenolone from cholesterol.[13]

We next tested 20-isocholesterol as an insect nutrient. It is well established that insects require exogenous sterol for normal growth and development, since they are devoid of *de novo* sterol biosynthesis. It has been indicated[2,3,14] that cholesterol and several structural analogs satisfy the silkworm sterol requirement. In the present experiments, silkworm larvae were reared on an artificial diet containing 20-isocholesterol as the sole sterol source. As shown in Table I, most larvae fed on this diet died within 10 d after hatching, while all the cholesterol-fed silkworms developed to the second instar. The inadequacy of 20-isocholesterol as an insect nutrient might be due to its inability to act as a biogenetic precursor of the molting hormone ecdysone. If this is so, the deleterious effect of 20-isocholesterol would be overcome by addition of a small amount of cholesterol to the diet, since this could be utilize for ecdysone biosynthesis. Therefore, the larvae were fed with a mixture of cholesterol and 20-isocholesterol in a various ratios. However, even a combination of the two sterols in a 3:2 ratio induced a serious retardation of insect growth (data not shown). It may be that 20-isocholesterol is not only inadequate as an ecdysone precursor but also leads to a disordered membrane structures. Nes *et al.* have recently reported that 20-isocholesterol prevents yeast growth[15] and sterol metabolism by *Tetrahymena pyriformis*.[16] Considering all of these results, 20-isocholesterol may be regarded as a physiologically inappropriate sterol.

Finally, 20-isocholesterol was incorporated into liposomes and the thermotropic behavior of this model membrane was explored. Multilamellar liposomes composed of dipalmitoylphosphatidyl choline (DPPC) and cholesterol or 20-isocholesterol in various molar ratios were prepared as described previously,[4] and the gel-to liquid crystalline phase transition was measured by differential scanning calorimetry (DSC). As shown in Fig. 2, the

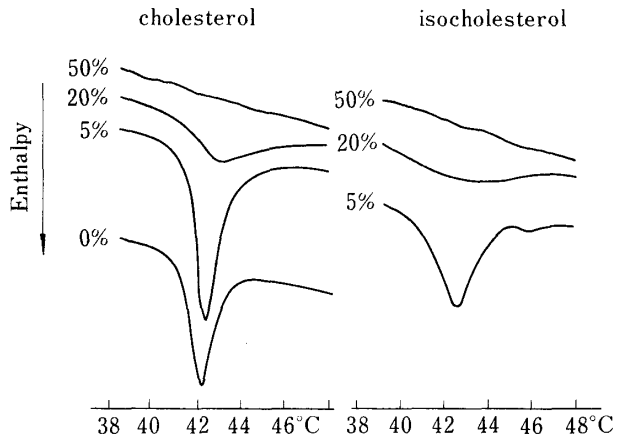


Fig. 2. Differential Scanning Calorimetry of Dipalmitoyl Phosphatidyl Choline Liposomes Containing Cholesterol or 20-Isocholesterol at Mole Concentrations of 0, 5, 20 and 50%

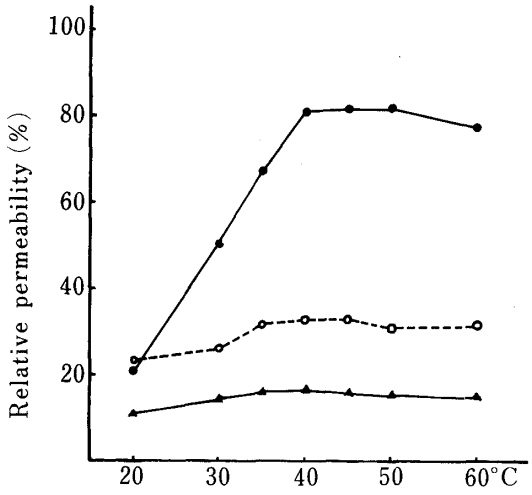


Fig. 3. Permeation of Carboxyfluorescein from DPPC Liposomes Containing Cholesterol or 20-Isocholesterol at 40 mol%

Relative fluorescence intensity was measured at various temperatures as described previously.[4] —●—, DPPC alone; ---○---, DPPC containing cholesterol; —▲—, DPPC containing 20-isocholesterol.

addition of increasing amounts of cholesterol to DPPC bilayers gradually diminished the transition, which became very broad and shifted to a higher temperature as compared with the original unperturbed transition. These results are in good accord with the reported ones.[17] Figure 2 indicates that the liposomes containing 20-isocholesterol behaved essentially in the same manner. We also measured the permeability of the liposomes containing carboxyfluorescein. As shown in Fig. 3, introduction of either cholesterol or 20-isocholesterol into liposomes at 40 mol% almost completely abolished the temperature dependence of carboxyfluorescein permeation seen with DPPC alone. These results are somewhat unexpected, because the above-mentioned *in vivo* results with insects, and results with yeast[15] and protozoa[16] seemed to suggest that complexing of 20-isocholesterol with phospholipid may be interfered with due to the different conformation of the side chain from that of cholesterol, and this might be one of the causes of the inappropriateness of 20-isocholesterol as a membrane sterol. One explanation of this discrepancy may be low sensitivity of the present methods to the alteration of sterol side chain structures. We have previously noticed[4] that cholesterol analogs with various lengths of side chain and even androst-5-en-3β-ol which is devoid of a side chain, behaved just like cholesterol in DPPC liposomes. Further investigations with alternative methodology will be needed to reach a definite conclusion.

