Review

Reminiscences of research on the chemistry and biology of natural sterols in insects, plants and humans

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Abstract: Natural sterols often occur as a heterogeneous mixture of homologs, which had disturbed the progress of steroid research. Development and application of GC methodology overcame this difficulty and enabled us to obtain detailed sterol profiles. Together, fine synthesis of stereo-defined isomers and homologs of steroids having oxygenated side chains allowed us to compare them with natural samples as well as to investigate structure-activity relationship. Advance of HPLC technology also facilitated the determination of the stereochemical structure of naturally occurring steroidal compounds, which were obtained only in minute amounts. This review highlights three topics out of our steroid research that have been performed mainly at Tokyo Institute of Technology around 1970–1990. These are sterol metabolism in insects focusing on the mechanism of the conversion of plant sterols to cholesterol and ecdysone biosynthesis, the synthesis and biochemical research of active forms of vitamin D_3 derivatives, and the synthesis and microanalysis of plant hormone brassinosteroids.

Keywords: sterol, ecdysteroid, vitamin D_3, brassinosteroid, structure determination, biological activity

Introduction

Since 1930s biologically active mammalian steroids had been paid attention to many researchers and tremendous studies on steroids had provided numerous brilliant results. On the other hand, plant steroids were known only as final products in the biosynthesis of isoprenoids and thus were not paid attention due to lack of knowledge on their biological activities. In 1957, discovery of cholesterol in algae by Tsuda *et al.* became a topic in this field.[1)] Before 1970s mammalian steroid hormones which have short or no side chains were major targets for the steroid research.[2)] Steroids originated from non-mammalian sources such as fungi, insects and plants showed interesting side chains oxygenated at C20, C22, C23, C25 and C26 as found in antheridiol,[3)] ecdysteroids[4)] and withanolides.[5)] These steroids have stigmastane (C29), cholestane (C27) and ergostane (C28) skeletons, respectively.

Chemical and biochemical studies on the steroids having side chains had experienced several difficulties in the isolation of the active steroids due to their faint amount in the biological pool and in the separation of the stereoisomers. Thus, we had established a novel gas-chromatography (GC) technology, which enabled us to cleanly separate natural steroids and also developed the chemical synthetic methods in conjunction with the biochemical methods to solve the problems, which interfered with the research on the steroids having functionalized side chains. These methods were applied to the investigation of plant and insect sterols.[6)]

GC technology enabled us the microanalysis of sterol mixtures and the synthetic organic chemistry made it possible to prepare all stereoisomers through the stereoselective reactions at the steroid side chains. Thus, we synthesized numerous kinds of the steroids having oxygenated side chains and revealed

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the roles of those steroids in their biological activities.[7)]

Sterol metabolism in insects

1. Dealkylation mechanism of plant sterols to cholesterol. Insects, unlike most plants and animals, are incapable of *de novo* sterol biosynthesis and therefore require a dietary or exogenous source of sterol for their normal growth and development. The sterol requirement of insects is, in most cases, satisfied by cholesterol (**1**), which is the principal sterol in insects and serves as the structural component of cell membranes and as biogenetic precursor of the molting hormone, 20-hydroxyecdysone (**2**). In phytophagous insects the requirement can be fulfilled also with plant sterols *e.g.*, sitosterol (**3**), campesterol (**4**) and stigmasterol (**5**), since these C-24 alkylated sterols are metabolically converted to cholesterol. Thus, dealkylation of phytosterols is an important metabolic step in many insects.

GC analysis of the sterol fraction of silkworm larvae indicated the presence of cholesterol, together with small amounts of campesterol and sitosterol.[8)] The sterol fraction of mulberry leaves contained sitosterol and a smaller amount of campesterol.[9)] It was thus suggested that these plant sterols were converted into cholesterol and we had an interest in the conversion mechanism. Indeed we showed that [^{3}H]-sitosterol was converted into cholesterol in the silkworm *Bombyx mori* in 1967.[10)] Meanwhile, fucosterol (**6**), 24-methylenecholesterol (**7**) and desmosterol (**8**) were postulated as intermediates of the dealkylative conversion principally by Svoboda's group utilizing tobacco hornworm.[11)]

However, the precise C-24–C-28 bond-cleavage mechanism of plant sterols has remained unanswered. We started the study on the dealkylation mechanism around 1968, since we possessed a sizable amount of fucosterol (**6**), available from brown algae such as *Sargassum ringgoldianum* through our search for marine resources.[12)] It was a lucky break to find out that BF_3 treatment of 3-acetate of fucosterol 24,28-epoxide (FE) (**9**) yielded desmosterol acetate together with usual epoxide-carbonyl rearrangement products during chemical studies of the fucosterol $\Delta^{24(28)}$-double bond.[13)] The unique rearrangement of FE was accompanied by the formation of acetaldehyde and thus expected to involve hydrogen migration from the C25 to C24 position (See Fig. 2).

The rather unexpected C-C bond cleavage reaction was regarded as a biomimetic version of the phytosterol dealkylation. We proved this hypothesis by several lines of evidence utilizing silkworm larvae. First, ^{3}H-label of fucosterol administered to larva was trapped in FE and ^{3}H-label of FE was incorporated into cholesterol.[14)] Secondly, hydrogen migration from C-25 to C-24 was verified by feeding [25-^{3}H]-24-ethylcholesterol followed by determining chemically the position of the label of the resulting desmosterol.[15)] Thirdly, the 24,28-imine analogue of FE was shown to be a potent inhibitor of the enzyme termed fucosterol epoxide lyase that catalyzes the conversion.[16)] The intermediacy of FE in sitosterol dealkylation was supported in three other insects.[17)] We subsequently indicated that the same hydrogen migration took place in the dealkylation of stigmasterol and campesterol, now utilizing [^{2}H]-labeled substrates coupled with GC-MS analysis.[18)] These studies have established the phytosterol dealkylation mechanism as summarized in Fig. 1 (Ikekawa pathway).

We then investigated several stereochemical issues of the dealkylation processes. Incubation with a cell free preparation obtained from midguts of silkworm larvae revealed that (24*R*,28*R*)-fucosterol epoxide and (24*R*,28*S*)-isofucosterol ($\Delta^{24(28)}$-*Z* isomer of **6**) epoxide, out of the four diastereomers at the C-24 and C-28 positions, were converted into desmosterol by the lyase.[19)] Similarly, (24*R*)-24-methylenecholesterol 24,28-epoxide (**10**), but not the (24*S*)-isomer, became a substrate for the enzyme.[20)] Both (24*R*,28*R*)- and (24*S*,28*S*)-FEs were, however, characterized from the whole body of silkworm larvae.[21)] Yet another stereochemical preference with respect to the epoxide isomers was reported for the insect *Tenebrio molitor*.[22)] These discrepancy needs to be clarified, including the question as to which epoxide diastereomers are formed from the $\Delta^{24(28)}$-olefinic substrates.

Metabolic fate of the diastereotopic C-26 and C-27 methyl groups of FE was investigated using a cell free preparation from midguts of silkworm larvae, and [*pro-R*]-methyl group (designated as open circle in Fig. 2) was turned out to become sterespecifically (*E*)-methyl of desmosterol.[23),24)] The stereochemistry in the desmosterol reduction was explored with the cell-free preparation in the presence of NADPH cofactor by applying deuterium-decoupled ^{1}H,^{13}C shift correlation NMR analysis of the biosynthesized cholesterol.[25)] The study established that the hydrogen addition by sterol-Δ^{24}-reductase took places in *anti* fashion from the *re*-face at C-24 and the *si*-face at C-25 (Fig. 2). The steric course of the hydrogen

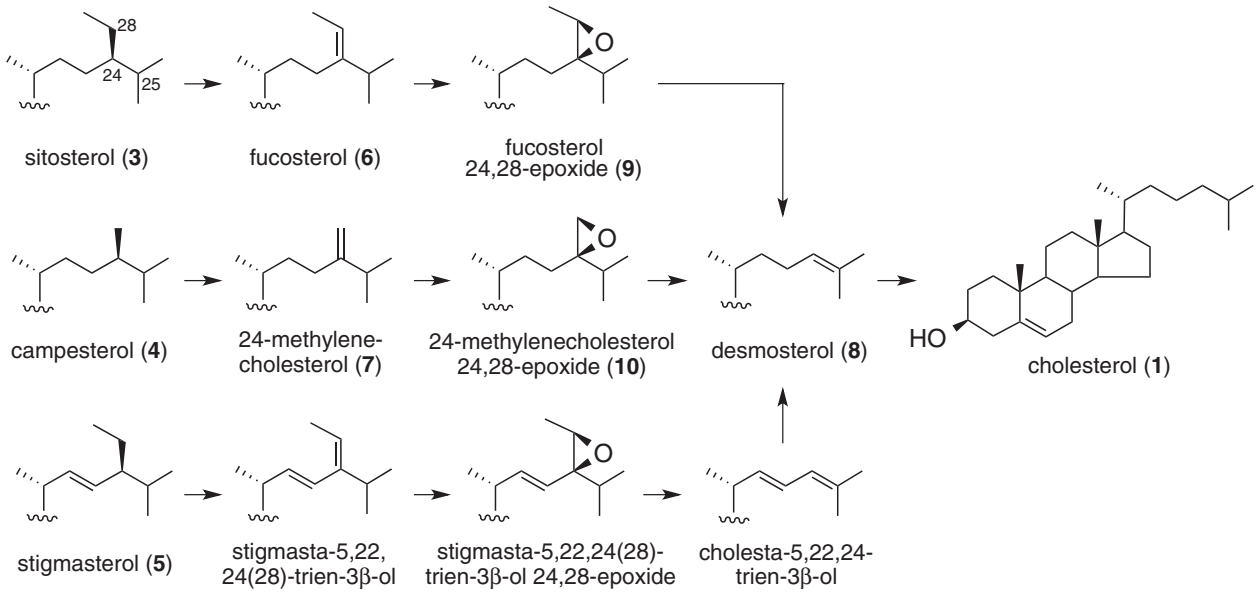

Fig. 1. Dealkylation mechanism of phytosterol.

