molecules

MDPI

Communication

Synthesis of Deuterium-Labeled Vitamin D Metabolites as Internal Standards for LC-MS Analysis

Akiko Nagata [1], Kazuto Iijima [1], Ryota Sakamoto [1], Yuka Mizumoto [1], Miho Iwaki [1], Masaki Takiwaki [2], Yoshikuni Kikutani [2], Seketsu Fukuzawa [2], Minami Odagi [1], Masayuki Tera [1] and Kazuo Nagasawa [1,*]

[1] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan; s193819x@st.go.tuat.ac.jp (A.N.); s206189q@st.go.tuat.ac.jp (K.I.); s218969x@st.go.tuat.ac.jp (R.S.); s200343y@st.go.tuat.ac.jp (Y.M.); s218363r@st.go.tuat.ac.jp (M.I.); odagi@cc.tuat.ac.jp (M.O.); tera@go.tuat.ac.jp (M.T.)

[2] Medical Equipment Business Operations, Management Strategy Planning Division, JEOL Ltd., Akishima, Tokyo 196-8558, Japan; mtakiwak@jeol.co.jp (M.T.); ykikutan@jeol.co.jp (Y.K.); sfukuzaw@jeol.co.jp (S.F.)

* Correspondence: knaga@cc.tuat.ac.jp; Tel.: +81-42-388-7295

Abstract: Blood levels of the vitamin D_3 (D_3) metabolites 25-hydroxyvitamin D_3 (25(OH)D_3), 24*R*,25-dihydroxyvitamin D_3, and 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) are recognized indicators for the diagnosis of bone metabolism-related diseases, D_3 deficiency-related diseases, and hypercalcemia, and are generally measured by liquid-chromatography tandem mass spectrometry (LC-MS/MS) using an isotope dilution method. However, other D_3 metabolites, such as 20-hydroxyvitamin D_3 and lactone D_3, also show interesting biological activities and stable isotope-labeled derivatives are required for LC-MS/MS analysis of their concentrations in serum. Here, we describe a versatile synthesis of deuterium-labeled D_3 metabolites using A-ring synthons containing three deuterium atoms. Deuterium-labeled 25(OH)D_3 (**2**), 25(OH)D_3-23,26-lactone (**6**), and 1,25$(OH)_2D_3$-23,26-lactone (**7**) were synthesized, and successfully applied as internal standards for the measurement of these compounds in pooled human serum. This is the first quantification of 1,25$(OH)_2D_3$-23,26-lactone (**7**) in human serum.

Keywords: vitamin D; deuterium labeling; liquid-chromatography tandem mass spectrometry; measurement of vitamin D metabolites in blood

Citation: Nagata, A.; Iijima, K.; Sakamoto, R.; Mizumoto, Y.; Iwaki, M.; Takiwaki, M.; Kikutani, Y.; Fukuzawa, S.; Odagi, M.; Tera, M.; et al. Synthesis of Deuterium-Labeled Vitamin D Metabolites as Internal Standards for LC-MS Analysis. *Molecules* **2022**, *27*, 2427. https://doi.org/10.3390/molecules27082427

Academic Editor: Giovanni Ribaudo

Received: 19 March 2022
Accepted: 6 April 2022
Published: 9 April 2022

1. Introduction

Vitamin D_3 (D_3) (**1**) is metabolized by members of the cytochrome P450 (CYP) family to generate more than 50 compounds in vivo. Among them, 25-hydroxyvitamin D_3 (25(OH)D_3) (**2**) is generated from D_3 (**1**) by CYP2R1 and/or CYP27A1-mediated hydroxylation at C25 in the liver, and the resulting 25(OH)D_3 (**2**) is further metabolized to the active form of D_3, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) (**3**), by CYP27B1-mediated oxidation at C1α in the kidneys (Figure 1). 1,25$(OH)_2D_3$ (**3**) plays a key role in the regulation of bone metabolism in vivo [1]. In normal conditions, production of **3** from **2** is strictly controlled by the concentrations of calcium and parathyroid hormone (PTH) in the blood, and thus the concentration of **3** in the blood is a useful indicator of functional status [2] and is helpful in the diagnosis of diseases such as hypercalcemia, hyperphosphatemia, rickets, and bone metabolism-related diseases [3,4]. The concentration of **2** in the blood is also useful as an indicator for the diagnosis of various vitamin D deficiency-related diseases [3,4]. Recently, various D_3 metabolites, mostly oxidized at the D-ring side chain, have also been found to show biological activities. For example, 20*S*,25$(OH)_2D_3$, which is generated by CYP11A1, inhibits the growth of keratinocytes, leukemia cells, and melanoma cells [5–9], while 24*R*,25$(OH)_2D_3$ (**4**), produced by CYP24A1, shows inhibitory activity against various cancer cell lines [10,11]. Further, 25(OH)D_3-23,26 lactone (**6**) and 1,25$(OH)_2D_3$-23,26 lactone (**7**), which are thought to be final metabolites of D_3, show antagonistic activity

towards 1,25(OH)$_2$D$_3$ [12–14], thereby, inhibiting bone formation and resorption. Recently, compound **7** was reported to inhibit fatty acid oxidation [15]. Thus, there is a need to measure the blood levels of these metabolites.

