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Optimization of Chemical Syntheses of Vitamin D C3-Epimers

LARS KATTNER and ERIK RAUCH

Endotherm Life Science Molecules, Saarbrücken, Germany

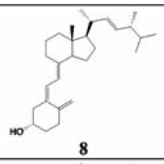
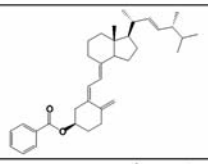
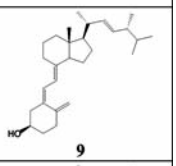
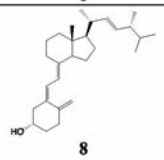
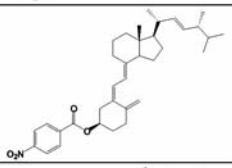
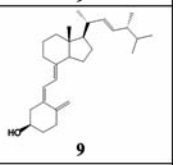
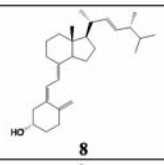
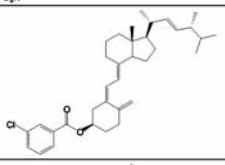
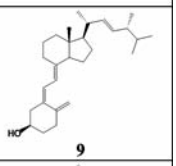
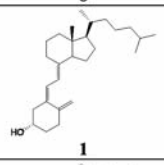
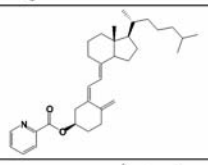
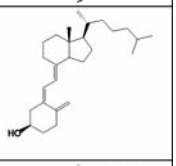
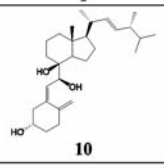
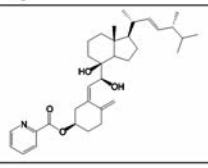
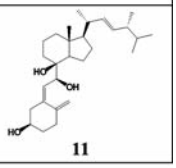
Abstract. *Due to the widespread impact of vitamin D on human health, the development of appropriate assays to detect deficiency of all vitamin D metabolites of pharmacological interest is being continuously improved. Although over 50 naturally-occurring metabolites of vitamin D are known to date, only very few are routinely detected in commercially available assays. This is particularly true regarding C3-epimers of vitamin D_3 and D_2, which not only may interfere in analytical measurements with other metabolites of interest, but also have controversial and not yet fully understood physiological functions. In this study we optimized a synthetic method to obtain various vitamin D_3 and D_2 C3-epimers in order to make them available in gram quantities for further evaluation and for their use in assay development or drug discovery. Particularly, the inversion of the C3-OH group at the A-ring of vitamin D_2, which, in turn, serves as a suitable starting material for most of chemical syntheses of vitamin D metabolites, can be converted to the corresponding C3-epimer under so-called "Mitsunobu conditions". Thus, the C3-OH group is converted into the corresponding ester by treatment with an aromatic acid, subsequent addition of an azodicarboxlate and triphenylphoshine, leading to the corresponding ester, concomitant to the inversion of the stereogenic center at C3. Reduction or saponification of the resulting ester finally leads to the corresponding C3-epimer, that may serve as starting material for a wide variety of vitamin D_3 and D_2 C3-epimers.*