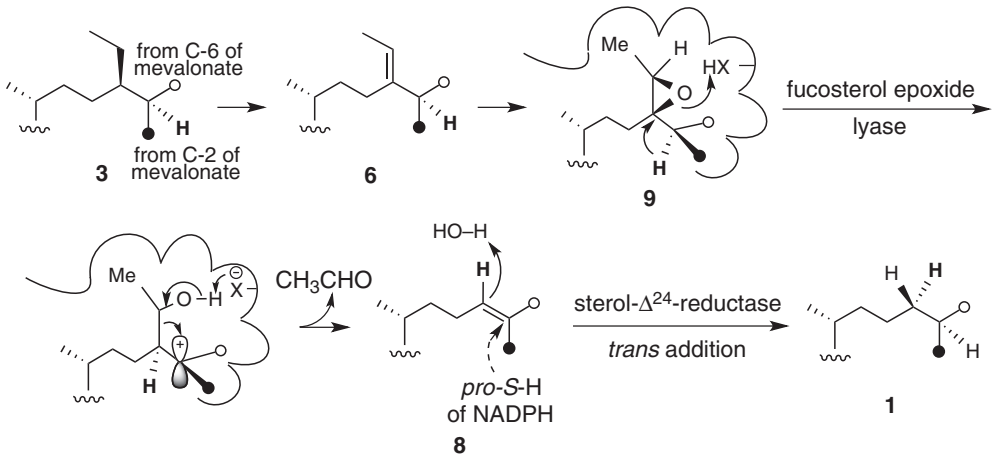

Fig. 2. Steric course of dealkylation of sitosterol.

addition was also investigated with rat liver cell-free preparation and plant callus, which confirmed the same *anti* addition. This required revision of *syn* addition mechanism originally reported by Caspi and co-workers in rat liver system more than 30 years ago. The stereochemistry of the sitosterol dehydrogenation (*cis*- vs. *trans*-dehydrogenation) leading to fucosterol, but not isofucosterol,[26] remains an open question. The gene encoding the insect sterol-Δ^{24}-reductase of *Bombyx mori* has been identified recently,[27] although those encoding the enzymes of the preceding three steps remain unidentified. Characterization of these genes may facilitate search for inhibitors of the phytosterol dealkylation, which would be environmentally friendly insecticides for agrochemical use. Also, they can be used for the conversion of plant sterols to useful sterols functionalized in the side-chain by the fermentation of engineered microbes.

2. Biosynthesis of insect molting hormones. At the beginning of the 1940s, Fukuda first proposed that prothoracic ground (PG) secrets a molting hormone.[28] In addition, our group reported a brain hormone-like activity of cholesterol administered to silkworm pupae as early as 1963 when the structure of ecdysone remained unclear.[29] Since then,

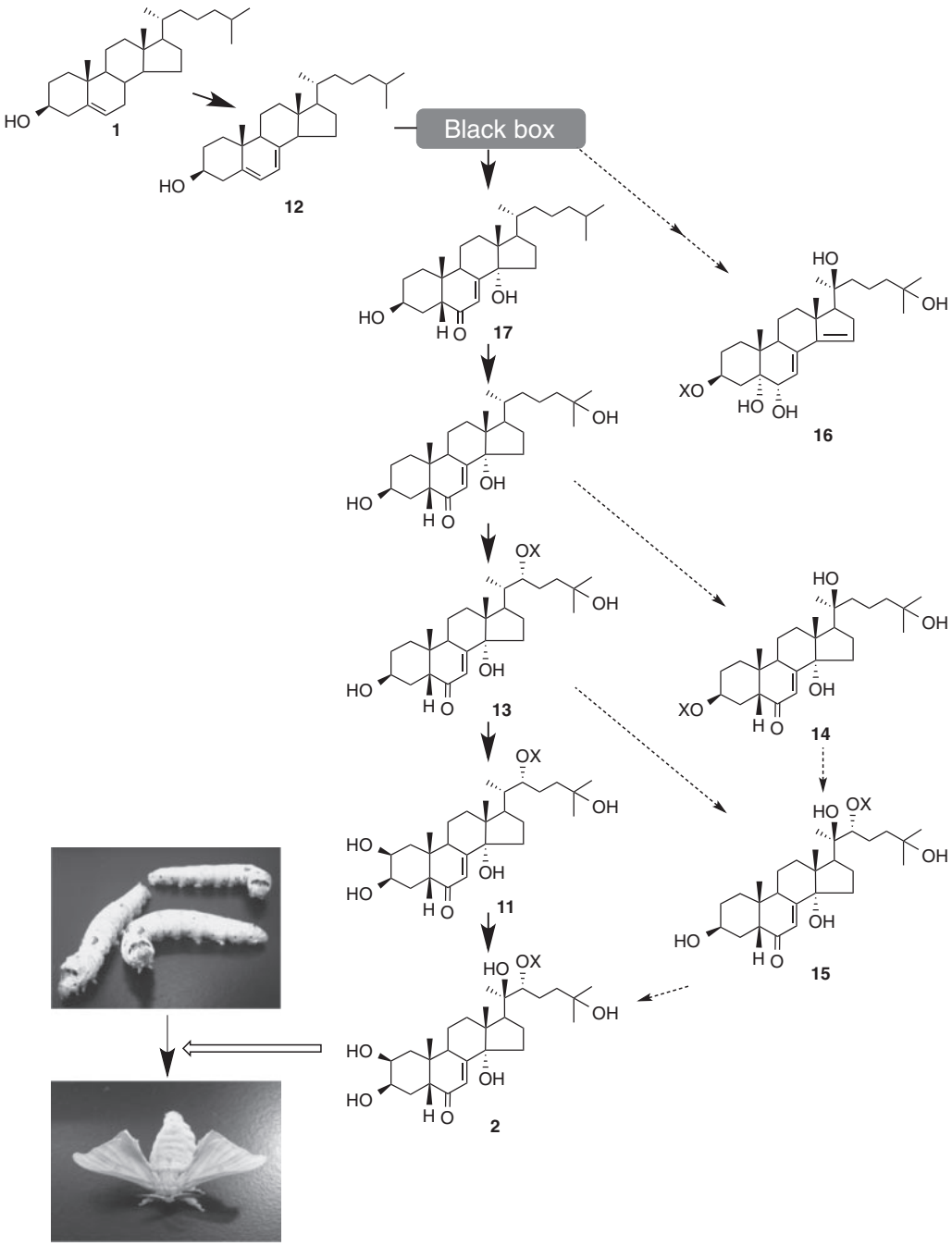

Fig. 3. Biosynthetic route of ecdysteroids (X = H or PO_3^{2-}).

α-ecdysone (ecdysone) (**11**) and β-ecdysone (20-hydroxyecdysone) (**2**) were isolated from the silkworm and their structures were elucidated.[4),30),31)] However, it had not been clear whether PG is a responsible organ that produces a molting hormone, and if so, which substance(s), either ecdysone and/or 20-hydroxyecsysone, is produced by PG.

Chino and co-workers challenged this problem and succeeded in culturing PG organs that produced a hormonally active substance.[32)] We had developed a micro-analytical method of ecdysteroids based on GC-MS (mass chromatography mode) analysis of their trimethylsilyl ether derivatives,[33)] and successfully characterized the substance released from the

cultured PGs to the medium as ecdysone in 1973.[32] Ecdysone thus formed is converted into 20-hydroxyecdysone by 20-hydroxylase distributed in peripheral tissues such as fat body.

GC analysis of a PG extract revealed the occurrence of 7-dehydrocholesterol (**12**) as well as 7-dehydrositosterol and 7-dehydrocampesterol, the amounts of which changed in proportional to that of ecdysone,[34] supporting a view that 7-dehydrocholesterol is a biosynthetic intermediate next to cholesterol.

Accumulation of highly polar ecdysteroid conjugates in the diapause eggs of the silkworm *Bombyx mori* was first reported by Ohnishi *et al.*[35] His group also noted that the ovaries of *B. mori* exhibited high molting hormone activity.[36] Since then, we worked jointly with his group on the structure analysis of ovarian ecdysteroids, and characterized six ecdysteroids, 2-deoxyecdysone (**13**),[37] 2,22-dideoxy-20-hydroxyecdysone (**14**),[39] ecdysone (**11**), 2-deoxy-20-hydroxyecdysone (**15**),[38] 20-hydroxyecdysone (**2**), and a unique ecdysteroid bombycosterol (**16**)[40] as major ecdysteroids in *Bombyx* ovaries. Their conjugate forms were elucidated to be all phosphate esters ($X = PO_3^{2-}$ in Fig. 3).[41] Figure 3 also shows the principle biosynthetic route ($X = H$ in Fig. 3) of 20-hydroxyecdysone, although the mechanism of an early stage (so-called black box), leading to the 14-hydroxy-5β-enone (**17**), has remained unsolved. Our characterization of various free ecdysteroids and their phosphate derivatives in *Bombyx* ovaries/eggs has contributed to establish the generally accepted view that the ecdysteroid conjugates are a physiologically inactive form that are stored in eggs and serve as a source of an active ecdysteroid like 20-hydroxyecdysone that is indispensable for embryonic developments. It is interesting to note that ecdysteroids work on a membrane receptor in addition to the nuclear ecdysone receptor (EcR).[42]

Synthesis and biochemical research of active forms of vitamin D_3 metabolites and their analogs

1. Structure determination of vitamin D_3 metabolites. New era of steroid research was opened by the identification of 25-(OH)-VD_3 (**18**, for the structure see Fig. 8) and 1,25-$(OH)_2$-VD_3 (**19**) as the active metabolites of VD_3.[43] During 1968–71, DeLuca and Tanaka mainly contributed to the discovery of the hydroxylated VD_3. The most intriguing findings were the importance of secosteroid derivatives having oxygenated side chains, although 1α-hydroxylation is essential to elicit their biological activity. After identification of 1,25-$(OH)_2$-VD_3, 24,25-$(OH)_2$-VD_3 (**20**) was identified by MS analysis.[44] However, the configuration at C24 of **20** remained unestablished.

