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Synthesis of vitamin D_3 analogues with A-ring modifications to directly measure vitamin D levels in biological samples

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ABSTRACT

C-3-substituted 25-hydroxyvitamin D_3 analogues were synthesized as tools to directly measure levels of vitamin D in biological samples. The strategy involves vinyloxycarbonylation of the 3β-hydroxy group and formation of a carbamate bond with a hydroxyl or amino group at the end of the alkyl chain. Biotinylated conjugates of synthesized derivatives were generated to be linked with vitamin D binding protein (DBP). The spacer group present in the alkyl chain is important in the binding of antibodies to the analogue–DBP complex. When compared to 25-hydroxyvitamin D_3-DBP, the binding of some antibodies to the analogue–DBP complex of the 25-hydroxyvitamin D_3 derivative **10** that posses an 8-aminoctyl alkyl chain is significantly reduced, but this analogue displaced [26,27-^{3}H]-25-hydroxyvitamin D_3 from DBP. In contrast, the 8-hydroxyoctyl alkyl chain analogue **9** showed less displacement.

1. Introduction

In the last decade there has been renewed interest in the vitamin D research area due to the evidence of the clinical benefits of adequate vitamin D levels. Vitamin D exists in two principal forms, vitamin D_2 (**1**, Chart 1) and vitamin D_3 (**2**). It was reported that vitamin D_3 was approximately 87% more potent in raising and maintaining serum vitamin D levels and produced a two to threefold greater storage of vitamin D than vitamin D_2.[1]

Vitamin D_3, formed in the skin after sunlight exposure or taken in dietary foods, is bound in the plasma to vitamin D binding protein (DBP),[2] a key component in the vitamin D endocrine system that carries vitamin D_3 and its metabolites through the circulatory system to their various target organs.[3] Vitamin D_3 is metabolized in the liver into 25-hydroxyvitamin D_3 (**4**, 25-OH-D_3), the prevailing vitamin D metabolite in the blood, and then in the kidney to its functional form, namely 1α,25-dihydroxyvitamin D_3 [**6**, 1α,25-$(OH)_2$-D_3].[4]

On a molar basis, DBP concentration is ordinarily 20-times that of vitamin D in the blood. Since DBP has very high affinity for vitamin D, most vitamin D is found bound to DBP.[5] Vitamin D can also be found bound to human serum albumin (HSA) and chylomicrons at lower levels and affinities.[6] The two main forms of vitamin D which can be measured are 25-OH-D_3 and 1α,25-$(OH)_2$-D_3.[7] 25-OH-D_3 is found at a higher concentration in the blood than 1α,25-$(OH)_2$-D_3 and has a relatively long half-live. Furthermore, being the active form of vitamin D, the plasma/serum levels of 1α,25-$(OH)_2$-D_3 tend to be maintained at a constant level. For these reasons, 25-OH-D_3 levels are commonly used to determine a patient's vitamin D status. However, the level of 1α,25-$(OH)_2$-D_3 serves as an indication of whether there is adequate production in the kidney of the active form of the compound.

Vitamin D_3 has been recognized to be essential to human health.[8] Discovery of a vitamin D receptor (VDR) in a variety of tissues and cells in the body hints a wide spectrum of activities of the vitamin D-VDR endocrine system. 1α,25-Dihydroxyvitamin D_3 is involved in the regulation of calcium homeostasis and bone metabolism, the inhibition of proliferation and increased differentiation of various malignant cells, as well as suppression of autoimmune diseases.[9] Vitamin D deficiency affect at least 50% of the population and there is strong evidence of its impact not only on bone and muscle health, but also in the prevention of other illnesses such as cardiovascular diseases, cancer (e.g., colorectal, breast, prostate), diabetes, infectious diseases and multiple sclerosis.[10] Although there is no consensus on how much calcium and vitamin D are needed to achieve desirable health outcomes, vitamin D deficiency is defined by most experts as a 25-hydroxyvitamin D level of less than 20 ng/mL (50 nmol/l).[11] Sun exposure should not be used as the only source of vitamin D. An adequate intake should combine

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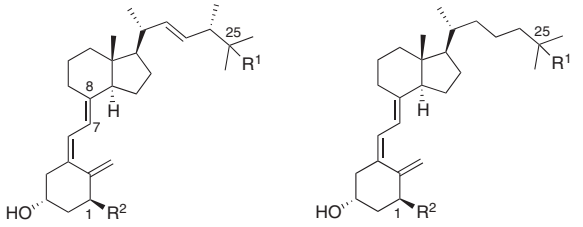


1, $R^1 = R^2 = H$ Vitamin D_2
3, $R^1 = OH$; $R^2 = H$ 25-OH-D_2
5, $R^1 = R^2 = OH$ 1α,25-$(OH)_2$-D_2

2, $R^1 = R^2 = H$ Vitamin D_3
4, $R^1 = OH$; $R^2 = H$ 25-OH-D_3
6, $R^1 = R^2 = OH$ 1α,25-$(OH)_2$-D_3

Chart 1. Vitamin D related structures.

sunlight exposure, dietary intake, and supplements whenever required.

In recent years several methods to determine vitamin D levels for diagnostic purposes were described and performance characteristics and limitations have been reviewed.[7,12] There are two types of methods: competitive immunoassay and those based on chromatography separation followed by non-immunological direct detection. Currently, immunoassays are performed on automated platforms, which use a chemiluminescent label. These methods are limited by specificity in relation to the nonequimolar recognition of the D_2 and D_3 forms of 25-OH-D. Meanwhile liquid chromatography/tandem mass spectrometry methods have the advantage of being able to measure both 25-OH-D_2 (**3**) and 25-OH-D_3 (**4**) independently. However, they require expensive equipment and restrict sample throughput in the large clinical laboratory. Current assays to determine 25-OH-D require initial dissociation from its DBP which may be achieved by denaturing and removing DBP or using a displacement reagent to release free 25-OH-D. Solvents are used to release the 25-OH-D and denature the DBP but subsequent separation of the denatured DBP and solvent extraction of the sample is required prior to assay. The hydrophobic nature of 25-OH-D introduces significant problems, for example the molecule has a propensity to bind to plastics surfaces thereby affecting the complete recovery of free 25-OH-D. The requirement for organic solvents is non-compatible with the use of protein molecules, such as antibodies, in the assay system, or with the specialised high throughput instrumentation present in most modern laboratories. High variability is a common issue with vitamin D assays[13] because of the multi-stage nature of the existing assays, including 25-OH-D displacement, liquid additions and removals, and multiple wash steps, precision suffers accordingly. Consequently, there is a need for a simplified vitamin D assay that is likely to provide higher precision.