Due to the widespread impact of vitamin D on human health, the development of appropriate assays to measure the status of vitamin D metabolites in human serum/plasma or relevant tissue is continuously being improved, mainly with the aim to detect and thus prevent vitamin D deficiency, that is considered to cause a wide variety of diseases, including cancer of the breast, colon and pancreas (1-3). Additionally, vitamin D metabolites may serve as starting points for the development of novel therapeutic rationales (4-6). Although over 50 natural metabolites of vitamin D are known to date (7, 8), only very few are routinely measured in commercially available assays (9-13), thus neglecting the impact of most other metabolites of potential relevance (14-16). Regarding its metabolism, vitamin D_3 (**1**) (Figure 1), generated mainly by UV irradiation of 7-dehydrocholesterol in the skin, is hydroxylated in the liver to 25-hydroxyvitamin D_3 (**2**), which is subsequently hydroxylated in the kidney to 1α,25-dihydroxyvitamin D_3 (**3**, calcitriol), in turn apparently the medicinally most relevant metabolite. **2** is metabolized to other oxidative products, such as 24(R),25-dihydroxyvitamin D_3 (**4**), mediated by the enzyme CYP24, followed by subsequent enzymatic degradation of the carbon side chain. **3** is degradated analogously in a parallel metabolism pathway. Additionally, presumably all vitamin D metabolites can be metabolized separately through a C3 epimerization pathway, leading to C3-*epi*-metabolites such as **5-7** with an inversion of the stereogenic center at position C3 of the respective molecule (17-22). Additionally, the corresponding metabolites of vitamin D_2 (**8**) have to be recognized, because food from plant origin and food supplements may contain vitamin D_2, and its metabolites are considered to have similar physiological functions comparing to their corresponding vitamin D_3 counterparts (23), although the metabolism products slightly vary due to an additional methyl group at C24 and a double bond at C22-23 (8), and their potency seems apparently lower. Interestingly, C3-*epi*-dihydroxyvitamin D_2 has been identified along with elevated concentrations of C3-*epi*-hydroxyvitamin D_3 in serum of young children (22, 24). Consequently, all naturally-occurring C3-epimers of vitamin D_3 and D_2 deserve attention, because some of them may not only interfere in analytical measurements with other metabolites of interest, but also have controversial and not yet fully understood physiological functions. Thus, a flexible approach towards the chemical synthesis of all relevant vitamin D C3 epimers is highly desirable in order to make them available in sufficient quantities for their evaluation.

Correspondence to: Lars Kattner, Endotherm Life Science Molecules, Science Park 2, 66123 Saarbrücken, Germany.

Key Words: Cancer prevention, vitamin D metabolites, epimers, assay development, stereoselective synthesis.

Table I. *Starting materials, reagents, reaction conditions, yields and products.*

Entry	Starting material	Reagents, reaction conditions, yield	Product A	Reagents, reaction conditions, yield	Product B
1	 **8**	benzoic acid, DIAD, PPh_3, toluene, RT, 19%		lithium aluminium hydride, diethyl ether, 48%	 **9**
2	 **8**	4-nitrobenzoic acid , DIAD, PPh_3, THF, RT, 18%-33%		lithium aluminium hydride, diethyl ether, 74%	 **9**
3	 **8**	3-chlorobenzoic acid, DEAD, PPh_3, toluene -THF (4:1), RT, 28%; 3-chlorobenzoic acid, DEAD, PPh_3, toluene, RT, 30%; 3-chlorobenzoic acid, DIAD, PPh_3, toluene, RT, 30%		KOH, methanol-diethyl ether (1:1), 88% / 57% / 75%	 **9**
4	 **1**	2-picolinic acid, DIAD, PPh_3, toluene, RT, 47%		$Cu(OAc)_2$, chloroform-methanol (95:5), 22% over 2 steps	
5	 **10**	2-picolinic acid, DIAD, PPh_3, toluene, RT, 64%		$Cu(OAc)_2$, chloroform-methanol (95:5), 64% over 2 steps	 **11**

DEAD: diethyl azodicarboxylate, PPh_3: triphenylphosphine, RT: room temperature, THF: tetrahydrofurane, DIAD: diisopropyl azodicarboxylate, KOH: potassium hydroxide, $Cu(OAc)_2$: copper(II) acetate.

Materials and Methods

Most routine assays, particularly RIA and ELISA, are competitive assays, where the metabolite of interest competes with a corresponding labeled metabolite for binding to assay specific antibodies or DBP. Although these techniques are suitable for automated high-throughput analysis of samples, they are often restricted to measure only one metabolite, *i.e.* 25-hydroxyvitamin D_3 **(2)** suffering from cross-reactivity (low specificity) and lacking sensitivity. For instance, the presence of 3-*epi*-25-hydroxyvitamin D_3 **(6)** in the sample may either not be detected at all, or lead to overestimated concentrations of 25-hydroxyvitamin D_3 (18), Low-abundant metabolites are widely neglected. By contrast, mass spectrometry, particularly liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is currently considered the "gold standard", allows measurement of various metabolites, including C3-epimers and other low-abundant metabolites, in one sample at the same time with high accuracy (11-16). Usually, chemically synthesized stable metabolites, in turn labeled with isotopes (^2H or ^{13}C) are used as internal standards for this purpose. However, advanced LC equipment and material is needed for accurate separation of all relevant metabolites.