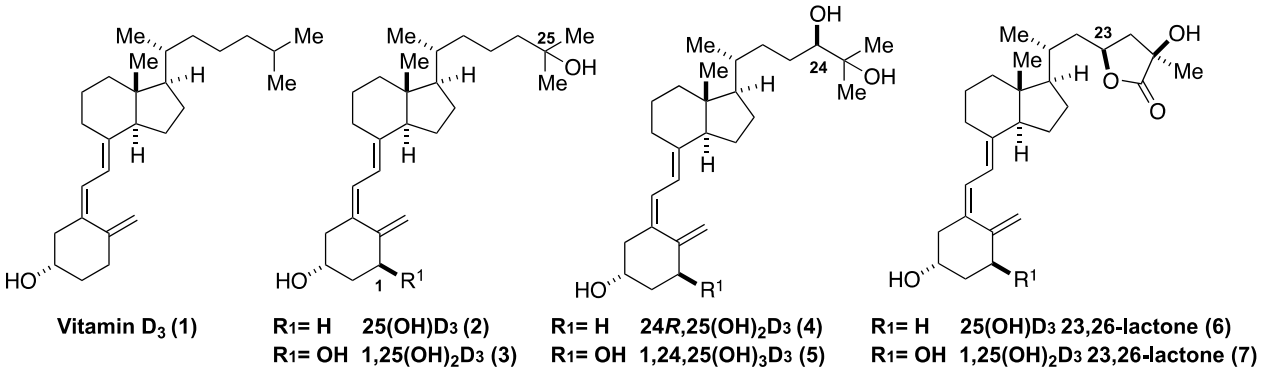


Figure 1. Structures of vitamin D$_3$ (**1**) and its metabolites (**2**)–(**7**).

The concentrations of metabolites **2** and **3** in blood have been measured for clinical purposes by radioimmunoassay (RIA) or chemiluminescent enzyme immunoassay (CLEIA) [16]. They, however, have disadvantages such as the need to handle radioactive materials, and insufficient discrimination of vitamin D metabolites by antibodies [17]. More recently, a liquid-chromatography tandem mass spectrometry (LC-MS/MS) method has been developed to determine the concentration of multiple vitamin D metabolites simultaneously in blood [18]. However, LC-MS/MS-based measurement also has some problems, such as the low ionization efficiency of vitamin D derivatives and interference by contaminants including multiple D$_3$ metabolites in the blood. To address these issues, several approaches have been investigated. Cookson-type reagents have been developed to improve the ionization efficiency of D$_3$ metabolites, affording high sensitivity even at low abundance [19,20]. The isotope dilution method has also been applied to avoid interference from contaminants in the blood. This method requires a stable isotope-labeled compound as an internal standard, and so far, deuterium-labeled 25(OH)$_2$D$_3$ (**2**), 1,25(OH)$_2$D$_3$ (**3**), and 24*R*,25(OH)$_2$D$_3$ (**4**), in which deuterium is introduced at C26, C27, C6, and C19, have been synthesized (Figure 2) [21–25].

In the synthesis of the deuterium-labeled metabolites **2**–**4**-d_6, deuterium was introduced into the side chain at C26 and C27 by reacting esters **8** with deuterated Grignard reagent, CD$_3$MgBr (Figure 2B) [21,22]. On the other hand, **2**–**3**-d_3 were synthesized by reacting SO_2 adducts of cyclic compounds **9** derived from D$_3$ with deuterium oxide (D$_2$O) [23–25]. In both strategies, the range of metabolites that can be synthesized is limited due to the restrictions imposed by the use of steroid precursors. Therefore, a more versatile approach is required. Convergent strategies, with coupling between CD-ring and A-ring moieties, have been widely applied for the synthesis of D$_3$ derivatives [26,27]. Since the CD-ring structures of the metabolites are diverse, whereas the A-ring structures are relatively constant, we considered that deuterium-labeled A-ring synthons would be suitable for the preparation of a variety of deuterium-labeled D$_3$ metabolites (Figure 2D). In addition, labeling in the A-ring has an advantage in metabolism studies because the side chains of the D3 are well known to be enzymatically metabolized easily. In this study, we have developed a synthesis of deuterium-labeled A-ring precursors **13**-d_3 and **16**-d_3 incorporating three deuterium atoms. These precursors were coupled with CD-ring moieties **17** and **18** to afford deuterium-labeled 25(OH)D$_3$-d_3 (**2**-d_3) and vitamin D lactones 25(OH)D$_3$-23,26-lactone-d_3 (**6**-d_3) and 1,25(OH)$_2$D$_3$-23,26-lactone-d_3 (**7**-d_3). We also confirmed that the concentrations of **2**, **6**, and **7** in human serum could be measured by LC-MS/MS using the corresponding deuterium-labeled compounds as the internal standards (IS) (see Supplementary Materials).