2. Results and discussion

With this background in mind, here we report a novel method to determine the relative amounts of the vitamin D–DBP complex (hereinafter referred to as holo-DBP, Fig. 1) and free DBP (referred to as apo-DBP) by binding apo-DBP to a binding molecule (analogue) to form a molecule-DBP complex, such that a recognition molecule (e.g., an antibody) binds preferentially to holo-DBP over the molecule-DBP complex. Since most of the 25-OH-D_3 is bound to DBP, using a recognition molecule, which preferentially binds holo-DBP over molecule-DBP complex, allows assessment of the vitamin D level in a sample. The relative holo-DBP/vitamin D level may be determined by reference to control measurements. The recognition molecule can be an antibody or antibody fragment, for example an anti-DBP antibody or fragment of antigen binding (Fab). In order to facilitate development or identification of recognition molecules that are specific for or bind preferentially

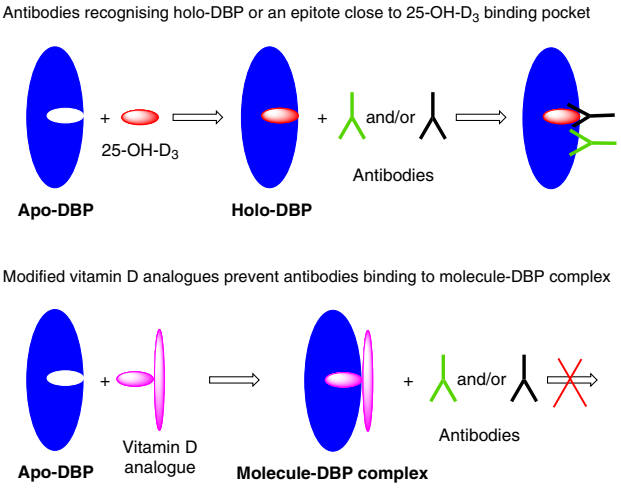
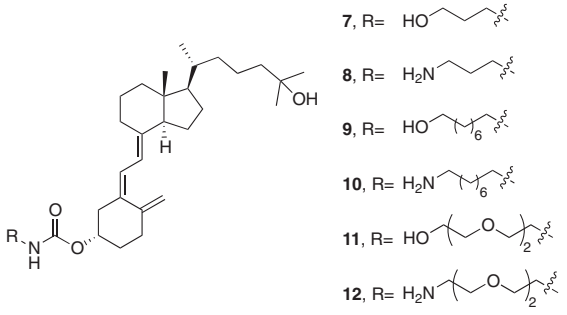


Figure 1. The modified vitamin D analogues when complexed to apo-DBP could prevent antibodies binding to holo-DBP and/or antibodies recognising epitotes close to the vitamin D binding pocket. The analogues could also be used to remove apo-DBP from the samples prior to measurement of holo-DBP using either an antibody capable of recognising either holo- or apo-DBP.



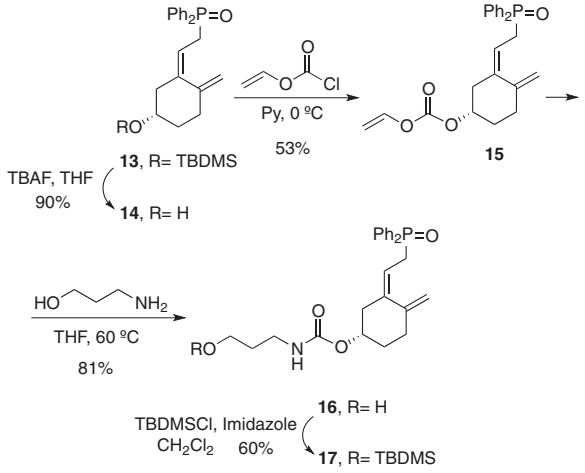
7, R= HO
8, R= H_2N
9, R= HO-(CH2)6
10, R= H_2N-(CH2)6
11, R= HO-(CH2CH2O)2
12, R= H_2N-(CH2CH2O)2

Chart 2. Target vitamin D analogues with C-3 A-ring modifications.

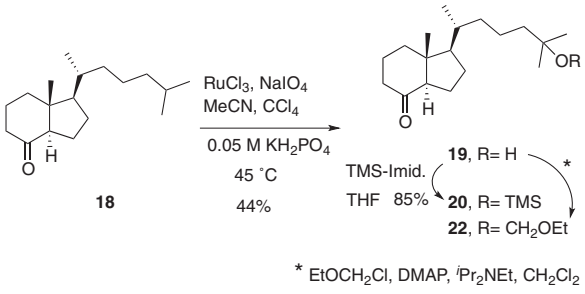
to holo-DBP or molecule-DBP complex, the latter may have an altered conformation of DBP, and/or introduces steric hindrance in the region around the vitamin D binding pocket of DBP, and/or an altered conformation of a region around the vitamin D binding pocket of DBP.

In our on going research related with vitamin[14] and previtamin D_3,[15] we have developed several vitamin D_3 derivatives (**7**–**12**, Chart 2) substituted at 3-position with a carbamate function as apo-DBP binding molecule. In holo-DBP the A-ring is closest to the opening of the binding pocket[16] and, therefore, modifying it may have a significant effect on the ability of molecules to bind DBP around the binding pocket, for example by impeding access to the binding pocket or creating a conformational change.

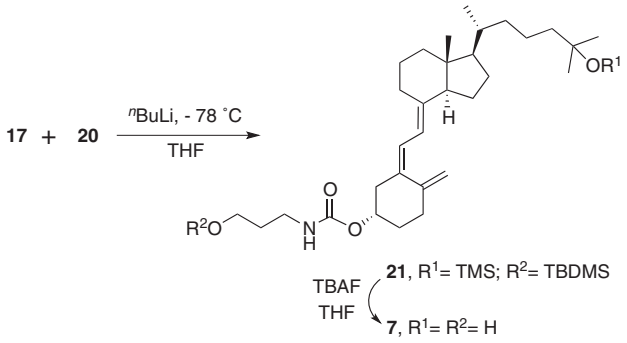
An advantage of these derivatives is that they contain hydrophilic groups such as alcohol or amino at the end of the exogenous alkyl chain of the carbamate and a spacer group of different length. Among the spacer arms, polyethylene glycol (PEG) has been incorporated to increase solubility and flexibility. Thus, the side chain could protrude from the surface of DBP upon formation of the molecule-DBP complex. In this way, it may inhibit binding of a holo-DBP recognition molecule. To further facilitate differentiation between holo-DBP and molecule-DBP complex the side chain will be coupled to biotin or streptavidin thus potentially further increasing the steric hindrance of the molecule when bound to DBP.



Scheme 1. Synthesis of A-ring synthon precursor **17**.



Scheme 2. Synthesis of CD-rings/side chain synthon precursors **20** and **22**.



Scheme 3. Synthesis of 25-OH-D_3 analogue **7** by direct coupling reaction.