In this study we explored several synthetic methods to invert the configuration of the stereogenic center at C3 of the intact vitamin D skeleton with the aim to apply the most efficient method to the synthesis of various vitamin D_3 and D_2 C3-epimers (Table I).

It has already been explored previously, that readily-available vitamin D_2 (**8**) is a most suitable starting material for the chemical synthesis of many vitamin D metabolites of interest (6, 7, 25) (Figure 2). The inversion of the configuration of the C3-OH group (from β to α) at the A-ring of vitamin D, leading to the corresponding C3-epimer, can be accomplished most appropriately under so-called "Mitsunobu conditions" (26). Thus, vitamin D_2 or a related derivative thereof is treated with an aromatic acid, an azodicarboxlate and triphenylphoshine, leading to formation of a corresponding ester, concomitant to the inversion of the configuration of the stereogenic center at C3. Reduction or saponification of the resulting ester finally leads to the corresponding C3-epimer, which may serve as starting material for a wide variety of other vitamin D_3 and D_2 C3-epimers. Two alternative strategies can be applied, either by leaving the vitamin D skeleton intact and proceed with **9** in the synthesis, or by conversion of **8** to bishydroxylated **10**, followed by inversion of C3 configuration leading to **11**, cleavage of the molecule in an A-ring **12** and CD ring **13**, appropriate chemical modification of these both building blocks,