Experimental

Melting points are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL-PS-100 in $CDCl_3$ with Me_4Si as an internal reference. GC-MS were run on a Shimadzu-LKB 9000S spectrometer with 3% OV-17. HPLC was carried out using a Shimadzu LC-4A on ODS (25 cm × 4.6 mm) with methanol (2.5 ml/min). "The usual work-up" refers to dilution with CH_2Cl_2, washing with dil. HCl, sat. $NaHCO_3$ and H_2O, drying over $MgSO_4$, filtration and solvent evaporation under vacuum.

Synthesis of 20-Isocholesterol——(20*R*)-3β-Acetoxybisnorcholenic acid[5] (276 mg) was refluxed in a mixture of 1% methanolic KOH (10 ml) and tetrahydrofuran (2 ml) for 2 h. Solvent was evaporated off and the residue was washed with dil. HCl and then water. Drying in a desiccator gave the (20*R*)-acid **3** (220 mg). A part (*ca.* 10 mg) of this sample and (20*S*)-bisnorcholenic acid (Steraloids) were separately treated with $LiAlH_4$ (30 mg) in tetrahydrofuran (3 ml) at ambient temperature for 1 h. The usual work-up gave the 3,20-diols, and these were treated with trimethylsilylimidazole (0.1 ml) to yield the bis-trimethylsilyl ethers. These samples were analyzed by GC-MS at

260 °C and showed single peaks at 4.4 and 4.9 min for the 20*R* and 20*S* isomers, respectively. MS *m/z*: 476 (M^+), 386 (M-TMSOH), 257, 129 and 73 (base peak). A mixture of the 20*R* acid **3** (197 mg), *p*-toluenesulfonic acid (5 mg), dihydropyran (0.35 ml) and CH_2Cl_2 (4 ml) was stirred at ambient temperature for 10 min. The crude product obtained by the usual work-up was treated with $LiAlH_4$ (50 mg) in tetrahydrofuran (6 ml) at ambient temperature for 0.5 h. The usual work-up gave the (20*R*)-22-alcohol **5** (170 mg), mp 124—126 °C (from hexane–ethyl acetate), NMR δ: 0.70 (3H, s, 13-Me), 0.95 (3H, d, J=6 Hz, 20-Me), 1.0 (3H, s, 10-Me), 4.8 (1H, m, 2′-H of tetrahydropyranyl) and 5.4 ppm (1H, m. 6-H). This alcohol **5** (170 mg) was treated with *p*-toluenesulfonyl chloride (150 mg) in pyridine (2 ml) at ambient temperature overnight. The usual work-up gave the tosylate **6** (199 mg), NMR δ: 0.60 (3H, s, 13-Me), 0.70 (3H, d, J=6 Hz, 20-Me), 2.45 (3H, s, tosyl-Me), 4.75 (1H, m, 2′-H of tetrahydropyranyl) and 5.4 ppm (1H, m, 6-H). An Li_2CuCl_4 solution (1.4 ml) [prepared from LiCl (85 mg), $CuCl_2$ (135 mg) and tetrahydrofuran (5 ml)] was added to a solution of Grignard reagent[prepared from magnesium ribbon (250 mg), isoamyl bromide (1.6 g) and tetrahydrofuran (10 ml)] under argon at 0 °C. Then the tosylate **6** (199 mg) in tetrahydrofuran (4 ml) was added at 0 °C and the mixture was stirred at 0 °C for 2 h then at ambient temperature overnight. The crude product obtained by the usual work-up was treated with one drop of conc. HCl in methanol (7 ml) at 50 °C for 20 min. Solvent was evaporated off and the residue was chromatographed on silica gel with benzene–ethyl acetate (6 : 1) to give 20-isocholesterol (**7**, 110 mg), mp 152—153 °C (from methanol–acetone), NMR δ: 0.70 (3H, s, 13-Me), 0.84 (3H, d, J=7 Hz, 20-Me), 0.88 (6H, d, J=6 Hz, 25-Me_2), 1.03 (3H, s, 10-Me), 3.5 (1H, m, 3-H) and 5.4 ppm (1H, m, 6-H). The trimethylsilyl ether showed a single peak at 5.0 min on GC at 273 °C (cholesterol trimethylsilyl ether, at 6.5 min), and *m/z* 458 (M^+), 368 (M-TMSOH), 329 (M-129) and 129 (base peak). The benzoate appeared at 36 min on HPLC (cholesterol benzoate, at 41 min).

Incubation with Cytochrome P-450$_{scc}$——Cholesterol or 20-isocholesterol (1 μg) was incubated and the product pregnenolone was analyzed by GC-MS with selected ion monitoring at *m/z* 298 of the trimethylsilyl ether as described previously.[11]

Insect Rearing——The newly hatched larvae (40 larvae in each group) of the silkworm *Bombyx mori* were reared on an artificial diet containing 0.1% sterol as described previously.[2,3]

Thermotropic Behavior of Liposomes——Preparation of liposomes composed of dipalmitoylphosphatidyl choline and sterols with or without carboxyfluorescein was described previously.[4] Differential scanning calorimetry with a Daini Seikosha SSC 540 and measurement of carboxyfluorescein permeation from liposomes were carried out as described previously.[4]

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