2.1. Preparation of A-ring modified 25-hydroxyvitamin D_3 derivatives

For the synthesis of 25-OH-D_3 carbamate derivatives **7**–**12** we used standard Lythgoe coupling[17] in which an anion of an allylic phosphine oxide A-ring **17** reacts with a CD-rings/side chain ketone **20** via the Wittig–Horner reaction with the concomitant construction of the vitamin D triene system (see Scheme 3). As starting compound for the synthesis of the A-ring fragment **17**, we used the commercially available vitamin D_2. Briefly, vitamin D_2 (**1**) was cleaved at the C7-C8 double bond (see Chart 1) by oxidation, and the lower fragment was converted in several steps into silyl-protected phosphine oxide **13** (Scheme 1) by a well-known procedure.[18] Cleavage of the silyl ether using TBAF gave alcohol **14** in a 90% yield. To synthesize the C-3 carbamate derivative **17** we used a two-step process. First, alkoxycarbonylation reaction of synthon **14** with vinyl chloroformate in pyridine gives place to carbonate **15** in an un-optimized 53% yield. The latter reacts with 3-amino-propan-1-ol in THF at 60 °C to obtain the corresponding carbamate **16**. In order to improve the yield of the coupling reaction, when this A-ring synthon is coupled with the CD-rings/side-chain fragment, hydroxyl group in **16** was conveniently protected as silyl ether with *tert*-butyldimethylsilyl chloride (TBDMSCl) giving place to phosphine oxide precursor **17**.

For the synthesis of the upper fragment **20** (Scheme 2), we used vitamin D_3 (**2**) as starting material. Grundmann's ketone **18** was prepared by ozonolysis of vitamin D_3 according to the published procedure.[19] The latter was oxidized at the 25-position (steroid numbering) to obtain alcohol **19**, which was protected as a trimethylsilyl (TMS) ether to afford synthon **20**.[20]

Wittig–Horner reaction of **20** with lithium phosphinoxy carbanion, generated from the phosphine oxide **17** and *n*-butyllithium, yielded the protected analogue **21**. Subsequent removal of the silyl protecting groups afforded the desired analogue **7** (Scheme 3) in 13% yield (for coupling and desilylation steps).

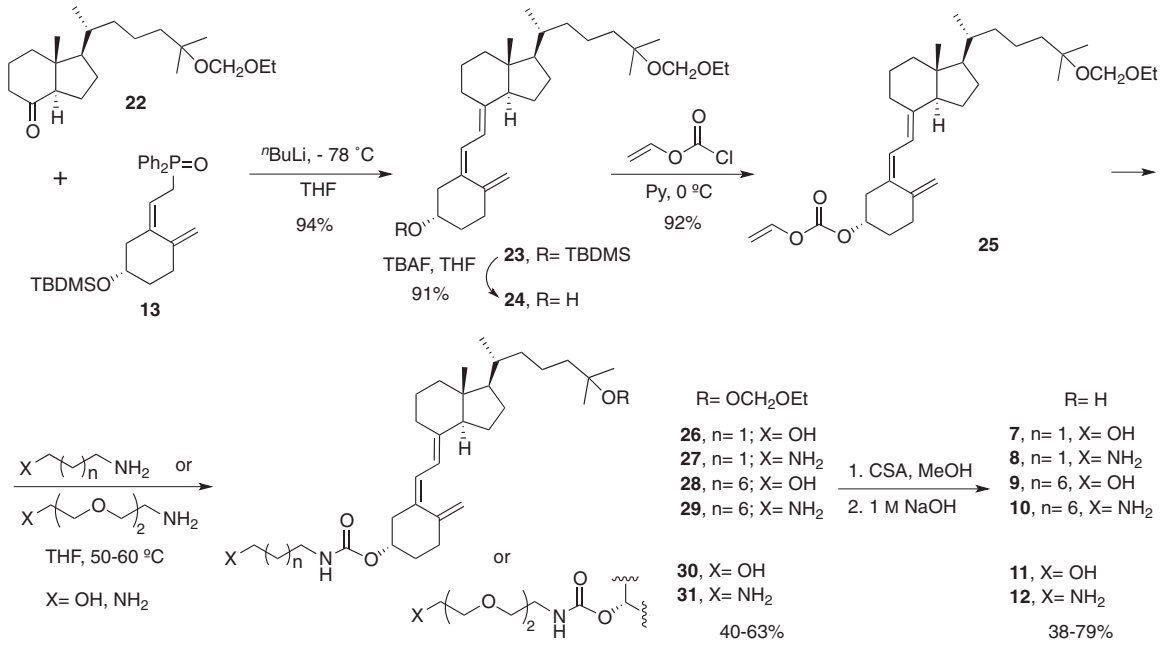
In an attempt to increase the yield of the coupled product, several reaction conditions were modified: different temperatures, times of reaction, etc. However, these alternative procedures provide **7** in a similar overall yield, probably due to acid-base side reactions with the C3-subtituted chain.

In order to avoid this drawback, we changed the synthetic strategy coupling the A-ring and CD-rings/side chain synthons before the introduction of the carbamate function at the A-ring fragment, that is, before modify the hydroxyl group at C-3. This implies that the C-3 and C-25 hydroxyl groups can be selectively deprotected. To first achieve selective C-3 deprotection, protecting group at C-25 position should be changed from trimethylsilyl to ethoxymethyl.[14a] Thus, new CD-rings/side chain synthon **22** was prepared as shown in Scheme 2.

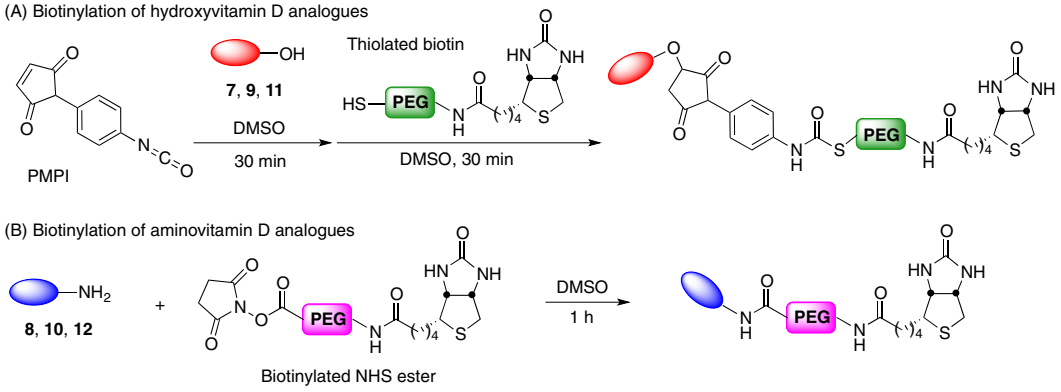
The 25-OH-D_3 analogues **7**–**12** were successfully synthesized according to the reaction sequence shown in Scheme 4. 25-Ethoxymethoxy protected Grundmann's ketone **22** was treated with the anion of A-ring synthon **13**, obtained from treatment with ^{n}BuLi in THF, affording the derivative **23**, which after silyl ether deprotection with TBAF in THF gave alcohol **24** in 92% yield. Reaction with vinyl chloroformate in pyridine at 0 °C provided the desired vitamin D carbonate **25** in high yield (92%). Subsequent reaction of **25** with linear amino alcohols or diamines of various lengths between the two different functionalities (amino and amino/alcohol) afforded carbamates **26**–**29**. To modulate the hydrophilicity of the vitamin D derivatives, a PEG group is attached to the carbamate chain. Thus, if amino alcohol or diamino PEG derivatives were used, analogues **30**–**31** were obtained in which the spacer arm was formed with a PEG chain. Next, deprotection of 25-position with (−)-camphor sulphonic acid [(−)-CSA] and basic treatment of the resulting solution with 1 M NaOH yielded 25-hydroxyvitamin D_3 analogues **7**–**12** in moderate to high yields (38–79%).