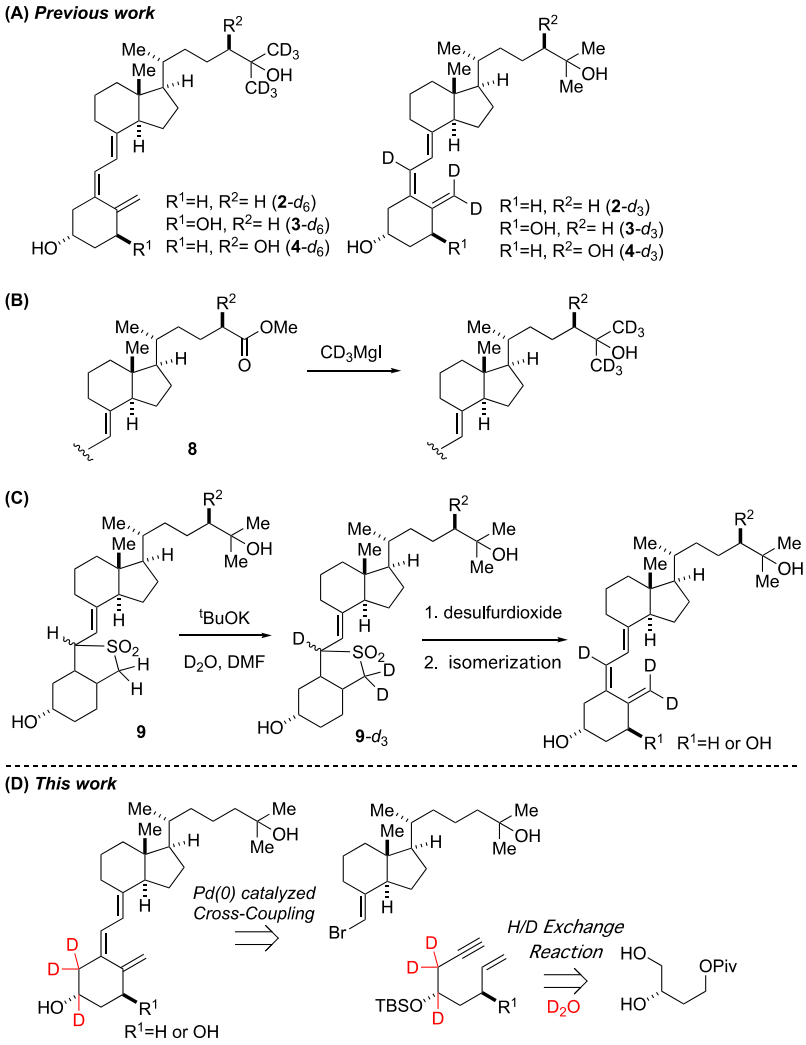
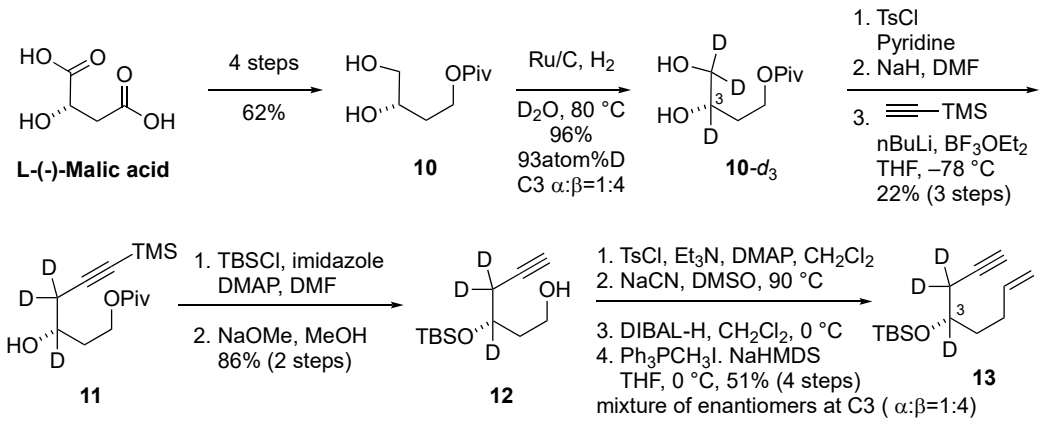


Figure 2. Synthetic strategy of deuterium-labeled D_3 metabolites. (**A**) Structures of reported deuterium-labeled D_3 metabolites. (**B**) Previous work on the synthesis of **2**–**4**-d_6. (**C**) Previous work on the synthesis of **2**–**4**-d_3 (**D**) This work: general synthesis of deuterium-labeled D_3 metabolites.