At this moment we had a large amount of desmosterol (**8**) chemically derived from fucosterol (**6**). Thus, our research work was started from the determination of the C24 configuration. At first, 24-OH cholesterol (**21**) was prepared from **8** and was separated into two isomers (**21a**/**21b**). Their C-24 configurations were determined with modified Horoeu's method using GC.[45]

Two C24-isomers of 24,25-$(OH)_2$ cholesterol (**22**) were prepared from **8** and the resulting mixture was separated by HPLC of the tri-TMS derivatives (Fig. 4). Both 24*S* and 24*R* isomers (**22a** and **22b**) were converted into VD_3 form, and assayed for VD_3 activity by DeLuca and Tanaka of Univ. of Wisconsin-Madison, showing that the 24*R* isomer exhibited potent activity (vide infra). This was a start of the long-term joint research between their groups and ours lasting nearly 20 years, and biological activities of our synthetic VD_3 samples were always evaluated by their group thereafter. HPLC comparison of the synthetic samples with natural 24,25-$(OH)_2$-VD_3 (**20**) revealed that 24*R* isomer (**20a**) was correspondent to the natural metabolite.[46]

Subsequently, 25,26-$(OH)_2$-VD_3 (**23**) was identified as a metabolite of 25-(OH)-VD_3.[47] Then, two C25-isomers of 25,26-$(OH)_2$ cholesterol were stereoselectively synthesized from (24*R*)- and (24*S*)-Δ^{25}-24-hydroxycholesterol derivative (**24a**/**24b**) as shown in Fig. 5. Interestingly, 25,26-$(OH)_2$-VD_3 had both 25*S* and 25*R* configuration (**23a**/**23b**) in an equal amount as revealed by HPLC comparison with the synthetic 25,26-$(OH)_2$-VD_3,[48] while 24*R* isomer was a sole metabolite for 24,25-$(OH)_2$-VD_3.

A further research on VD_3 metabolism using [^{14}C]-25-(OH)-VD_3 led to identification of 23,25-$(OH)_2$-VD_3 (**25**)[49] and 25-(OH)-VD_3 26,23-lactone (calcidiol lactone, **26**),[50] which are 23-hydroxylated VD_3 derivatives.

The 23S configuration of **25** was elucidated as follows. (23*S*)-23,25-$(OH)_2$-VD_3 and its (23*R*)-isomer were synthesized according to Fig. 6. Coupling of the aldehyde (**27**) with metallylmagnesium chloride yielded the C-23 hydroxy derivative as an epimeric mixture, which was separated into the epimers (**28a** and **28b**) in the form of 3,23-dibenzoate (R=Bz).

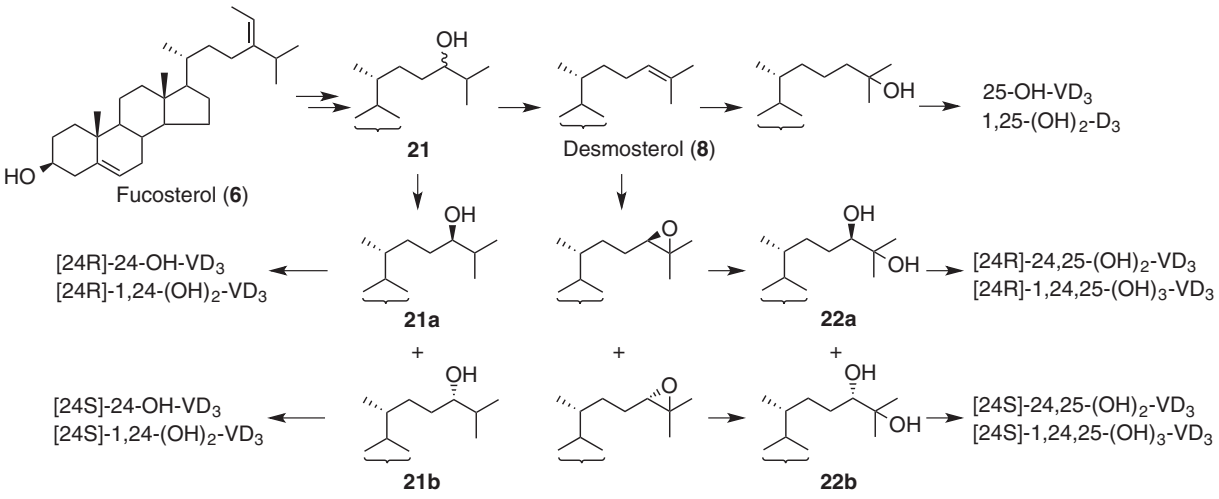

Fig. 4. Synthesis of 24-hydroxy and 24,25-dihydroxycholesterols.

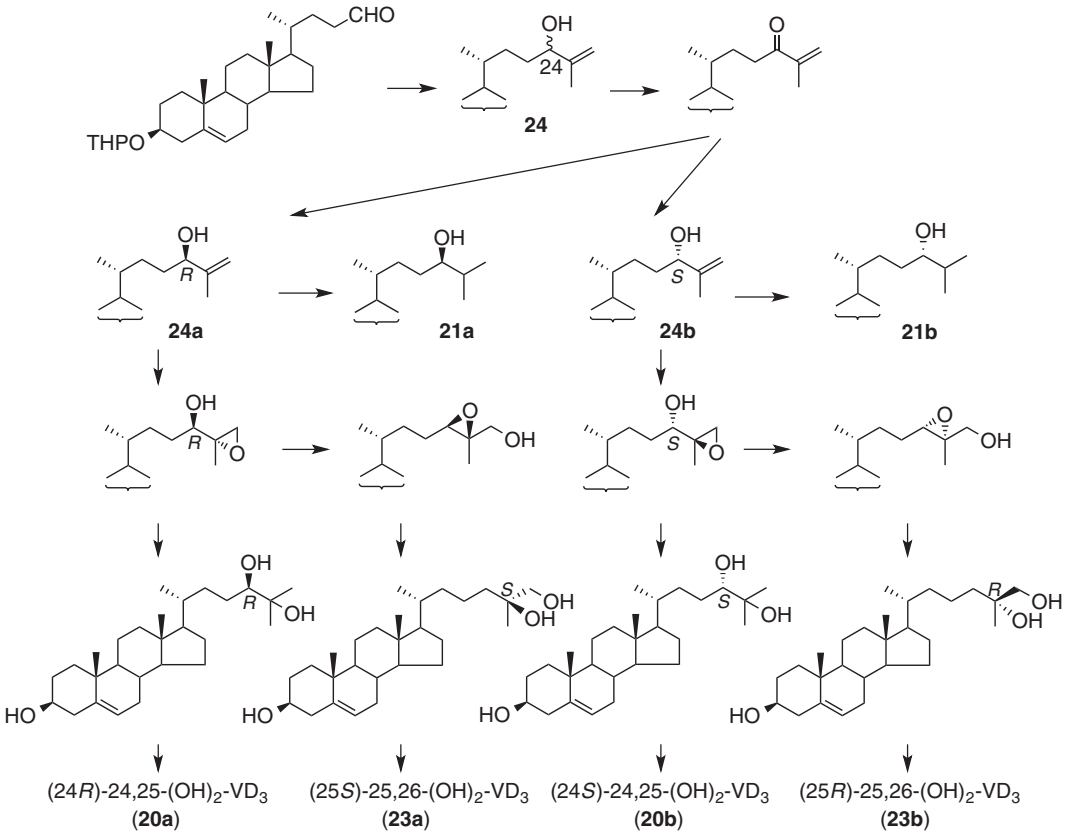

Fig. 5. Stereoselective synthesis of 24,25- and 25,26-dihydroxycholesterols.

Compounds **28a** and **28b** were converted to the 3,23*S*,25-triol (**29a**, R=H) and its 23*R*-isomer (**29b**), respectively. The configuration at C-23 of **29a** was determined to be *S* by X-ray analysis. Compounds **29a** and **29b** (R=Bz) were further converted into VD_3 form. HPLC comparison of the stereo-defined synthetic samples with the natural metabolite established the 23*S* configuration of **25**.[51)]

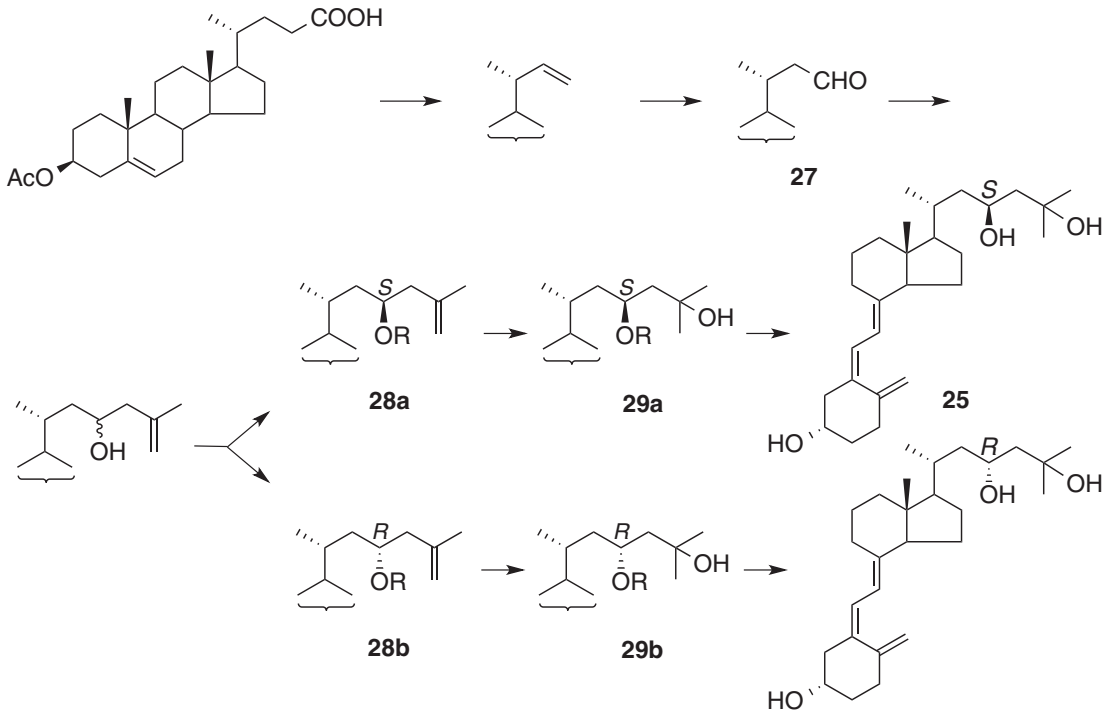

Fig. 6. Synthesis of (23*S* and 23*R*)-23,25-$(OH)_2$-VD_3.