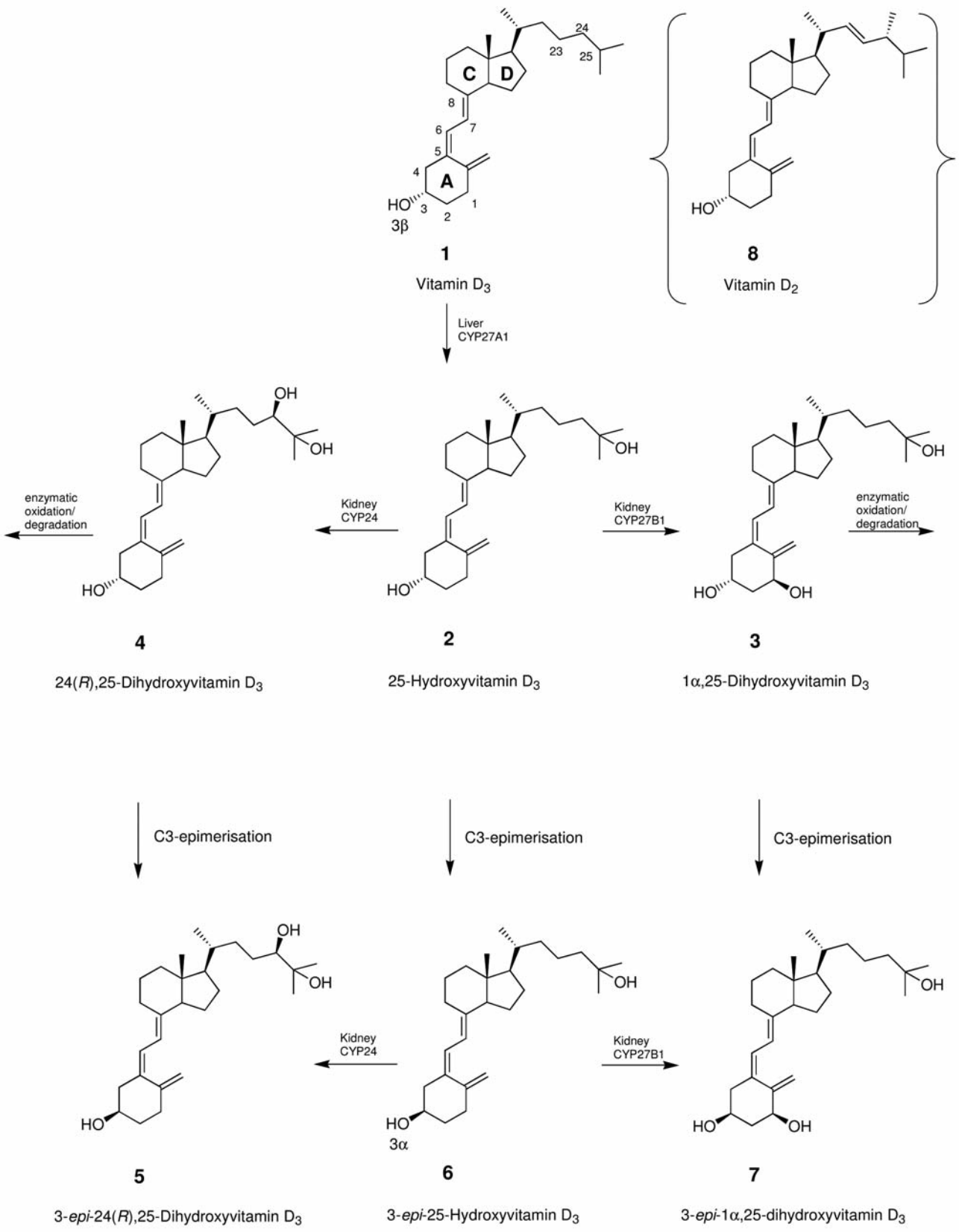


Figure 1. *Metabolic pathways of vitamin D*.