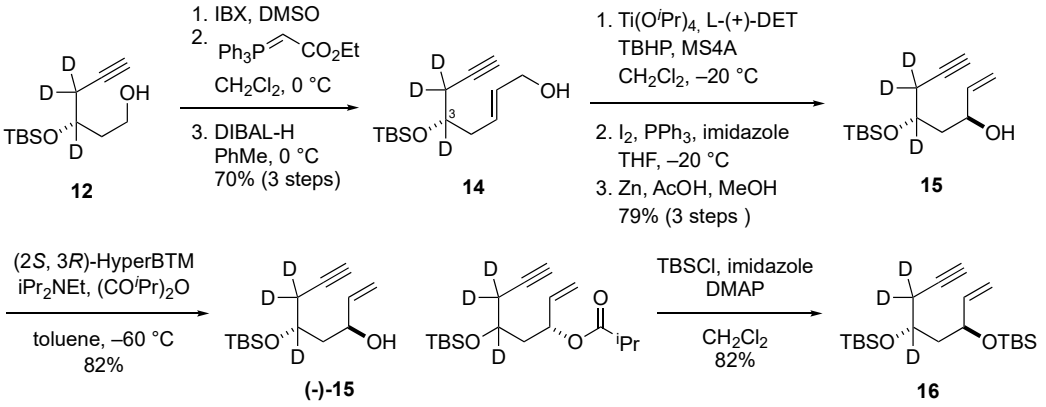
2. Results

We employed a convergent strategy using the palladium-catalyzed coupling reaction of enyne-type deuterium-labeled A-ring precursors **13**-d_3 and **16**-d_3 with bromoolefins **17** and **18** as the CD-ring moieties. The deuterium atoms in **13**-d_3 and **16**-d_3 were introduced by the H/D exchange at the a-position of the alcohol, as reported by Sajiki et al. [28]. Our synthesis of deuterium-labeled enyne **13** commenced with the H/D exchange reaction of alcohol **10**, which was obtained from L-(-)-malic acid in 4 steps (Scheme 1) [29]. The alcohol **10** was subjected to the H/D exchange reaction with a catalytic amount of Ru/C in D_2O at 80 °C under an H_2 atmosphere to afford **10**-d_3 deuterium-labeled at C3 and C4 in a 96% yield with over 93% deuteride content [28]. In this reaction, the stereochemistry at C3 was isomerized (4:1 ratio of α-**10a** and β-**10b**). The deuterium-labeled alcohol **10** (enantiomeric mixture) was converted into alkyne **11** by tosylation of the primary alcohol followed by epoxidation with NaH and reaction with TMS-acetylene (22% yield from **10**-d_3). The hydroxyl group in alkyne **11** was protected with TBS ether, followed by deprotection of the TMS and pivaloyl groups with NaOMe in MeOH to give the alcohol **12** in an 86% yield from **11**. Enyne **13** was obtained in a 51% yield from **12** via 4 steps, (i) tosylation of the primary alcohol; (ii) cyanation with NaCN; (iii) reduction of the nitrile group with DIBAL-H to aldehyde; and (iv) a Wittig reaction with Ph_3PCH_3I and NaHMDS. It was confirmed by ^{1}H-NMR that the deuteration rate did not decrease in these reaction steps [30].



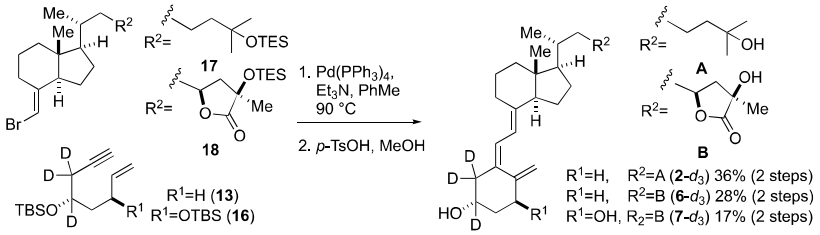
Scheme 1. Synthesis of deuterium-labeled enyne **13**-d_3.

Next, the deuterium-labeled enyne **16** bearing a hydroxyl group at C1α was synthesized (Scheme 2). The alcohol moiety in **12** was oxidized with an IBX and the resulting aldehyde was reacted with a HWE Wittig reagent to give an unsaturated ester, whose ester group was reduced with a DIBAL-H to give allyl alcohol **14** in a 70% yield from **12** [31,32]. The allyl alcohol **14** was subjected to a Sharpless asymmetric epoxidation with a TBHP in the presence of $Ti(OiPr)_4$ and L-(+)-DET [33], and the resulting epoxy alcohol was subjected to iodination with iodine and triphenylphosphine followed by treatment with zinc to give a secondary alcohol **15** in a 79% yield (3 steps) [31,32]. The diastereomer ratio at C1 in **15** was 10:1, and the undesired C1β diastereomer was removed by kinetic resolution, using acylation with isopropyl acid anhydride in the presence of (2*S*,3*R*)-HyperBTM [34], to give (-)-**15** in an 82% yield as a single diastereomer. The undesired diastereomer at C3 was also removed via silica gel column purification. The deuterium-labeled enyne **16**, in which the secondary alcohol was protected as the TBS ether, was obtained in an 82% yield.



Scheme 2. Synthesis of deuterium-labeled enyne **16**-d_3.

The palladium-catalyzed coupling reaction of **13**-d_3 and bromoolefin **17** followed by deprotection of the silyl groups provided 25(OH)D_3-d_3 (**2**-d_3) in a 36% yield [35]. Next, 25(OH)D_3-23,26-lactone-d_3 (**6**-d_3) and 1,25$(OH)_2D_3$-23,26-lactone-d_3 (**7**-d_3) were similarly synthesized by reacting bromoolefin **18** and enynes **13**-d_3 and **16**-d_3, respectively [36]. In the synthesis of **2**-d_3 and **6**-d_3, the undesired diastereomers at C3α were separated by an HPLC (Scheme 3).



Scheme 3. Synthesis of vitamin D_3 metabolites-d_3 (**2**-d_3, **6**-d_3, **7**-d_3).

2.1. Derivatization of **2**, **6**, **7** *for LC-MS/MS, and Preparation of Calibration Curves*

With the deuterium-labeled D_3 metabolites of **2**-d_3, **6**-d_3, and **7**-d_3 in hand, we next examined the quantitative analysis of the three D_3 metabolites in pooled human serum by LC-MS/MS. First, we confirmed that our deuterium-labeled D_3 metabolites were suitable as the internal standards for the isotope dilution method in an LC-MS/MS analysis. As described above, D_3 and its metabolites have low ionization efficiency in an LC-MS/MS, and derivatization is necessary to improve the ionization efficiency. Thus, the D_3 metabolites **2**, **6**, and **7**, as well as **2**-d_3, **6**-d_3, and **7**-d_3, were derivatized with a recently developed reagent DAP-PA (4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione-phenyl anthracene) [20], and the ion peaks of the DAP adducts were detected by selective reaction monitoring (SRM) under the LC-MS/MS conditions shown in Table 1 (Figure 3).

Table 1. Parameters for the LC/MS/MS analysis.