The synthesis of four isomers at C23 and C25 of calcidiol lactone was accomplished by the stereospecific iodolactonization or selenolactonization of the *trans*- and *cis*-Δ^{22}-26-carboxylic acids (**30** and **31**). The introduction of the 25-OH group was achieved through the oxidation at the Cα-position of the 26-ester or lactone moiety. The stereoselective introduction of the hydroxyl group at C25 was dependent on the configuration at C23 of the lactone and thus, the hydroxyl group of the other configuration was introduced in the ester moiety before the lactonization as shown in Fig. 7.[52] The 23*S*,25*R* configuration of calcidiol lactone was also established by HPLC comparison with the natural metabolite.[53]

2. Biological activity for vitamin D_3 analogs having hydroxylated side chain. Vitamin D_3 biosynthesized at the skin is transformed at liver and then kidney to the hydroxylated derivative, 1α,25-$(OH)_2$-VD_3 (**19**). This activated hormone binds vitamin D_3-binding protein in blood and is transferred to the target cells. Then, 1α,25-$(OH)_2$-VD_3 binds vitamin D_3 receptor (VDR) in the cell nucleus. It also forms RXR-VDR-1α,25-$(OH)_2$-VD_3 complex with 9-cis-retinoic acid receptor (RXR), which binds vitamin D-responding element (VDRE) present at the upstream of vitamin D_3-dependent gene. Then, translation-coupled co-activator binds this complex to regulate the gene expression. Thus, the complex in the process will become the targets of vitamin D_3 derivatives.

In relation to the configuration at the 24 position of 24,25-$(OH)_2$-VD_3 (**20**), we prepared two isomers of 24-(OH)-VD_3 and found that (24*R*)-24-(OH)-VD_3 has the same mobilization of bone Ca^{2+} and calcification activity as 25-(OH)-VD_3 and a similar activity to 24,25-$(OH)_2$-VD_3, while the 24*S* isomer showed little activity.[54] Although both isomers were metabolized to the respective 24,25-$(OH)_2$-VD_3, only 24*R* isomer was further converted to 1,24,25-$(OH)_3$-VD_3 (**32**).[55] Thus, 1α-hydroxylase recognizes only 24*R* isomer to give the active metabolite. When we assume that a particular residue in the enzyme requires the interaction with the hydroxyl group at C24 in the substrate-binding site, the conformation of the C25–C27 side chain moiety should affect the binding of the steroid skeleton. This is a unique example that the stereochemistry at the side chain affects the enzymatic reaction at A-ring of steroids. Furthermore, only 25*S*-isomer of 25,26-$(OH)_2$-VD_3 was converted to 1,25,26-$(OH)_3$-VD_3 (**33**) by 1α-hydroxylase. When the 3D-structure of 1α-hydroxylase is available, it will be revealed whether the

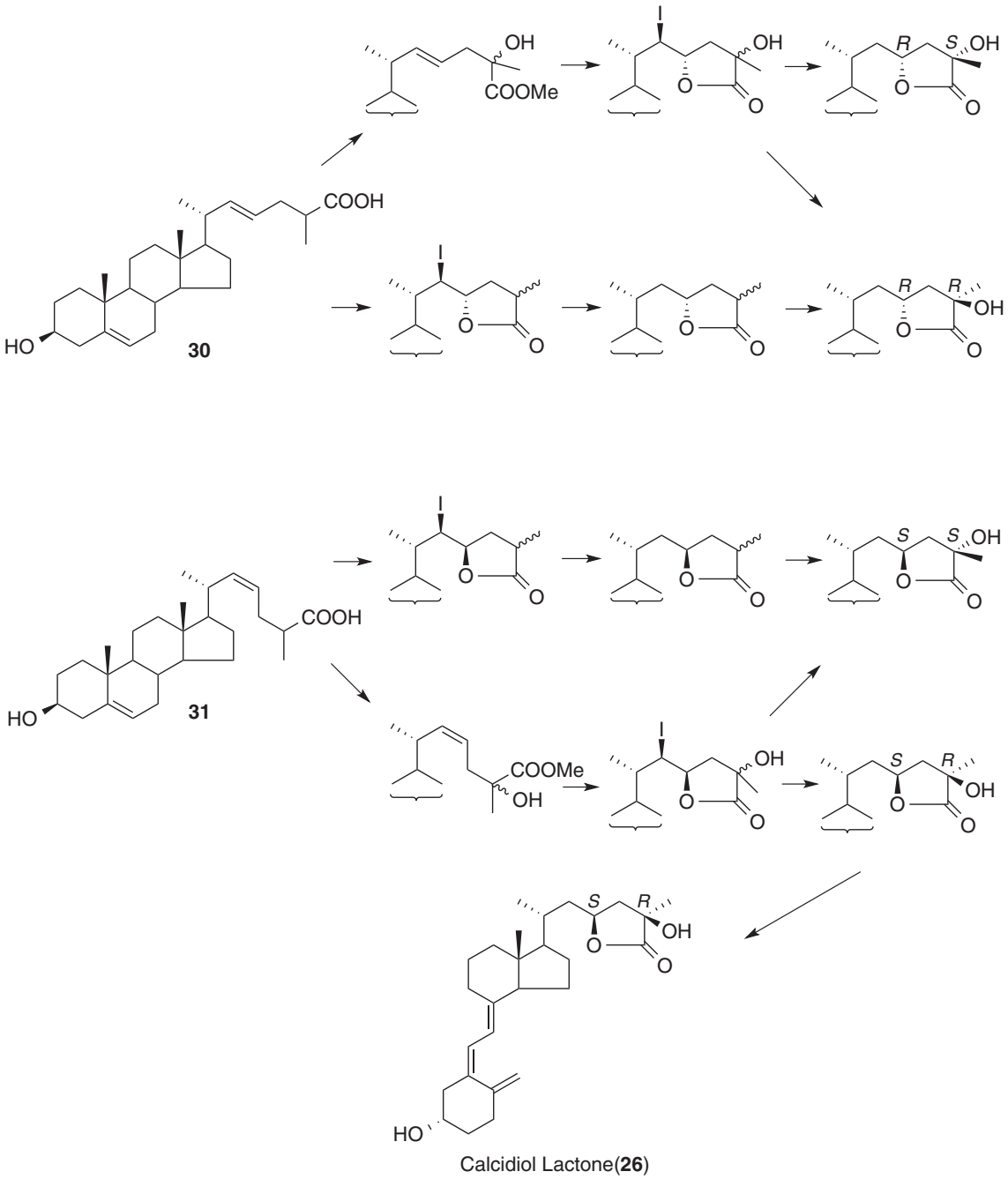

Fig. 7. Synthesis of calcidiol lactone isomers.

same residues are contributed to the highly specific recognition of the stereochemistry of the hydroxylated side chains. According to the VD_3 metabolites identified so far, the plausible metabolic pathway can be summarized in Fig. 8.

The activity of calcification of these hydroxylated VD_3 derivatives is as follows: 24,25-$(OH)_2$-VD_3 is almost equal to 25-(OH)-VD_3 and 25,26-$(OH)_2$-VD_3 is weaker than 24,25-$(OH)_2$-VD_3. Furthermore, 23,25-$(OH)_2$-VD_3 is weaker than 25,26-$(OH)_2$-VD_3. These results indicate that the hydroxylation at 23, 24 and 26 is a deactivation step of 25-(OH)-VD_3. Interestingly, the C22-hydroxylated (*R*)- and (*S*)-22,25-$(OH)_2$-VD_3 lost VD_3 activity, although these