2.2. Specificity for vitamin D analogue–DBP complexes and competitive displacement studies

To carry out measurements of vitamin D levels on blood or serum samples, biotinylated derivatives of vitamin D_3 analogues were prepared as shown in Scheme 5. In biotinylation of hydroxyvitamin D derivatives, heterofunctional crosslinker *N*-(*p*-maleimidophenyl)isocyanate (PMPI) is used to couple a thiolated PEG-biotin molecule to a hydroxyvitamin D analogue (**7**, **9**, **11**). The coupling is performed in DMSO with a 1.1 molar ratio of PMPI to the hydroxyvitamin D molecule. After a 30 min reaction, with mixing, the thiolated PEG-biotin, in DMSO, is added at 1.1 molar ratio to the PMPI-vitamin D molecule reaction and mixed for an additional 30 min. In case of aminovitamin D derivatives, a biotinylated



Scheme 4. Syntheses of 25-OH-D_3 analogues **7**–**12** by coupling reaction and subsequent C-3 modification.



Scheme 5. Preparation of biotinylated derivatives of vitamin D_3 analogues using: (A) hydroxyvitamin D analogues **7**, **9**, and **11**; (B) aminovitamin D analogues **8**, **10**, and **12**.

N-hydroxysuccinimide (NHS) ester containing a hydrophilic polyethylene glycol spacer arm is coupled to an aminovitamin D analogue (**8**, **10**, **12**). The coupling is performed in DMSO with a 1.5 molar excess of the biotin and the reaction is incubated for 1 h.

To prepare the biotinylated molecule-DBP complexes, which can be used to test against antibodies, a 5–10 molar excess of corresponding biotinylated vitamin D analogue is incubated with mixed type vitamin D binding protein overnight at 2–8 °C. Biotinylated molecule-DBP complex is separated from unreacted apo-DBP using a chromatofocusing technique on a mono P column (GE Healthcare) equilibrated with a 0.025 M methylpiperazine pH 5.7 start buffer. The biotinylated molecule-DBP complex mixture, which is pre-adjusted to the same pH as the start buffer, is applied and eluted with 10% polybuffer 74 pH 4.0. The biotinylated molecule-DBP complex fractions are collected while running the elution buffer. Similar procedure is followed to form holo-DBP, but using 25-OH-D_3 (**4**) instead of vitamin D analogues **7**–**12**.

The first derivative synthesised was biotinylated molecule **7** complex (**7** is the short hydroxyvitamin D analogue of 25-OH-D_3), which was then used to develop the assay for screening the binding ability of the antibodies or Fabs to holo-DBP and molecule-DBP complex. The data are collected in Tables 1 and 2, and were obtained when either holo-DBP or molecule-DBP (50–100 μg/mL at pH 4.5) is covalently coupled onto a COOH sensor for use on the SensiQ, which is a surface plasmon resonance technology based instrument for measuring protein interaction. Streptavidin (0.1 mg/mL) is optionally passed over the holo-DBP or molecule-DBP coated sensor.

An increase in signal is observed for the molecule-DBP coated sensor when the streptavidin binds the biotin on the molecule-DBP, as expected. Antibodies for DBP, which are commercially available, or Fabs isolated from a phage display screen using the MorphoSys's HuCAL® library (supplied by AbD Serotec) with holo-DBP, are tested at a concentration of 0.5–5 μM in a HEPES buffered saline pH 7.4 solution, for binding to both the holo-DBP and molecule-DBP complex.

Table 1 shows the specificity of the antibodies tested for holo-DBP and the first analogue developed that is analogue 7-DBP. As can be seen from the data in the Table, some antibodies can bind both the holo-DBP and molecule-DBP complex with similar affinity (Table 1, entries 1 and 4) whilst other antibodies bind the molecule-DBP complex with lower affinity than holo-DBP (Table 1, entries 2, 3, and 5), indicating that the molecule-DBP complex can reduce antibody binding/recognition as compared to holo-DBP.

Table 1
Specificity of antibodies for holo-DBP and analogue **7**-DBP complex[a]

Entry	Ab/Fab ID[b]	Binding RU[c] (refractive units)		Difference[d] (%)
		Endogeneous holo-DBP	**7**-DBP complex + streptavidin	
1	AbD Serotec 11732.1	240	240	0
2	AbD Serotec 11636.1	202	106	−48
3	AbD Serotec 11634.1	150	41	−73
4	AbD Serotec 11631.1	247	263	6
5	AbCam 23484	868	166	−81

[a] Data produced using sensor coated with biotinylated analogue **7** which had streptavidin (0.1 mg/mL) passed over it.
[b] 11 μg of each antibody was used.
[c] Holo-DBP and molecule-DBP complexes were coated at a concentration of 50 μg/mL.
[d] In percentage compared to holo-DBP.

However for the purposes of developing an assay for measuring vitamin D there remains a 19% binding of the antibody to DBP with the short hydroxyvitamin D analogue of 25-OH-D_3, which would artificially increase the apparent concentration of holo-DBP in a sample. This would not be acceptable for an on-market assay.

Based on the assay model, we hypothesised that an analogue with a longer arm could result in an increased steric barrier to DBP antibody binding in the region of the vitamin D binding pocket. To test this hypothesis a biotinylated long aminovitamin D analogue of 25-OH-D_3 (named **10**) in complex with DBP was screened against the antibodies and Fabs. Binding of some antibodies to the complex is reduced as compared to their binding of holo-DBP (Table 2, entries 1–7 and 9–10). Further, when streptavidin is passed over the biotinylated aminovitamin D analogue **10**, the level of antibody binding/recognition can be even further reduced in the case of seven antibodies and Fabs (Table 2, entries 2, 4–7 and 9–10). Data from Table 2 suggests that an analogue with a longer linker is better at inhibiting antibody binding, two antibodies show less than 6% binding to molecule-DBP.

As previously mentioned, it would be desirable that the vitamin D analogues do not displace endogenous 25-OH-D_3 from vitamin D binding protein. Thus, additional experiments, using tritiated [^{3}H]25-OH-D_3 to monitor displacement, were performed in order to know if 25-OH-D_3, the natural molecule that complex with DBP, is displaced from holo-DBP by vitamin D analogues. Having established that the long analogues had better potential at inhibiting binding of antibodies to DBP, we tested the capacity of the hydroxyl and amine long linker analogues (analogues **9** and **10**) to displace endogenous vitamin D from DBP. These analogues were also selected to investigate the effect of the amine and hydroxyl group on the displacement of 25-OH-D_3 from holo-DBP. These data are presented in Table 3.