Compound	SRM Transition (*m*/*z*)	Cone Voltage (kv)	CE (eV)
25(OH)D_3-DAP (**2-DAP**)	619.4 > 341.2 $[M + H]^+$ $[A]^+$	48	28
25(OH)D_3-23,26-lactone-DAP (**6-DAP**)	647.4 > 341.2	48	28
1,25(OH)$_2$$D_3$-23,26-lactone-DAP (**7-DAP**)	663.4 > 357.2	48	28
25(OH)D_3-d_3-DAP (**2**-d_3**-DAP**)	622.4 > 344.2	48	28
25(OH)D_3-23,26-lactone-d_3-DAP (**6**-d_3**-DAP**)	650.4 > 344.2	48	28
1,25(OH)$_2$$D_3$-23,26-lactone-$d_3$-DAP (**7**-$d_3$**-DAP**)	666.4 > 360.2	48	28

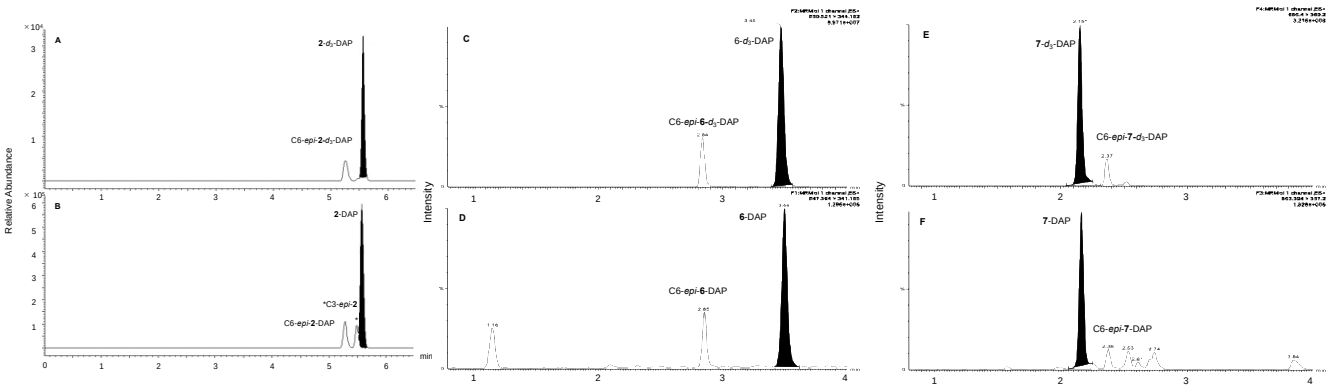


Figure 3. SRM chromatograms for the DAP adducts of D_3 metabolites **2**, **6**, and **7**, as well as **2**-d_3, **6**-d_3, and **7**-d_3.

In the case of the DAP-adducts of **2** and **2**-d_3 (Figure 3A,B), we observed identical ion peaks at the retention time of 5.60 min (abbreviated as t_R: 5.60 min). Similarly, **6** and **6**-d_3 showed the same t_R of 3.50 min, and **7** and **7**-d_3 showed the same t_R of 2.15 min, indicating that the deuterium-labeled compounds are suitable as internal standards for the

isotope dilution method. We also observed small peaks at the retention times of 5.20 min (Figure 3A,B), 2.65 min (Figure 3C,D), and 2.37 min (Figure 3E,F) for **2**/**2**-d_3, **6**/**6**-d_3, and **7**/**7**-d_3, respectively. These peaks are due to the epimers at C6 of the DAP adducts, because DAP-PA reacts from both the α- and β-faces.

Next, the calibration curves were prepared as follows (Figure 4). A total of 100 µL of each one of the calibrator solutions was mixed with 200 µL of the internal standard solution and evaporated to dryness. After derivatization with DAP-PA, an LC-MS/MS analysis of the unlabeled and labeled DAP-adducts was performed, and the calibration curves were prepared by plotting the concentration of unlabeled DAP-adduct against the ion peak area ratio of unlabeled versus labeled DAP-adduct. All of the calibration curves showed good linearity.

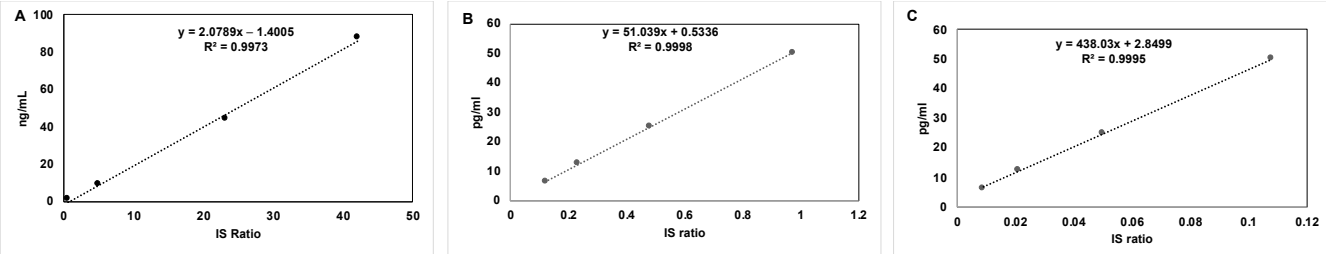


Figure 4. Calibration curve of D_3 metabolites; (**A**) 25(OH)D_3 (**2**); (**B**) 25(OH)D_3-23,26-lactone (**6**); and (**C**) 1,25$(OH)_2D_3$-23,26-lactone (**7**).