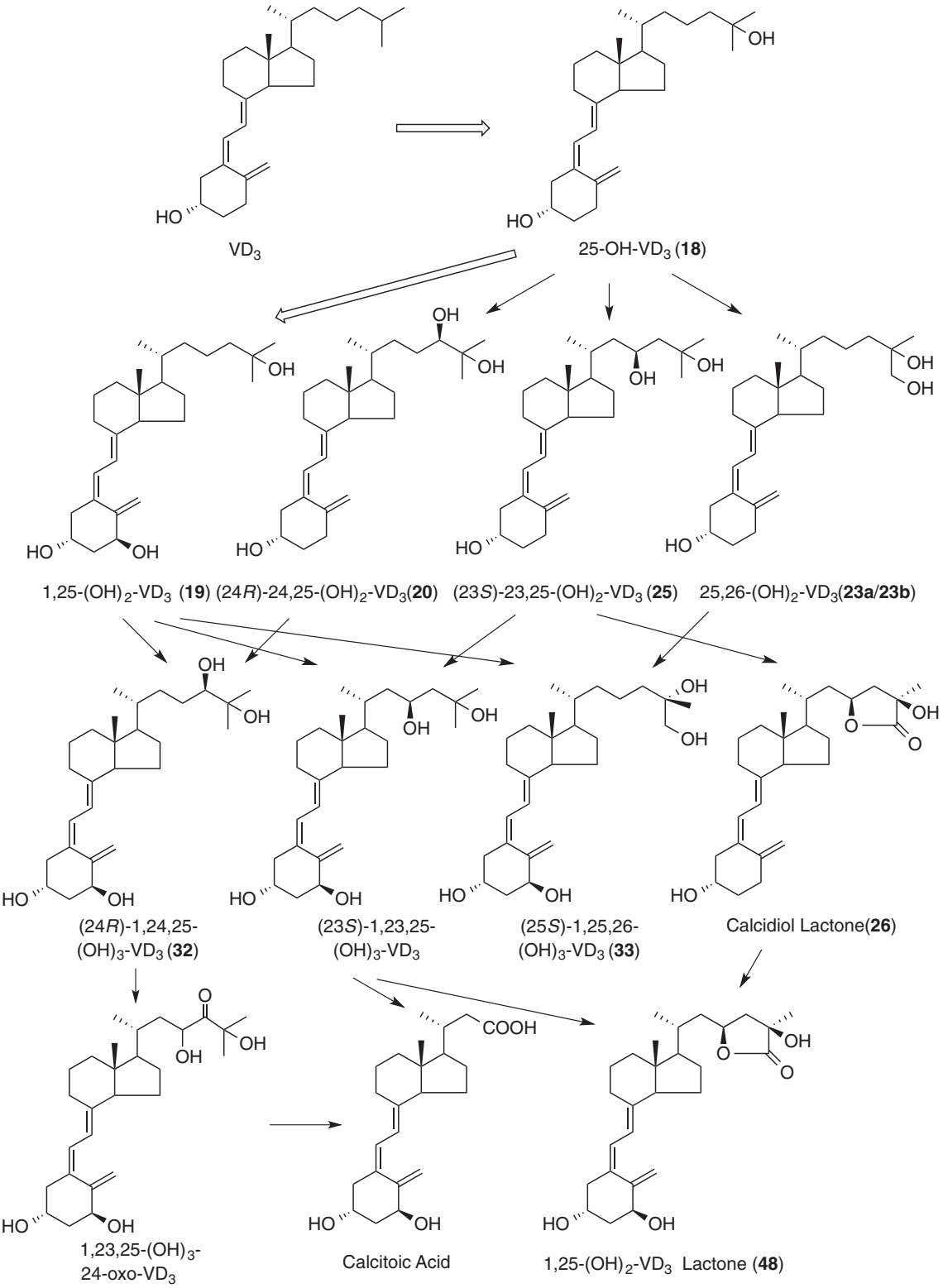

Fig. 8. Major metabolic map of vitamin D_3.

derivatives have not been found in the metabolites. This suggests that the hydroxyl group at the C22 position disturbs their binding or affects the binding mode to the VD_3 receptor.

3. Fluorine derivatives and the enhancing activities of VD_3. We prepared 26,27-F_6-, 24-F_2-, and 23-F_2-1,25-$(OH)_2$-VD_3 derivatives (**34**, **35**, **36**, respectively) to block the hydroxylation (Fig. 9). As we expected, 24-F_2-1,25-$(OH)_2$-VD_3 (**35**) showed 5–10 times higher activity than 1,25-$(OH)_2$-VD_3 and 26,27-F_6-1,25-$(OH)_2$-VD_3 (**34**) showed 10 times higher activity than 1,25-$(OH)_2$-VD_3, which maintained its activity for a week.[56] This is because compound **34** underwent 23-hydroxylation and the metabolite remained in cells for a longer time. 23-F_2-1,25-$(OH)_2$-VD_3 derivative (**36**) did not enhance the activity of 1,25-$(OH)_2$-VD_3.

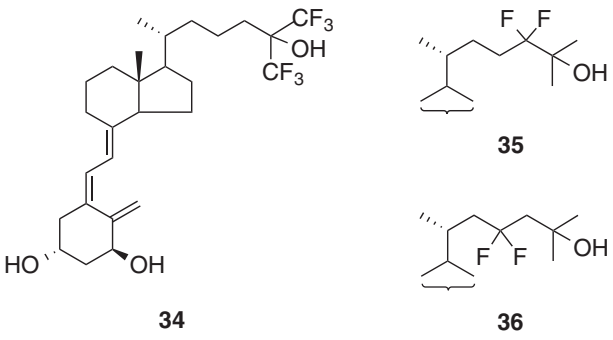

Fig. 9. 1,25-$(OH)_2$-Vitamin D_3 derivatives with fluorinated side chain.

4. A new rearrangement of the side chain—Biological activity of side chain carbon homologues. It was reported that 1,25-$(OH)_2$-VD_3 receptor is distributed in not only intestine, kidney, and bone, but also stomach, skin, pituitary gland, parathyloid gland and lung cancer cells.[57] Furthermore, 1,25-$(OH)_2$-VD_3 was reported to strongly facilitate the cell differentiation of human myeloid leukemia cells.[58] In order to develop anticancer and anti-leukemia drugs, it is thus required to seek for derivatives that have potent cell differentiation activity (10^{-7}–10^{-8} M for 1,25-$(OH)_2$-VD_3) but much more weak calcium regulating activity (10^{-9}–10^{-10} M for 1,25-$(OH)_2$-VD_3), *i.e.*, separation of the activities.

DeLuca and Tanaka found a new metabolite that strongly bound to the cytosol VD_3 receptor in addition to the 24- and 1-hydroxylated metabolites when they incubated 24-epi-25-OH-VD_2 ((24*R*)-24-methyl 25-(OH)-VD_3) (**37**) with avian kidney. The mass spectrum of the metabolite suggested that it could be either Δ^{22}-26-methyl-1,25-$(OH)_2$-VD_3 (**38**) or 24-homo-Δ^{22}-1,25-$(OH)_2$-VD_3 (**39**). Thus, the two compounds were synthesized and the following comparison with the metabolite established that the metabolite was **38**.[59] Although the exact mechanism of this unique reaction is not clear, it might involve the cleavage of the C23–C24 bond in the metabolism as reported by Djerassi *et al.* in the biosynthesis of the marine sterols (Fig. 10).[60]

In addition to the unique formation of the 26-homo skeleton, the metabolite showed 10 times

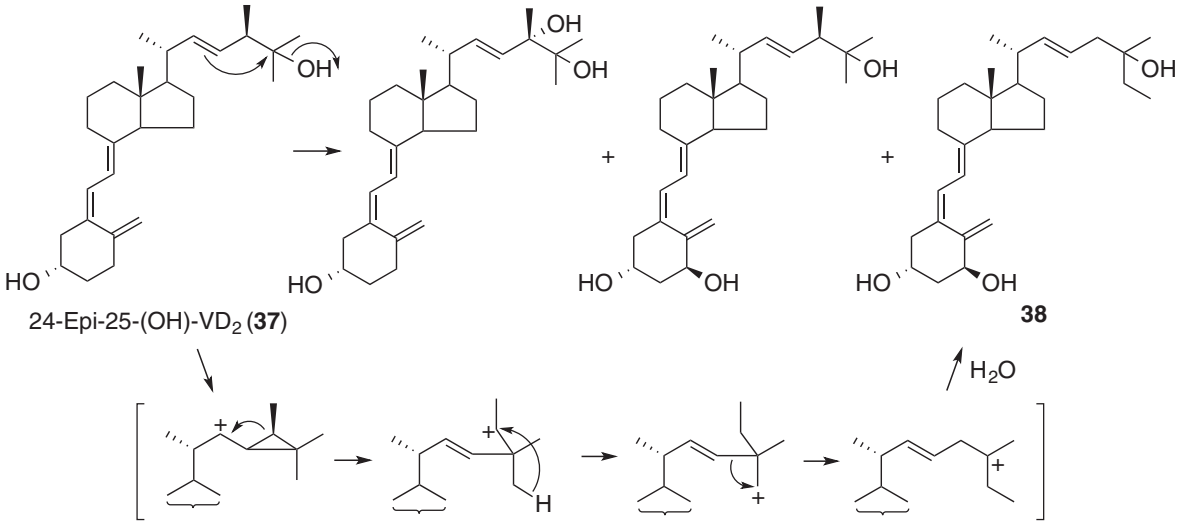

Fig. 10. Metabolism of 24-epi-25-hydroxyvitamin D_2.

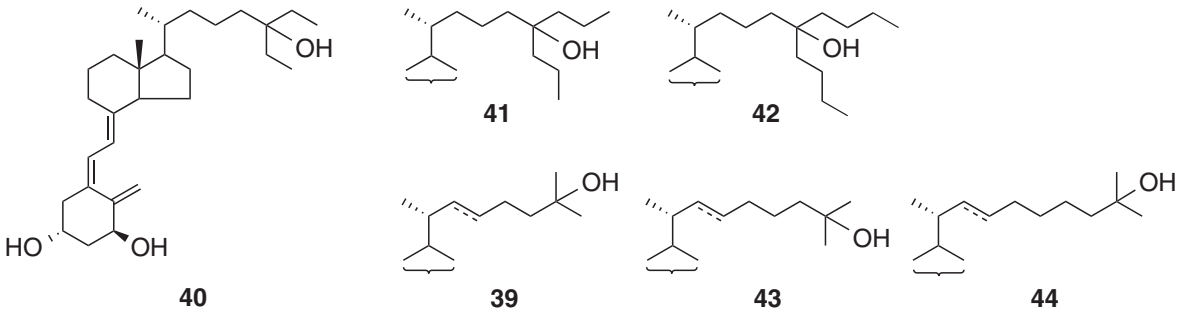

Fig. 11. Side-chain homologs of VD_3.