The reference sample was prepared as follow: to 1 mg of apo-DBP was added 750 μL of phosphate buffer saline (PBS) pH 7.4, 50 μL of 25-OH-D_3 (1 mg/mL), and 200 μL of [26,27-^{3}H]25-OH-D_3. This mixture was incubated overnight at 2–8 °C, and a 10 μL aliquot was taken to obtain a radioactivity reading by scintillation counting (Table 3, entry 2). Free 25-OH-D_3 and [26,27-^{3}H]25-OH-D_3 were removed using Zeba desalt spin column. Approximately 850 μL of flowthrough was recovered from the column and a 100 μL aliquot was taken to obtain a radioactivity reading (Table 3, entry 3).

All other entries of Table 3 were obtained for incubation of 15 μL of samples (1 mg/mL) with 100 μL of [^{3}H]holo-DBP. The structures analyzed in this experiment were non-biotinylated versions of the long hydroxy-25-OH-D_3 analogue **9** and the long amino-25-OH-D_3 analogue **10**. The results of the radioactivity reading are shown in Table 3. In the column, 67.6% is retained, which is close to what would be expected as normally, being only about 25–30% of apo-DBP converted to holo-DBP. Entries 6–9 of Table 3 shown averaged data for duplicated samples of analogues **9** and **10** and indicated the percentage of [^{3}H]vitamin D displacement. The data suggest that the presence of the hydroxyvitamin D analogue **9** is actually protecting against displacement (Table 3, entries 6 and 7). The PBS data would suggest that there is equilibrium of the 25-OH-D_3 linked and in solution, and the free 25-OH-D_3 is removed in the Zeba desalt (Table 3, entry 4). In this experiment, the endogenous 25-OH-D_3 either replaces or does not help drive the equilibrium to maintain holo-DBP. However, there is less displacement/equilibrium of the tritiated 25-OH-D_3 with the hydroxy analogue **9**. This is not the case with the aminovitamin D analogue **10**, which appears to have displaced all of the tritiated 25-OH-D_3. Therefore it is evident that the different analogues described have different characteristics for displacement of 25-OH-D_3 from holo-DBP, which could be exploited in various assay formats.

The analogues prepared will enable the future development of novel assay(s) for vitamin D measurement. Examples of potential assay formats for indirect measurement of holo-DBP, and thus

Table 2
Specificity of antibodies for holo-DBP and analogue **10**–DBP complex[a]

Entry	Ab ID	Ab used (μg)	Binding RU[b] (refractive units)			Difference[c] (%)	
			Endogeneous holo-DBP	**10**-DBP complex	**10**-DBP complex + streptavidin (strep)	No strep	Strep
1	AbD serotec 17384	15	55	25	49	−55	−11
2	AbD serotec 17385	15	385	77	25	−80	−94
3	Abcam 65636	7.5	1111	472	—	−58	—
4	AbD serotec 17354	15	465	136	68	−71	−85
5	AbD serotec 17355	7.5	473	35	26	−93	−95
6	AbD serotec 17358	15	353	87	49	−75	−86
7	Abcam 23484	7.5	145	113	49	−22	−66
8	Abcam 89765	7.5	0	249	15	—	—
9	AbD serotec 11732	11	431	183	99	−58	−77
10	AbD serotec 11634	15	144	34	23	−76	−84

[a] Data produced using sensor coated with biotinylated analogue **10**, both with and without streptavidin (0.1 mg/mL) passed over it.
[b] Holo-DBP and molecule-DBP complex were coated at a concentration of 50 μg/mL.
[c] In percentage compared to holo-DBP without and with streptavidin, respectively.

Table 3
Vitamin D analogues displacement measurements using radiolabeled [26,27-^{3}H]25-OH-D_3[a]

Entry	Sample	Cpm[b]	Average	Dilution	Total	Displaced [^{3}H] vitD (%)
1	Blank	10				
2	Apo-DBP[c]	1469		100	146,900	
3	Apo-DBP[d]	7321		6.5	47586.5	70
4	PBS[e]	2217				
5	25-OH-D_3	1747				76
6	**9**	5257	5254			28
7	**9**	5250				
8	**10**	190	202			97
9	**10**	213				

[a] The samples were prepared as follow: to a 100 μL of [^{3}H]holo-DBP solution, 15 μL of PBS buffer or 25-OH-D3 (1 mg/mL) or analogue (1 mg/mL) was added and then incubated at 20 °C for 60 min.
[b] Cpm, counts per minute.
[c] Aliquot taken before the use of Zeba desalt spin column.
[d] Aliquot taken after the use of Zeba desalt spin column.
[e] PBS, phosphate buffer saline.

the vitamin D levels in plasma or serum samples, include radioimmunoassays (RIA), enzyme linked immunosorbent assays (ELISA), and microparticle enzyme immunoassays (MEIA). To perform the assay, a sample could be incubated with excess vitamin D analogue and allowed to bind to sample apo-DBP. Holo-DBP could be measured by capturing the holo-DBP, but not the molecule-DBP complex on a solid surface of a microtiter plate or magnetic or latex microparticle pre-coated with holo-DBP recognition molecule (e.g., an antibody or antibody fragment). Holo-DBP could be measured using a holo-DBP recognition molecule, which may also recognize apo-DBP but binds holo-DBP preferentially over molecule-DBP complex, conjugated to a label or signal molecule. Alternatively, the molecule-DBP complex can be removed from the sample using a recognition molecule, capable of binding molecule-DBP complex, pre-coated onto a solid surface such as a magnetic or latex microparticle or the analogue can be pre-coated onto a solid surface and used to remove the apo-DBP from the sample. A label or signal molecule can be conjugated to the recognition molecule such as enzymes or chemiluminiscent compounds.

An antibody such as AbD Serotec 17355 could potentially be used in direct detection of holo-DBP as this molecule preferentially binds holo-DBP over molecule-DBP complex. Thus, techniques such as Surface Plasmon Resonance,[21] Surface Acoustic Wave,[22] and Quartz Crystal Microbalance[23] methodologies can be used.

3. Conclusions

We designed and synthesized six 25-hydroxyvitamin D_3 derivatives substituted at 3-position with a carbamate function that contain hydrophilic groups such as alcohol or amino at the end of the exogenous alkyl chain of the carbamate and a spacer group of different length. The synthetic strategy was based on the Wittig–Horner reaction to couple 25-protected Grundmann's ketone and A-ring phosphine oxide precursor. Modification at C-3 position of 25-hydroxyvitamin D_3 was introduced by a two-step protocol that involves vinyloxycarbonylation of the C-3-hydroxyl group and subsequent treatment with linear aminoalcohols or diamines of different length including a polyethylene glycol spacer arm. Conjugation of corresponding biotinylated derivatives of synthesized 25-hydroxyvitamin D_3 analogues with vitamin D binding protein (DBP) were carried out as suitable complexes to measure vitamin D levels in blood or serum samples. In this regard, screening of antibodies for their ability to bind 25-hydroxyvitamin D_3-DBP (holo-DBP) over the molecule-DBP complex was performed. The results of the assays performed show that the presence of a long aminoalkyl chain in the 25-hydroxyvitamin D_3 analogue **10** resulted in a reduced binding to some antibodies, and the level of antibody binding/recognition can be even further reduced when streptavidin is passed over the biotinylated analogue. However, analogue **10** displaces 25-hydroxyvitamin D_3 from DBP. Better results are observed with the long hydroxylalkyl chain analogue **9** of 25-hydroxyvitamin D_3, which exhibits less displacement of the tritiated 25-hydroxyvitamin D_3 from holo-DBP. These results make these new 25-hydroxyvitamin D_3 analogues promising candidates for measurement of vitamin D levels via indirect or direct assay formats in the clinical laboratory.