2.2. Quantification of the D_3 Derivatives in Human Serum

The levels of **2**, **6**, and **7** in pooled human serum were quantified by the LC-MS/MS using the isotope dilution method with the constructed calibration curves. The serum was pretreated as follows. An aliquot of serum (100 µL) was mixed with the internal standards solution (200 µL). Each sample was loaded onto a supported liquid extraction column (ISOLUTE SLE+ 300 µL sample Volume, Biotage, Uppsala, Sweden) and eluted three times with 600 mL hexane/ethyl acetate (1/1, *v*/*v*) using a PRESSURE+48 positive pressure manifold (Biotage, Uppsala, Sweden). The combined eluates were evaporated to dryness in a centrifugal evaporator. The ion peaks of the metabolites matched well with those of the corresponding internal standards in the pretreated samples (Figure 5).

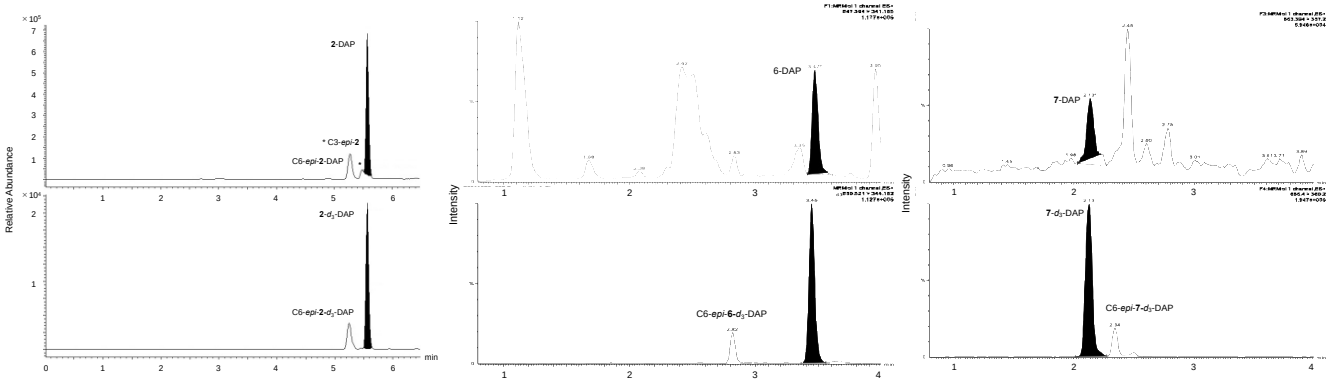


Figure 5. SRM chromatograms for D_3 derivatives **2**, **6**, and **7** in pooled human serum.

The concentrations of **2**, **6**, and **7** in human serum were calculated to be 5.1 ng/mL, 38.3 pg/mL, and 8.9 pg/mL, respectively, based on the area ratios of the detected peaks. The concentrations of **2** and **6** were in agreement with previously reported values [37], while this is the first quantification of 1α-lactone **7** in human serum.

3. Conclusions

We synthesized deuterium-labeled A-ring-d_3 synthons **13** and **16** and utilized them for the convergent synthesis of deuterium-labeled D_3 derivatives 25(OH)D_3 (**2**), 25(OH)D_3-23, 26-lactone (**6**), and 1,25$(OH)_2D_3$-23, 26-lactone (**7**). These deuterium-labeled D_3 metabolites were successfully applied as internal standards for the quantification of the metabolites in pooled human serum by LC-MS/MS using the isotope dilution method. This is the first quantification of 1,25$(OH)_2D_3$-23, 26-lactone (**7**) in human serum.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27082427/s1, Experimental procedures for synthesis and characterization of compounds, Experimental procedure for LC-MS/MS analysis using the isotope dilution method, ^{1}H and ^{13}C NMR spectra.

Author Contributions: Conceptualization, A.N., S.F. and K.N.; investigation, A.N., K.I., R.S., Y.M. and M.I.; analysis, M.T. (Masaki Takiwakiand), Y.K. and S.F.; writing—original draft preparation, A.N. and K.N.; resources, M.O., M.T. (Masayuki Tera) and K.N.; funding acquisition, K.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: This work was supported by the Japan Agency for Medical Research and Development (AMED- CREST).

Conflicts of Interest: The authors declare no conflict of interest.