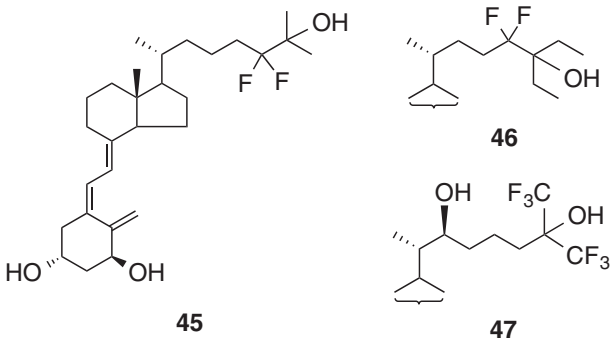

Fig. 12. Structures of drug candidates.

higher myeloid leukemia cell differentiation activity with little VD_3 activity than 1,25-$(OH)_2$-VD_3. This suggested that some homo VD_3 side-chain skeletons have different activity from the native VD_3 skeleton. In this course, we have synthesized 26,27-dimethyl-, 26,27-diethyl-, 26,27-dipropyl-1,25-$(OH)_2$-VD_3 (**40**, **41** and **42**).[61] The dimethyl and diethyl analogs showed 2.5 and 10 times higher myeloid leukemia cell differentiation activity, respectively, than 1,25-$(OH)_2$-VD_3. The dipropyl analog did not show the activity. A series of 24-homologated 1,25-$(OH)_2$-VD_3 analogs were also synthesized. Evaluation of the cell differentiation activity revealed that 1- or 2-carbon homologation (**39**, **43**) increased the activity by 10-fold, but 3-carbon homologation (**44**) reduced the activity by one half (Fig. 11).

5. Development of new drugs. As described in the above two sections, the effects of fluorine substitution and carbon homologation in the side chain have been elucidated. We, therefore, synthesized side chain modified analogues, 24,24-F_2-24-homo-1,25-$(OH)_2$-VD_3 (**45**) and 24,24-F_2-26,27-dimethyl-VD_3[62] (**46**) (Fig. 12). The latter showed more potent VD_3 activity than 1,25-$(OH)_2$-VD_3, whereas the former was much less active. However, the former was at least ten times more active than 1,25-$(OH)_2$-VD_3, for cell growth inhibitor of HL-60 cells. (22S)-24-Homo-26,27-F_6-1,22,25-$(OH)_2$-VD_3 (DD-003, **47**) was also synthesized as a possible drug candidate. This compound exhibited 10-fold greater inhibiting effect on the growth of HT-29 human colonic adenocarcinoma cells than 1,25-$(OH)_2$-VD_3, without calcium regulating activity.[63]

(24*R*)-1,24-$(OH)_2$-VD_3, which was synthesized at the early stage of our research in considering the importance of side chain hydroxylation, is an excellent antipsoriasis drug. This compound binds to chick intestinal cytosol receptor for 1,25-$(OH)_2$-VD_3 as equally as 1,25-$(OH)_2$-VD_3, is as active as 1α-OH-VD_3, and exhibits reduced toxicity due to rapid clearance. (24*R*)-1,24-$(OH)_2$-VD_3 is metabolized to 1,24,25-$(OH)_3$-VD_3 upon the action of 25-hydroxylase. 26,27-Cyclo-Δ^{22}-1,24-$(OH)_2$-VD_3 developed by Leo Pharmaceutical Products is a drug also for antipsoriasis.[66] 22-Oxa-1,25-$(OH)_2$-VD_3 has been developed as a drug for antipsoriasis and hyperparathyroidism by Chugai Pharmaceutical Co. Ltd.[64] 26,27-F_6-1,25-$(OH)_2$-VD_3 (**34**) has been developed as a drug of hyperparathyroidism. (23*S*,25*R*)-1,25-$(OH)_2$-VD_3-26,23-lactone (**48**) shows only osteogenesis activity without bone resorption. Furthermore, (24*R*)-24,25-$(OH)_2$-VD_3 (**20**) was found to have osteogenesis activity.[65] Thus, the compounds synthesized for investigation of the side chain structure of the VD_3 metabolites have contributed to not only biochemical and biomedical research but also the development of new drugs.

The recent research on hydroxylated steroids and their receptors opens up a new horizon of the research on biological function of steroid and its related compounds. A further investigation of VDR/NR ligands, in particular, molecular recognition of ligand and receptor would end up with a fruitful result.

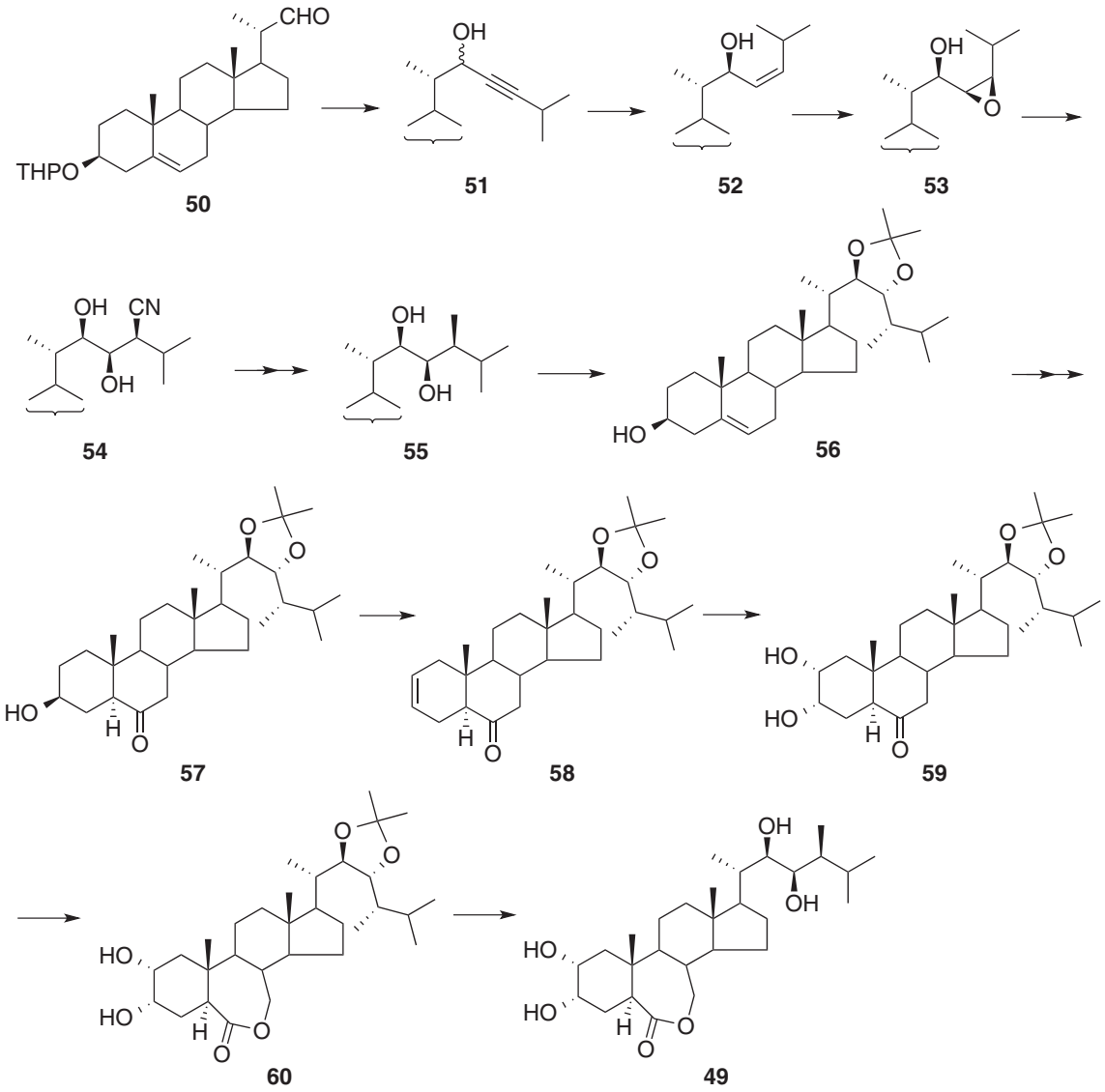

Fig. 13. Stereoselective synthesis of brassinolide.

Plant hormone brassinosteroids

1. Synthesis of brassinosteroids. In 1970, Mitchell and coworkers of the US Department of Agriculture reported that pollen of rape (*Brassica napus*) contains a new growth-promoting factor.[67] Their further efforts culminated in the isolation and structure elucidation of brassinolide (**49**) from 500 lbs. of bee-collected rape pollen in 1979.[68] A recent investigation on its receptor revealed that brassinolide binds a membrane protein which belongs to Toll-like receptor family alike mammal nuclear receptors for steroid hormones.[69]

The synthesis of brassinolide was completed by the use of Sharpless epoxidation as a key reaction to introduce the 23*R*-hydroxyl group together with 24*R*-methyl group as outlined in Fig. 13.[70] Starting from 22-aldehyde (**50**), the reaction with Grignard reagent generated from 3-methyl-1-butyn gave a mixture of 22*R* and 22*S*-hydroxy derivatives (**51**) in a similar ratio. Then, 22*R*-hydroxy derivative was converted to 23-*cis* allyalcohol (**52**). The Sharpless oxidation using vanadyl oxidant specifically provided the desired epoxyalcohol (**53**). Although direct introduction of the methyl group at C24 by opening the 23,24-epoxide was not successful, a cyano group

was introduced specifically at C24 to give 22*R*,23*R*-diol (**54**). The conversion of the cyano group to the methyl group (**55**) was successfully carried out via the aldehyde.