4. Experimental section

All chemical reagents were bought from Aldrich at highest commercial quality and used without further purification. All non-aqueous reactions were carried out under anhydrous conditions in dry, freshly distilled solvents. Reactions were monitored by TLC developed on 0.25 mm Merck silica gel plates (60F-254) using UV light as visualizing agent and/or heating after spraying with a 5% aqueous sulfuric acid solution containing cerium(IV) sulfate (1%) and molybdophosphoric acid (2.5%). Flash chromatography was performed using silica gel 60 (230–400 mesh). IR spectra were recorded as thin films on NaCl plates on an Infrared FT spectrophotometer. ^{1}H, ^{13}C NMR, and DEPT were obtained using 300.13 or 400.13 MHz for ^{1}H, and 75.5 or 100.61 MHz for ^{13}C NMR instruments. High resolution mass spectra (HRMS) were recorded on a VG 7070 HS mass spectrometer under electron spray ionization (ESI) conditions. Mixed type vitamin D binding protein was purchased from Merck Chemicals Ltd, catalogue number 345802. Zeba desalt spin column was acquired at Thermo, catalogue number 89891–5 mL. The antibodies/Fabs used in this work were obtained from AbCam and AbD Serotec. Radio labeled hydroxyvitamin D_3, [26,27-^{3}H]25-OH-D_3 [5μCi (185 kBq)], was bought from Perkin Elmer.

4.1. General procedure for preparation of 7–12

(−)-CSA (54 mg, 0.232 mmol) was added to a solution of **26–31** (0.046 mmol) in anhydrous MeOH (1.2 mL) at 0 °C and the mixture was stirred at rt for 1 h. The mixture was then poured into a separatory funnel, which contained 1 M NaOH (aq) (saturated $NaHCO_3$ for **11**) and extracted with EtOAc. The residue was purified by column chromatography (50% EtOAc/hexane for **7**; 0.5% NH_4OH/MeOH for **8**; 40% EtOAc/hexane for **9**; gradient eluent MeOH −0.1% NH_4OH/MeOH for **10**; 80% EtOAc/hexane for **11**; the residue was poured into the column in 1% NH_4OH/MeOH and eluted with MeOH for **12**).

4.1.1. 3-*O*-[*N*-(3-Hydroxypropyl)carbamoyl]-25-hydroxyvitamin D_3 (7)

Yield: 46%. R_f: 0.2 (50% EtOAc/hexane); IR (NaCl): ν 3383, 2945, 2850, 1698, 1531 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, Me_{18}), 0.94 (d, 3H, Me_{21}, J = 6.3 Hz), 0.79–2.46 (several m), 1.22 (s, 6H, Me_{26}+Me_{27}), 2.58 (dd, 1H, J = 13.3, 3.6 Hz), 2.83 (d, 1H, J = 10.5 Hz), 3.34 (dd, 2H, $H_{2'}$, J = 12.0, 6.1 Hz), 3.67 (t, 2H, $H_{4'}$, J = 5.7 Hz), 4.84 (d, 1H, H_{19}, J = 2.3 Hz), 4.85–5.01 (m, 2H, H_3+*NH*), 5.06 (s, 1H, H_{19}), 6.04 and 6.22 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 18.9 (CH_3), 20.9 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 27.8 (CH_2), 29.2 (CH_2), 29.3 (CH_3), 29.5 (CH_3), 32.2 (CH_2), 32.3 (CH_2), 32.9 (CH_2), 36.2 (CH), 36.5 (CH_2), 37.3 (CH_2), 40.7 (CH_2), 42.6 (CH_2), 44.5 (CH_2), 46.1 (C), 56.5 (CH), 56.6 (CH), 59.4 (CH_2), 71.3 (C), 72.4 (CH), 112.8 (CH_2), 117.6 (CH), 122.5 (CH), 134.7 (C), 142.5 (C), 144.9 (C), 157.4 (C) ppm; MS (ESI^+, m/z): 524 [$(M+Na)^+$, 100%]; HRMS (ESI^+, m/z) calcd for $C_{31}H_{51}NNaO_4$ $(M+Na)^+$: 524.3710, found: 524.3686.

4.1.2. 3-*O*-[*N*-(3-Aminopropyl)carbamoyl]-25-hydroxyvitamin D_3 (8)

Yield: 79%. R_f: 0.2 (1% NH_4OH/MeOH); IR (NaCl): ν 3356, 2942, 2850, 1698, 1532 cm^{-1}; ^1H NMR (300.13 MHz, MeOH-d_4): δ 0.57 (s, 3H, Me_{18}), 0.97 (d, 3H, Me_{21}, J = 6.3 Hz), 0.85–2.90 (several m), 1.19 (s, 6H, Me_{26}+Me_{27}), 3.19 (m, 2H, $H_{2'}$), 4.79 (br s, 1H, H_{19}), 5.07 (br s, 1H, H_{19}), 6.04 and 6.22 (2d, 2H, H_6+H_7, J = 11.1 Hz) ppm; ^{13}C NMR (75.5 MHz, MeOH-d_4): δ 12.4(CH_3), 19.4 (CH_3), 21.9 (CH_2), 23.3 (CH_2), 24.6 (CH_2), 28.7 (CH_2), 29.1 (CH_3), 29.3 (CH_3), 30.0 (CH_2), 30.5 (CH_2), 33.0 (CH_2), 33.4 (CH_2), 37.5 (CH), 37.8 (CH_2), 38.5 (CH_2), 38.7 (CH_2), 41.9 (CH_2), 43.5 (CH_2), 45.3 (CH_2), 47.0 (C), 57.5 (CH), 58.0 (CH), 71.5 (C), 73.4 (CH), 113.0 (CH_2), 118.8 (CH), 123.2 (CH), 136.3 (C), 142.9 (C), 146.6 (C), 158.8 (C) ppm; MS (ESI^+, m/z): 501 [$(M+H)^+$, 100%]; HRMS (ESI^+, m/z) calcd for $C_{31}H_{53}N_2O_3$ $(M+H)^+$: 501.4051, found: 501.4038.