References and Notes

1. Fleet, J.C. Regulation of intestinal calcium and phosphate absorption. In *Vitamin D*; Elsevier: Amsterdam, The Netherlands, 2018; pp. 329–342.
2. David, V.; Quarles, L.D. FGF23/Klotho new regulators of vitamin D metabolism. In *Vitamin D*; Elsevier: Amsterdam, The Netherlands, 2011; pp. 747–761.
3. Lips, P. Relative Value of 25(OH)D and 1,25$(OH)_2$D measurements. *J. Bone Miner. Res.* **2007**, *22*, 1668–1671. [CrossRef] [PubMed]
4. Holick, M.F. Vitamin D status: Measurement, interpretation, and clinical application. *Ann. Epidemiol.* **2009**, *19*, 73–78. [CrossRef] [PubMed]
5. Janjetovic, Z.; Tuckey, R.C.; Nguyen, M.N.; Thorpe, E.M.; Slominski, A.T. 20,23-dihydroxyvitamin D3, novel P450scc product, stimulates differentiation and inhibits proliferation and NF-kB activity in human keratinocytes. *J. Cell. Physiol.* **2010**, *223*, 36–48. [PubMed]
6. Janjetovic, Z.; Zmijewski, M.A.; Tuckey, R.C.; DeLeon, D.A.; Nguyen, M.N.; Pfeffer, L.M.; Slominski, A.T. 20-Hydroxycholecalciferol, product of vitamin D_3 hydroxylation by P450scc, decreases NF-κB activity by increasing IκBα levels in human keratinocytes. *PLoS ONE* **2009**, *4*, e5988. [CrossRef]
7. Wang, J.; Slominski, A.T.; Tuckey, R.C.; Janjetovic, Z.; Kulkarni, A.; Chen, J.; Postlethwaite, A.E.; Miller, D.; Li, W. 20-Hydroxyvitamin D_3 inhibits proliferation of cancer cells with high efficacy while being non-toxic. *Anticancer Res.* **2012**, *32*, 739–746.
8. Li, W.; Chen, J.; Janjetovic, Z.; Kim, T.K.; Sweatman, T.; Lu, Y.; Zjawiony, J.; Tuckey, R.C.; Miller, D.; Slominski, A.T. Chemical synthesis of 20*S*-hydroxyvitamin D_3, which shows antiproliferative activity. *Steroids* **2010**, *75*, 926–935. [CrossRef]
9. Slominski, A.T.; Janjetovic, Z.; Fuller, B.E.; Zmijewski, M.A.; Tuckey, R.C.; Nguyen, M.N.; Sweatman, T.; Li, W.; Zjawiony, J.; Miller, D.; et al. Products of vitamin D_3 or 7-dehydrocholesterol metabolism by cytochrome P450scc show anti-leukemia effects, having low or absent calcemic activity. *PLoS ONE* **2010**, *5*, e9907. [CrossRef]
10. Verma, A.; Cohen, D.J.; Schwartz, N.; Muktipaty, C.; Koblinski, J.E.; Boyan, B.D.; Schwartz, Z. 24*R*,25-Dihydroxyvitamin D_3 regulates breast cancer cells in vitro and in vivo. *Biochim. Biophys. Acta BBA—Gen. Subj.* **2019**, *1863*, 1498–1512. [CrossRef]
11. Verma, A.; Cohen, D.J.; Jacobs, T.W.; Boyan, B.D.; Schwartz, Z. The relative expression of ERα Isoforms ERα66 and ERα36 controls the cellular response to 24*R*,25-Dihydroxyvitamin D3 in breast cancer. *Mol. Cancer Res.* **2021**, *19*, 99–111. [CrossRef]
12. Kiyoki, M.; Kurihara, N.; Ishizuka, S.; Ishii, S.; Hakea, Y.; Kumegawa, M.; Norman, A.W. The unique action for bone metabolism of 1α,25-$(OH)_2D_3$-26,23-lactone. *Biochem. Biophys. Res. Commun.* **1985**, *127*, 693–698. [CrossRef]
13. Ishizuka, S.; Miura, D.; Eguchi, H.; Ozono, K.; Chokki, M.; Kamimura, T.; Norman, A.W. Antagonistic action of novel 1α,25-Dihydroxyvitamin D_3-26,23-lactone analogs on 25-hydroxyvitamin-D_3-24-hydroxylase gene expression Induced by 1α,25-dihydroxy-vitamin D_3 in human promyelocytic leukemia (HL-60) cells. *Arch. Biochem. Biophys.* **2000**, *380*, 92–102. [CrossRef] [PubMed]
14. Ishizuka, S.; Norman, A.W. The differenece of biological activity amang four diastereoisomers of 1α,25dihydoroxycholecalciferol-26,23-lactone. *J. Steroid Biochem.* **1986**, *25*, 505–510. [PubMed]