The AB ring was then derived to the 6,7-secolactone moiety. At first, hydroboration of 5,6-double bond for the acetonide derivative (**56**) and the following oxidation of the resulting 6α-hydroxyl group into the oxo group gave 6-oxo derivative (**57**). Then, dehydration of the hydroxyl group at C3 gave 2,3-olefin (**58**), which was oxidized to afford the 2α,3α-diol derivative (**59**). The Baeyer-Villiger oxidation with *m*-chloroperbenzoic acid gave the desired lactone (**60**) as a major product, which was converted to brassinolide through the following deprotection. The selectivity observed in this Baeyer-Villiger reaction is rather unusual, but the electronegative 2,3-diol group would control the selectivity.

The other groups reported similar synthetic routes for the synthesis.[71),72)] Furthermore, we have developed the method for synthesizing brassinosteroids such as castasterone (**61**) and dolicholide (**62**).[73)]

2. Microanalysis of brassinosteroid derivatives. Microanalytical methods for brassinosteroids were developed based on GC-MS and HPLC techniques. For GC analysis, bis-methaneboronate (BMB) ester derivative (**63**) of brassinosteroids was used.[74)] A series of the BMB derivatives were separated with detection limits at nanogram levels (Fig. 15).[75)] For HPLC, the bis-naphthaleneboronates (**64**) were preferably used for monitoring by a UV or a fluorimetric detector which enabled us to measure the amount of the derivatives down to 10–20 pg.[76)]

The structures of brassinosteroids were determined by means of MS using several ionization techniques such as FD, CI, EI and FAB methods. The selective ion monitoring in GC-MS revealed the presence of brassinone (28-norcastasterone) in the leaves of green tea and the insect galls of *Distylium racemosum*, and the presence of castasterone in the leaves and the insect galls.[77)] Dolichosterone (**65**) and castasterone were detected in the rice plant[78)] and brassinone, castasterone (**61**), brassinolide and 24-epibrassinolide (**66**) were identified in bee pollen of the broad bean.[79)] The analysis of various plant sources indicated that brassinosteroids are widely distributed in the plant kingdom as a mixture of several chemical entities (Fig. 14).[80),81)]

The microanalytical method also enabled us to quantitatively measure the location and amounts of brassinosteroids in plants. For example, brassinolide and 28-norbrassinolide were detected in leaves of *Distylium racemosum* in 23 and 156 ng/kg, respectively, but not in its insect galls, while castasterone and dolichosterone were detected in the insect galls in 2500 and 5000 ng/kg, respectively, and in the leaves in 133 and 16 ng/kg, respectively.

3. Structure-activity study and practical use of the analogs. The synthetic methods developed by us enabled to prepare more than 50 analogs of brassinosteroids for the study of structure-activity relationship analysis.[82)] Since a structure of brassinosteroids/receptor complex is not available yet, it is not clear how the hydroxyl groups in the side chain are recognized at the ligand-binding site of the receptor. However, we found that the 22,23-vicinal diol groups with 22*R*,23*R* or 22*S*,23*S* configuration are important for the receptor activation. In contrast, the isomers with 22*R*,23*S* or 22*S*,23*R* configuration were inactive. With respect to the C24 substituent, 28-norbrassinolide was equally active as brassinolide, while 28-homobrassinolide was 10-fold less active than brassinolide. Interestingly, a side chain truncated analog, 26,27-bisnorbrassinolide, was active as brassinolide.

24-Epibrassinolide (**66**) and 24-epicastasterone are rare natural brassinosteroids that have 24*S* configuration. The activity of 24-epibrassinolide was comparable to that of brassinolide. Since our group had found a considerable amount of brassicastrol (**67**) in rape seed oil by GC analysis of sterol content, we developed a production method of 24-epibrassinolide (**66**) in industrial scale (Fig. 16) as a promising candidate for agricultural application.[83)] Field testing on brassinosteroids such as 24-epibrassinolide and homobrassinolide for increasing crop yield was carried out in various countries and promising results were obtained not only for wheat, rice, soybean, and corn, but also for several vegetables.[84)]

Collaborative studies between Japan and China on the practical application of 24-epibrassinolide in agriculture were started in 1985. A series of preliminary green house tests using 24-epibrassinolide supplied by our group were carried out at the Shanghai Institute of Plant Physiology. These early studies showed that 24-epibrassinolide accelerated the growth of cereals (wheat and corn), vegetables (watermelon, cucumber and grape), and tobacco. Follow-up field trials have been pursued in Shanghai, Henan and Zhejiang Provinces over a five-years period and the significant effects of 24-epibrassinolide have been observed in this trials. 24-Epibrassinolide

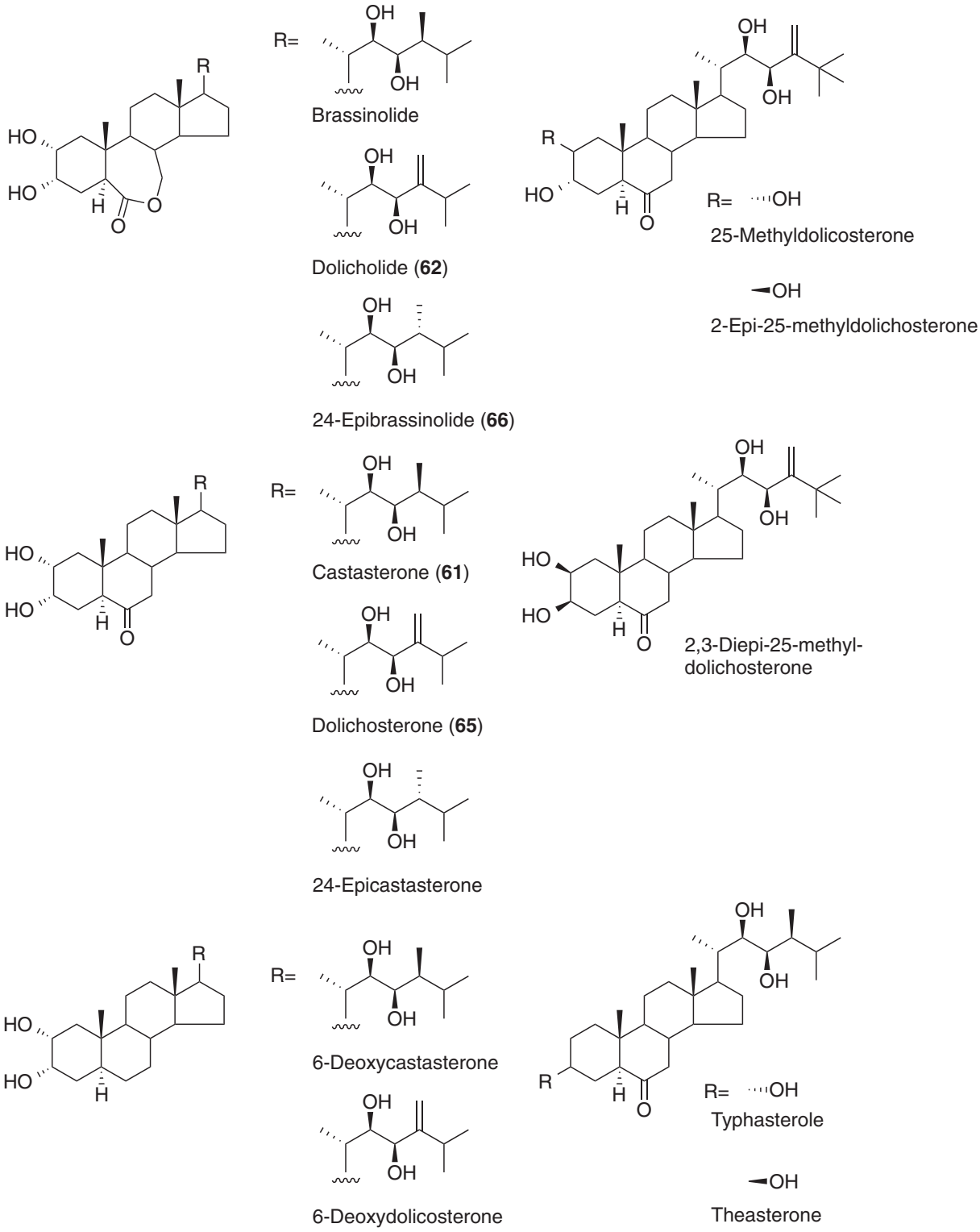

Fig. 14. Naturally occurring brassinosteroids.

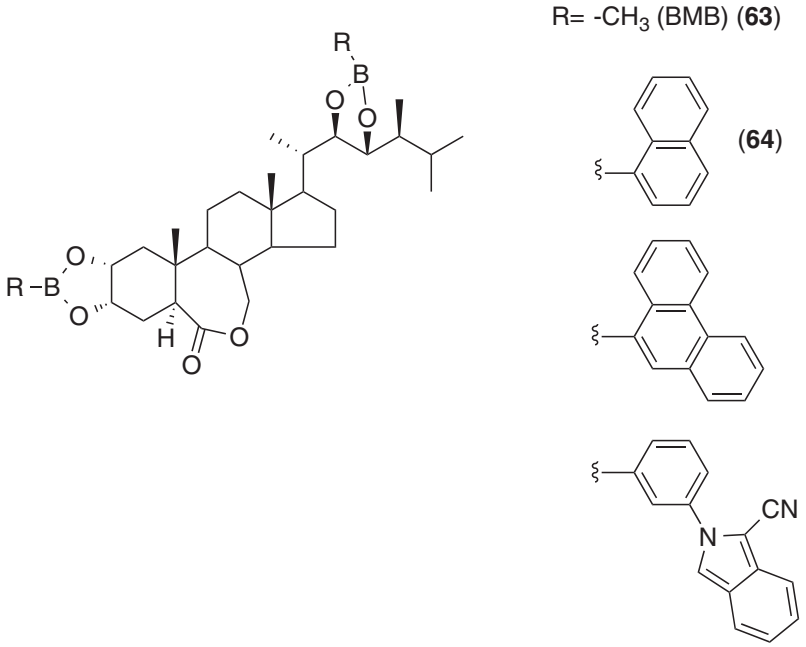

Fig. 15. Structures of bis-boronate derivatives of brassinolide.