4.1.3. 3-*O*-[*N*-(8-Hydroxyoctyl)carbamoyl]-25-hydroxyvitamin D_3 (9)

Yield: 40%. R_f: 0.3 (50% EtOAc/hexane); IR (NaCl): ν 3383, 2930, 2855, 1694 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, Me_{18}), 0.94 (d, 3H, Me_{21}, J = 6.3 Hz), 0.79–2.51 (several m), 1.22 (s, 6H, Me_{26}+Me_{27}), 2.58 (dd, 1H, J = 13.3, 3.3 Hz), 2.82 (d, 1H, J = 11.3 Hz), 3.16 (dd, 2H, $H_{2'}$, J = 12.5, 6.3 Hz), 3.64 (t, 2H, $H_{9'}$, J = 6.6 Hz), 4.66 (m, 1H, *NH*), 4.84 (s, 1H, H_{19}), 4.87 (m, 1H, H_3), 5.06 (s, 1H, H_{19}), 6.04 and 6.22 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 19.0 (CH_3), 20.9 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 25.8 (CH_2), 26.8 (CH_2), 27.8 (CH_2), 29.2 (CH_2), 29.3 (CH_3), 29.4 (CH_2), 29.5 (CH_3), 29.8 (CH_2), 30.1 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 32.9 (CH_2), 36.2 (CH), 36.5 (CH_2), 40.7 (CH_2), 41.0 (CH_2), 42.7 (CH_2), 44.5 (CH_2), 46.1 (C), 56.5 (CH), 56.6 (CH), 63.1 (CH_2), 71.3 (C), 71.8 (CH), 112.6 (CH_2), 117.7 (CH), 122.3 (CH), 135.0 (C), 142.4 (C), 145.1 (C), 156.3 (C) ppm; MS (ESI^+, m/z): 594 [$(M+Na)^+$, 100%]; HRMS (ESI^+, m/z) calcd for $C_{36}H_{61}NNaO_4$ $(M+Na)^+$: 594.4493, found: 594.4500.

4.1.4. 3-*O*-[*N*-(8-Aminooctyl)carbamoyl]-25-hydroxyvitamin D_3 (10)

Yield: 38%. R_f: 0.2 (1% NH_4OH/MeOH); IR (NaCl): ν 3370, 2930, 2848, 1702, 1538 cm^{-1}; ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, Me_{18}), 0.94 (d, 3H, Me_{21}, J = 6.4 Hz), 0.83–2.51 (several m), 1.21 (s, 6H, Me_{26}+Me_{27}), 2.57 (dd, 1H, J = 13.3, 3.0 Hz), 2.68 (br s, 2H,), 2.81 (d, 1H, J = 12.2 Hz), 3.15 (d, 2H, $H_{2'}$, J = 6.1 Hz), 4.69 (m, 1H, *NH*), 4.83 (s, 1H, H_{19}), 4.87 (br s, 1H, H_3), 5.05 (s, 1H, H_{19}), 6.04 and 6.21 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 19.0 (CH_3), 20.9 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 26.8 (CH_2), 27.0 (CH_2), 27.8 (CH_2), 29.2 (CH_2), 29.3 (CH_3), 29.4 (CH_2), 29.5 (CH_3), 30.1 (CH_2), 32.2 (CH_2), 32.4 (CH_2), 36.2 (CH), 36.5 (CH_2), 40.7 (CH_2), 41.0 (CH_2), 42.7 (CH_2), 44.6 (CH_2), 46.0 (C), 56.5 (CH), 56.7 (CH), 71.2 (C), 71.8 (CH), 112.6 (CH_2), 117.7 (CH), 122.3 (CH), 135.0 (C), 142.3 (C), 145.1 (C), 156.2 (C) ppm; MS (ESI^+, m/z): 571 [$(M+H)^+$, 100%]; HRMS (ESI^+, m/z) calcd for $C_{36}H_{63}N_2O_3$ $(M+H)^+$: 571.4833, found: 571.4866.

4.1.5. 3-*O*-[*N*-(8-Hydroxy-3,6-dioxaoctyl)carbamoyl]-25-hydroxyvitamin D_3 (11)

Yield: 51%. R_f: 0.2 (80% EtOAc/hexane); IR (NaCl): ν 3383, 2944, 2871, 1704, 1531 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, Me_{18}), 0.94 (d, 3H, Me_{21}, J = 6.3 Hz), 0.77–2.48 (several m), 1.21 (s, 6H, Me_{26}+Me_{27}), 2.58 (dd, 1H, J = 13.3, 3.5 Hz), 2.80 (dd, 1H, J = 10.5, 3.5 Hz), 3.37 (m, 2H, $H_{2'}$), 3.50–3.69 (several m, 8H, $H_{3'-6'}$), 3.72 (t, 2H, $H_{7'}$, J = 4.0 Hz), 4.83 (d, 1H, H_{19}, J = 2.4 Hz), 4.87 (m, 1H, H_3), 5.05 (d, 1H, H_{19}, J = 2.2 Hz), 5.41 (m, 1H, *NH*), 6.03 and 6.21 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1(CH_3), 18.9 (CH_3), 20.9 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 27.8 (CH_2), 29.2 (CH_2), 29.3 (CH_3), 29.5 (CH_3), 32.2 (CH_2), 32.3 (CH_2), 36.2 (CH), 36.5 (CH_2), 40.7 (CH_2), 40.8 (CH_2), 42.7 (CH_2), 44.5 (CH_2), 46.0 (C), 56.5 (CH), 56.6 (CH), 61.8 (CH_2), 70.3 (CH_2), 70.4 (CH_2), 70.5 (CH_2), 71.2 (C), 72.1 (CH), 72.7 (CH_2), 112.7 (CH_2), 117.6 (CH), 122.4 (CH), 135.0 (C), 142.4 (C), 145.0 (C), 153.3 (C) ppm; MS (ESI^+, m/z): 598 [$(M+Na)^+$, 100%]; HRMS (ESI^+, m/z) calcd for $C_{34}H_{57}NNaO_6$ $(M+Na)^+$: 598.4078, found: 598.4049.

4.1.6. 3-*O*-[*N*-(8-Amino-3,6-dioxaoctyl)carbamoyl]-25-hydroxyvitamin D_3 (12)

Yield: 50%. R_f: 0.2 (2% NH_4OH/MeOH): IR (NaCl): ν 3383, 2941, 2870, 1703, 1537 cm^{-1}; ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, Me_{18}), 0.93 (d, 3H, Me_{21}, J = 6.4 Hz), 0.75–2.41 (several m), 1.21 (s, 6H, Me_{26}+Me_{27}), 2.58 (d, 1H, J = 13.4 Hz), 2.73–2.94 (m, 3H), 3.36 (m, 2H, $H_{2'}$), 3.43–3.69 (several m, 8H, $H_{3'-6'}$), 4.82 (s, 1H, H_{19}), 4.87 (m, 1H, H_3), 5.05 (s, 1H, H_{19}), 5.44 (m, 1H, *NH*), 6.03 and 6.21 (2d, 2H, H_6+H_7, J = 11.1 Hz) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 12.1 (CH_3), 19.0 (CH_3), 20.9 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 27.8 (CH_2), 29.2 (CH_2), 29.3 (CH_3), 29.5 (CH_3), 32.2 (CH_2), 32.3 (CH_2), 36.2 (CH), 36.6 (CH_2), 40.7 (CH_2), 40.8 (CH_2), 41.8 (CH_2), 42.7 (CH_2), 44.6 (CH_2), 46.1 (C), 56.5 (CH), 56.7 (CH), 70.2 (CH_2), 70.3 (CH_2), 70.4 (CH_2), 71.2 (C), 72.0 (CH), 73.6 (CH_2), 112.7 (CH_2), 117.7 (CH), 122.4 (CH), 135.0 (C), 142.3 (C), 145.0 (C), 156.4 (C) ppm; MS (ESI^+, m/z): 575 [$(M+H)^+$, 100%]; HRMS (ESI^+, m/z) calcd for $C_{34}H_{59}N_2O_5$ $(M+Na)^+$: 575.4418, found: 575.4408.