15. Mendoza, A.; Takemoto, Y.; Cruzado, K.T.; Masoud, S.S.; Nagata, A.; Tantipanjaporn, A.; Okuda, S.; Kawagoe, F.; Sakamoto, R.; Odagi, M.; et al. Controlled lipid β-oxidation and carnitine biosynthesis by a vitamin D metabolite. *Cell Chem. Biol.* **2021**. [CrossRef] [PubMed]
16. Shah, I.; Akhtar, M.K.; Hisaindee, S.; Rauf, M.A.; Sadig, M.; Ashraf, S.S. Clinical diagnostic tools for vitamin D assessment. *J. Steroid Biochem. Mol. Biol.* **2018**, *180*, 105–117. [CrossRef]
17. It is difficult to develop an antibody that specifically recognizes the structure of the side chain of vitamin D metabolites, see; Kobayashi, N.; Higashi, T.; Saito, K.; Nlurayama, T.; Douya, R.; Shimada, K. Specificity of polyclonal antibodies raised against a novel 24,25-dihydroxyvitamin D_3- bovine serum albumin conjugant linked through the C-11α or C-3 position. *J. Steroid Biochem. Mol. Biol.* **1997**, *62*, 79–87.
18. Müller, M.J.; Volmer, D.A. Mass spectrometric profiling of vitamin D metabolites beyond 25-hydroxyvitamin D. *Clin. Chem.* **2015**, *61*, 1033–1048. [CrossRef]
19. Higashi, T.; Shimada, K. Application of Cookson-type reagents for biomedical HPLC and LC/MS analyses: A brief overview: Cookson-type reagents for biomedical HPLC and LC/MS analyses. *Biomed. Chromatogr.* **2017**, *31*, e3808. [CrossRef]
20. Seki, M.; Sato, M.; Takiwaki, M.; Takahashi, K.; Kikutani, Y.; Satoh, M.; Nomura, F.; Kuroda, Y.; Fukuzawa, S. A novel caged Cookson-type reagent toward a practical vitamin D derivatization method for mass spectrometric analyses. *Rapid Commun. Mass Spectrom.* **2020**, *34*, e8648. [CrossRef]
21. Nicoletti, D.; Mouriño, A.; Torneiro, M.S. Synthesis of 25-hydroxyvitamin D_3 and 26,26,26,27,27,27-hexadeutero-25-hydroxyvitamin D_3 on solid support. *J. Org. Chem.* **2009**, *74*, 4782–4786. [CrossRef]
22. Perlman, K.; Schnoes, H.K.; Tanaka, Y.; DeLuca, H.F.; Kabayashi, Y.; Taguchi, T. Chemical synthesis of (24R)-24,25-dihydroxy[26,27-^{3}H]vitamin D_3 of high specific activity. *Biochemistry* **1984**, *23*, 5041–5048. [CrossRef]
23. Ray, R.; Vicchio, D.; Yergey, A.; Holick, M.F. Synthesis of 25-hydroxy-[6,19,19′-$^2H_3$vitamin D_3 and 1α,25-dihydroxy-[6,19′-$^2H_3$]vitamin D_3. *Steroids* **1992**, *57*, 142–146. [CrossRef]
24. Yamada, S.; Shimizu, M.; Fukushima, K.; Niimura, K.; Maeda, Y. Syntheses of 24*R*,25-dihydroxy-[6,19,19-^{3}H]vitamin D_3 and 24*R*,25-dihydroxy-[6,19,19-^{2}H]vitamin D_3. *Steroids* **1989**, *54*, 145–157. [CrossRef]
25. Yamada, S.; Suzuki, T.; Takayama, H. Novel regioselective C-6 and C-19 alkylation of vitamin D_3 via its sulfur-dioxide adducts. *Tetrahedron Lett.* **1981**, *22*, 3085–3088. [CrossRef]
26. Zhu, G.D.; Okamura, W.H. Synthesis of vitamin D (Calciferol). *Chem. Rev.* **1995**, *95*, 1877–1952. [CrossRef]
27. Gu, J.; Rodriguez, K.; Kanda, Y.; Yang, S.; Ociepa, M.; Wilke, H.; Abrishami, A.; Joergensen, L.; Skak-Nielsen, T.; Chen, J.; et al. Convergent total synthesis of (+)-Calcipotriol: A scalable, modular approach to vitamin D analogs. *ChemRxiv* **2021**. [CrossRef]
28. Maegawa, T.; Fujiwara, Y.; Inagaki, Y.; Monguchi, Y.; Sajiki, H. A convenient and effective method for the regioselective deuteration of alcohols. *Adv. Synth. Catal.* **2008**, *350*, 2215–2218. [CrossRef]
29. Pattenden, G.; González, M.A.; Little, P.B.; Millan, D.S.; Plowright, A.T.; Tornos, J.A.; Ye, T. Total synthesis of (±)-phorboxazole A, a potent cytostatic agent from the sponge *Phorbas* sp. *Org. Biomol. Chem.* **2003**, *1*, 4173–4208. [CrossRef]
30. The enantiomers at C3 were separated after coupling the CD rings by HPLC purification.
31. Akagi, Y.; Usuda, K.; Tanami, T.; Yasui, K.; Asano, L.; Uesugi, M.; Nagasawa, K. Synthesis of 1α- and 1β-amino-25-hydroxyvitamin D_3. *Asian J. Org. Chem.* **2016**, *5*, 1247–1252. [CrossRef]
32. Venkanna, A.; Sreedhar, E.; Siva, B.; Babu, K.S.; Prasad, K.R.; Rao, J.M. Studies directed towards the total synthesis of koshikalide: Stereoselective synthesis of the macrocyclic core. *Tetrahedron Asymmetry* **2013**, *24*, 1010–1022. [CrossRef]
33. Gao, Y.; Klunder, J.M.; Hanson, R.M.; Masamune, H.; Ko, S.Y.; Sharpless, K.B. Catalytic asymmetric epoxidation and kinetic resolution: Modified procedures including in situ derivatization. *J. Am. Chem. Soc.* **1987**, *109*, 5765–5780. [CrossRef]
34. Joannesse, C.; Johnston, C.P.; Concellón, C.; Simal, C.; Philp, D.; Smith, A.D. Isothiourea-catalyzed enantioselective carboxy group transfer. *Angew. Chem. Int. Ed.* **2009**, *48*, 8914–8918. [CrossRef] [PubMed]
35. Trost, B.M.; Dumas, J.; Villa, M. New strategies for the synthesis of vitamin D metabolites via palladium-catalyzed reactions. *J. Am. Chem. Soc.* **1992**, *114*, 9836–9845. [CrossRef]
36. Nagata, A.; Akagi, Y.; Masoud, S.S.; Yamanaka, M.; Kittaka, A.; Uesugi, M.; Odagi, M.; Nagasawa, K. Stereoselective synthesis of four calcitriol lactone diastereomers at C23 and C25. *J. Org. Chem.* **2019**, *84*, 7630–7641. [CrossRef] [PubMed]
37. Kaufmann, M.; Schlingmann, K.; Berezin, L.; Molin, A.; Sheftel, J.; Vig, M.; Gallagher, J.C.; Nagata, A.; Masoud, S.S.; Sakamoto, R.; et al. Differential diagnosis of vitamin D–related hypercalcemia using serum vitamin D metabolite profiling. *J. Bone Miner. Res.* **2021**, *36*, 1340–1350. [CrossRef]