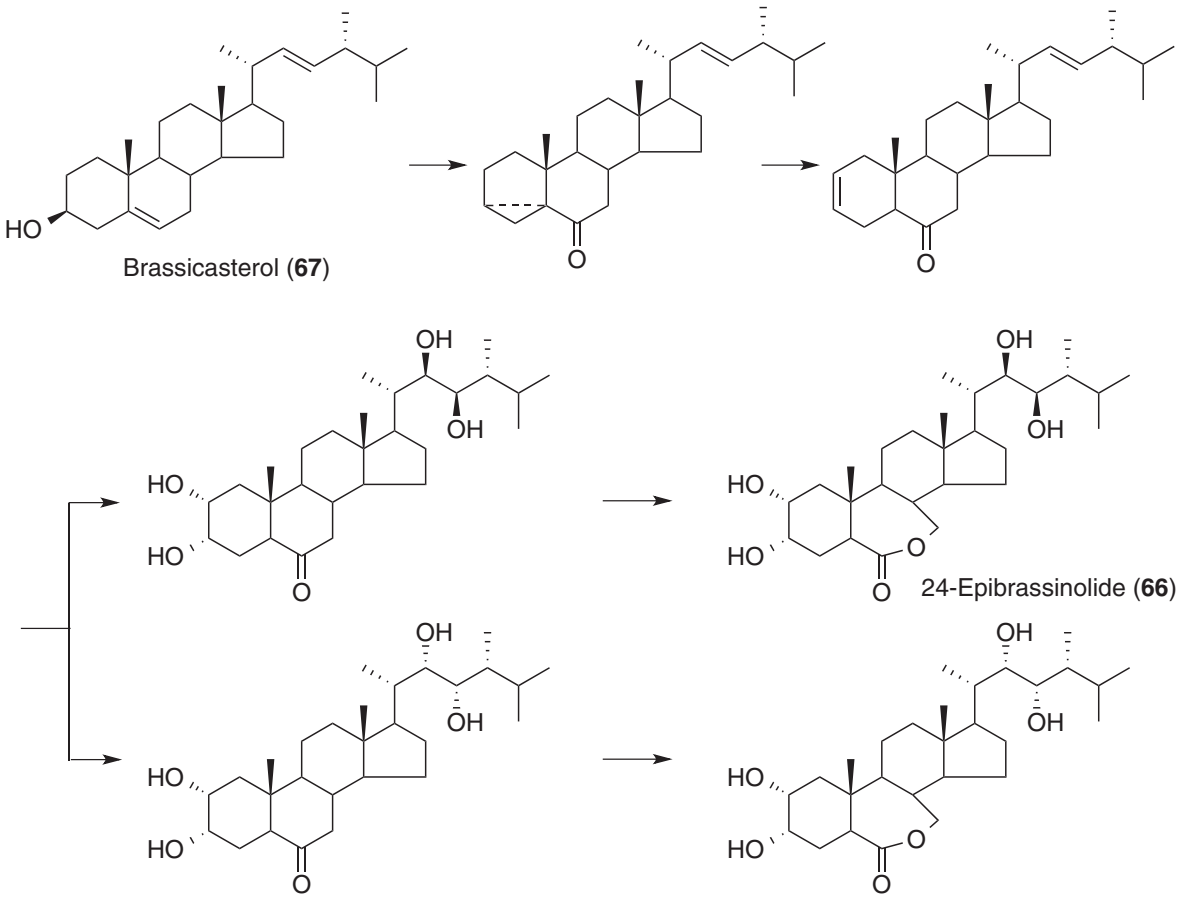

Fig. 16. Synthesis of epibrassinolide in industrial scale.

as a mixture with its 22*S*,23*S* isomer are currently used in China, Belarus and other countries.

Conclusion

Progress in chemistry and biology of the major three steroidal hormones that have oxygenated side chains has been summarized.

In insect, it has been established that fucosterol 24,28-epoxide is a key intermediate in the dealkylative conversion of sitosterol to cholesterol. An ecdysteroid biosynthesized and released in prothoracic gland was unambiguously determined as ecdysone by the application of GC-MS analysis developed by us. Furthermore, detailed analysis of free and conjugated ecdysteroids in silkworm ovaries led to the findings that the phosphate form is an inactive stocked form and converted to the free ecdysteroid during embryonic development.

The stereo-structures of vitamin D_3 metabolites such as 24,25-$(OH)_2$-VD_3 and calcidiol lactone were unambiguously determined by HPLC comparison of the natural metabolites with synthetic isomers. These basic studies have revealed the details of the metabolism of vitamin D_3 and clarified the physiological importance of the stereochemistry at the side chain of vitamin D_3. These understandings of the roles of the side chains were extended to the design and synthesis of more potent analogs having a specific and enhanced activity, which was eventually linked to the development of drugs for antipsoriasis and hyperparathyroidism.

More than 50 brassinosteroids, including the first synthesis of brassinolide, were synthesized in our laboratory and used as authentic standards in GC-MS microanalysis for natural brassinosteroids and in the study of their structure-activity relationships. These studies have ended up with the development of 24-epibrassinolide, which is an active and useful analog in agriculture.

For more than 40 years, we have investigated biologically active steroids focusing on those bearing oxygenated side chains and have clarified the importance of the position and configuration of hydroxyl groups and number of carbons in the oxygenated side chains. It is interesting that simple sterols such as cholesterol and campesterol serve as the substrates of oxidative metabolisms and the resulting oxygenated metabolites, 20-hydroxyecdysone, brassinolide and 1,25-$(OH)_2$-VD_3 commonly play roles of physiologically important hormones in their growth in insects, plants and human, respectively. Thus, the modification and utilization of steroid side chains commonly occur and are important in maintenance of the physiological conditions of the life. It should be finally mentioned that intimate collaborations with prominent biologists enabled us to bring fruitful results mentioned above.

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Profile

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He is the author and co-author of more than 400 original papers and close 100 review articles, dealing mainly with investigation of naturally occurring biologically important substances. He is a pioneer in the field of the separation and isolation of a number of steroids and also contributed to the synthesis of natural and designed steroids.

He edited four-volume book on Modern Gas Chromatography (Hirokawa Pub.) and served on the editorial board of journals of the Pharmaceutical Society of Japan.

He received Sato Memorial Award (1979), Society Award (1981) and Member of Merit Award (1998) from Pharmaceutical society of Japan, and Order of the Rising Sun, Gold Rays with Neck ribbon (2002) from the Japanese Government. He also received M. S. Tswett Chromatography Award (1982), Shimadzu Award (1992) and Career Recognition Award of Vitamin D (1994).

He was a board of directors of Pharmaceutical Society of Japan (1984–1991) and also a director of Niigata Bio-Research Center (2004–2012).

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Profile

Yoshinori Fujimoto was born in Yamaguchi prefecture in 1949. He completed his Ph.D. for a thesis entitled "Studies on the mechanism of the conversion of phytosterol to cholesterol in insects" under the supervision of Prof. Nobuo Ikekawa in Faculty of Science and Engineering at Tokyo Institute of Technology in 1979. In the same year, he joined Prof. Charles J. Sih's laboratory, School of Pharmacy, University of Wisconsin-Madison as a research fellow and spent three years there, where he worked on the mechanism of microbial degradation of sitosterol side chain and quantitative analyses of biochemical kinetic resolutions of enantiomers. He was appointed to an assistant professor in Department of Chemistry of Tokyo Institute of Technology in 1984 and then promoted to an associate professor in 1990. In 1996, he was promoted to professor, and joined a newly organized Department of Chemistry and Materials Science, Graduate School of Science and Engineering of the same university in 1997. He has been carried out researches in the field of natural product chemistry including biosynthesis of plant and insect sterols, isolation and structure elucidation of new secondary metabolites from plants, insects and marine invertebrates, and synthesis of steroidal compounds having unique side chains. His current research is focused on the identification of genes encoding enzymes that catalyze sterol metabolism in insects as well as plants.

Profile

Masaji Ishiguro was born in 1950 and received his Ph.D. in 1977 from Tokyo Institute of Technology. He worked at the laboratory of Prof. E. J. Corey in Harvard University as postdoctoral fellow in 1977 through 1979. While he worked on the synthesis of natural products in Harvard, he was greatly influenced on the computer-aided chemistry developed in the laboratory and thus applied the technology to designing biologically active molecules based on the structures of target biomolecules. Since he joined Suntory Institute for Biomedical Research in 1980, he directed the computer-aided drug design and succeeded in development of new β-lactam antibiotics. A penem β-lactam named Farom (Faropenem) was launched in the market in 1995. For this work, he was the first recipient of The Pharmaceutical Society of Japan Award for Drug Research and Development (2001). In 1995 he moved to Suntory Institute for Bioorganic Research (SUNBOR) and started the investigation of receptor-ligand interactions using computational methods focusing on G protein-coupled receptors (GPCR). In 2006, he was appointed to a professor in chemical biology laboratory of Niigata University of Pharmacy and Applied Life Sciences (NUPALS), continuing his research in SUNBOR as senior research fellow until his retirement in 2010. His major subject in chemical biology field has been molecular recognition in vision, odor, and taste. The crystal structure of squid rhodopsin, a member of GPCR, was solved in collaboration with Dr. S. Miyano's group of RIKEN in Harima and the molecular base of recognition of sweet taste molecules by sweet taste receptor (T1R2/T1R3) was established with collaboration with Prof. K. Abe's group of the university of Tokyo. Currently, he is a professor and dean of the faculty of Applied Life Sciences of NUPALS.