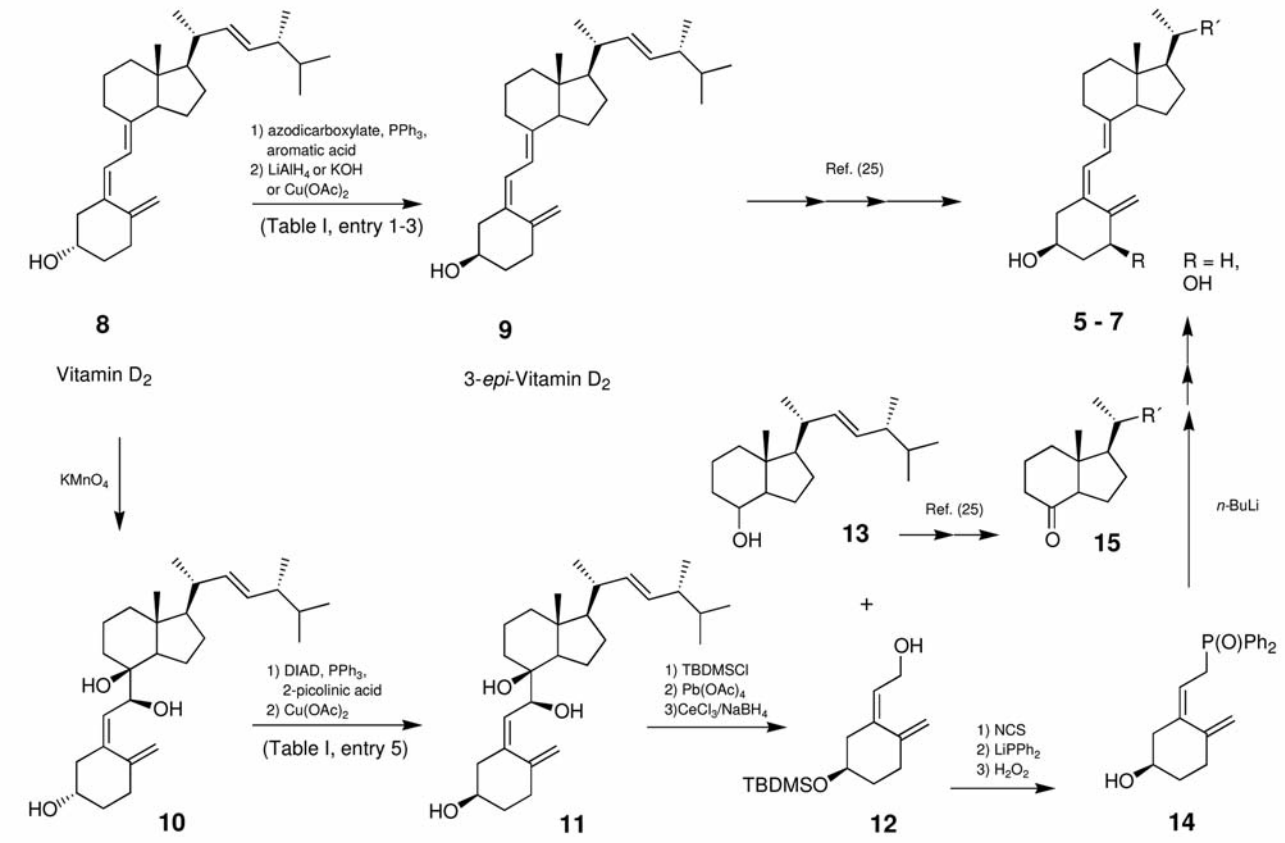


Figure 2. *General strategies for the synthesis of vitamin D C3-epimers. PPh_3: Triphenylphosphine, $LiAlH_4$: lithium aluminium hydride, KOH: potassium hydroxide, $Cu(OAc)_2$: copper(II) acetate, $KMnO_4$: potassium permanganate, DIAD: diisopropyl azodicarboxylate, TBDMSCl: tert-butyldimethylsilyl chloride, $Pb(OAc)_4$: lead tetraacetate, $CeCl_3$: Cer(III)-chloride, $NaBH_4$: sodium borohydride, NCS: N-chlorosuccinimide, $LiPPh_2$: lithium diphenylphosphide, H_2O_2: hydrogen peroxide, n-BuLi: n-butyllithium.*

and connection of the A-ring as a phosphine oxide **14** with an appropriate CD-ring ketone **15**.

Results and Discussion

The results of exploration of various starting materials, reagents and reaction conditions towards the synthesis of C3-*epi*-vitamin D derivatives are shown in Table I.

Vitamin D_2 (**8**), vitamin D_3 (**1**), and 7,8-bishydroxylated vitamin D_2 (**10**) served as starting material. Different acids (benzoic acid, 3-chlorobenzoic acid, 4-nitrobenzoic acid, 2-picolinic acid), various azodicarboxylates, such as diethyl- and diisopropyl-azodicarboxlate (DEAD, DIAD), as well as different solvents were employed. Additionally, reaction time and temperature were optimized.

Reaction of Vitamin D_2 (**8**) with benzoic acid, 3-chlorobenzoic acid and 4-nitrobenzoic acid (Table I, entries 1-3) gave just moderate yields (18%-33%) of the corresponding esters, mainly due to elimination reaction, leading to a presumably favored product containing a conjugated 3,4-5,6-7,8-*all-trans*-triene system. Although, cleavage of the esters by reduction with lithium aluminium hydride (Table I, entries 1-2) or saponification with potassium hydroxide (Table I, entry 3) could be carried out in reasonable yields (48%-88%). The most suitable acid for ester formation was picolinic acid, which gave the corresponding ester of vitamin D_3 (**1**) as a starting material in 47% yield (Table I, entry 4). In order to avoid the formation of a triene system by elimination in the course of esterification, 7,8-bishydroxylated vitamin D_2 (**10**) was employed as a starting material for the reaction with picolinic acid (27). Indeed, the corresponding ester could be obtained in good yield (64%) (Table I, entry 5). The cleavage of the ester with copper(II) acetate was optimized to yield 64% of the corresponding alcohol **11**. It has to be recognized that these conditions are quite mild, making them suitable for highly sensitive substrates. This approach is favored to proceed in a connective synthesis using building blocks **12-15** (Figure 2). By contrast, the use of vitamin D_2 (**8**) as a starting material, 4-nitro benzoic acid for esterification, and saponification with potassium hydroxide for ester cleavage appeared as most suitable for practical reasons to

obtain C3-*epi* derivatives to proceed in a non-connective synthesis *via* **9**, leaving the vitamin skeleton intact.

Conclusion

Inversion of the configuration at the C3 stereogenic center of vitamin D_2 or another appropriate vitamin D derivative under "Mitsunobu conditions" was optimized and can finally be carried out in gram scale, leading to products suitable for the synthesis of a wide variety of natural 3-*epi* vitamin D metabolites and analogs. Measurement of these low-abundant metabolites, favorably by LC-MS/MS, and thus assessment of their distribution in human blood or relevant tissue may open up a new avenue for physicians and clinicians for diagnosis, treatment and risk prediction of vitamin D-dependent diseases.

Acknowledgements

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