4.2. (*S*)-(*Z*)-[2-[2-Methylene-5-[(vinyloxy)carbonyloxy] cyclohexylidene]ethyl]diphenylphosphine oxide (15)

To a solution of phosphine oxide **14** (60 mg, 0.177 mmol) in anhydrous pyridine (440 μL) at 0 °C was added dropwise vinyl chloroformate (40 μL, 0.443 mmol). The reaction was stirred at room temperature overnight. The crude was diluted with EtOAc and filtered over Celite. Solvents were evaporated under vacuum and the residue purified by column chromatography (gradient eluent of 50–70% EtOAc/hexane) to give **15** in a 53% yield. R_f: 0.5 (100% EtOAc). ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.66–2.65 (several m, 6H, $2H_{3'}$, $2H_{4'}$, $2H_{6'}$), 3.31 (dd, 2H, H_1, $|^2J_{PH}|$ = 14.5 Hz, $|^3J_{HH}|$ = 7.8 Hz), 4.57 (dd, 1H, $H_{3''\text{-}cis}$, J = 6.2, 2.0 Hz), 4.70 (m, 1H, $H_{5'}$), 4.85 (s, 1H, $H_{1''}$), 4.89 (dd, 1H, $H_{3''\text{-}trans}$, J = 13.9, 2.0 Hz), 5.01 (s, 1H, $H_{1''}$), 5.42 (apparent q, 1H, H_2, J = 7.7, 6.5 Hz), 7.06 (dd, 1H, $H_{2''}$, J = 13.9, 6.2 Hz), 7.50 (m, 6H, *Ph*) and 7.72 (m, 4H, *Ph*) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): 31.1 (CH_2, d, J = 67.5 Hz), 31.5 ($2CH_2$), 41.7 (CH_2), 75.9 (CH), 97.5 (CH_2), 112.7 (CH_2), 115.1 (CH, d, J = 8.1 Hz), 128.4–133.1 (C_{arom}), 140.2 (C, d, J = 12.5 Hz), 142.4 (CH), 143.5 (C), 151.9 (C) ppm; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 29.86 ppm.

4.3. (*S*)-(*Z*)-[2-[5-[[*N*-(3-Hydroxypropyl)carbamoyl]oxy]-2-methylenecyclohexylidene]ethyl]diphenylphosphine oxide (16)

A solution of vinyl carbonate **15** (34 mg, 0.084 mmol) in anhydrous THF (1 mL) was treated with 3-amino-1-propanol (20 μL, 0.262 mmol) and the mixture was stirred at 60 °C until TLC (10% EtOAc/hexane) showed complete consumption of starting material (48 h). Then, solvent was evaporated and the residue purified by column chromatography (gradient eluent 100% EtOAc-5% MeOH/EtOAc) to give **16** in 81% yield. R_f: 0.15 (100% EtOAc); IR (NaCl): ν 3393, 3079, 2943, 1695, 1537 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.47–2.50 (several m, 8H, $2H_{3'}$, $2H_{4'}$, $2H_{6'}$, $2H_{3''}$), 3.02–3.53 (several m, 4H, $2H_1$, $2H_{2''}$), 3.62 (t, 2H, $H_{4''}$, J = 5.8 Hz), 4.76 (br s, 1H, *NH*), 4.77 (s, 1H, $H_{1''}$), 4.99 (s, 1H, $H_{1'''}$), 5.15 (m, 1H, $H_{5'}$), 5.34 (ap q, 1H, H_2, J = 7.9, 6.8 Hz), 7.48 (m, 6H, *Ph*) and 7.70 (m, 4H, *Ph*) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 31.2 (CH_2, d, J = 69.9 Hz), 31.8 ($2CH_2$), 32.4 (CH_2), 37.5 (CH_2), 42.4 (CH_2), 59.4 (CH_2), 71.1 (CH), 112.0 (CH_2), 114.0 (CH, d, J = 8.5 Hz), 128.5–133.3 (C_{arom}), 141.7 (C, d, J = 12.5 Hz), 145.0 (C), 156.8 (C) ppm; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 30.64 ppm; MS (ESI^+, m/z): 462 [$(M+Na)^+$, 100%].

4.4. (*S*)-(*Z*)-[2-[5-[[*N*-[(3-*tert*-Butyldimethylsilyl)oxypropyl]carbamoyl]oxy]-2-methylenecyclohexylidene]ethyl]-diphenylphosphine oxide (17)

To a solution of **16** (29 mg, 0.066 mmol) in anhydrous CH_2Cl_2 (250 μL) at 0 °C, were added imidazole (6 mg, 0.086 mmol) and *tert*-butyldimethylsilyl chloride (12 mg, 0.079 mmol). Afterwards, the reaction was stirred at room temperature for 2 h. Reaction was stopped by addition of water followed by extraction with CH_2Cl_2. The residue was purified by column chromatography (gradient eluent 60–70% EtOAc/hexane) to give protected alcohol **17** in a 60% yield. R_f: 0.3 (100% EtOAc); ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.05 (s, 3H, Si*Me*), 0.08 (s, 3H, Si*Me*), 0.89 (s, 6H, 2SiC*Me*), 0.91 (s, 3H, SiC*Me*), 1.50–2.51 (several m, 8H, $H_{3'}$, $H_{4'}$, $H_{6'}$, $H_{3''}$), 3.03–3.39 (several m, 4H, H_1, $H_{2''}$), 3.66 (m, 2H, $H_{4''}$), 4.66 (m, 1H, *NH*), 4.79 (s, 1H, $H_{1''}$), 4.98 (s, 1H, $H_{1'''}$), 5.00 (br s, 1H, $H_{5'}$), 5.35 (ap q, 1H, H_2, J = 7.1, 6.8 Hz), 7.48 (m, 6H, *Ph*) and 7.71 (m, 4H, *Ph*) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ −3.4 ($2CH_3$), 18.1 (C), 25.8 ($3CH_3$), 30.9 (CH_2, d, J = 69.7 Hz), 31.84 (CH_2), 32.5 (CH_2), 37.6 (CH_2), 42.4 (CH_2), 59.5 (CH_2), 71.2 (CH), 112.0 (CH_2), 114.1 (CH_2, d, J = 9.3 Hz), 128.6–133.5 (C_{arom}), 141.7 (C, d, J = 12.2 Hz), 145.1 (C), 156.9 (C) ppm; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 30.37 ppm.

4.5. 3-*O*-[*N*-[(3-*tert*-Butyldimethylsilyl)oxypropyl]carbamoyl]-25-trimethylsilyoxyvitamin D_3 (21)

To a solution of phosphine oxide **17** (15 mg, 0.027 mmol) in anhydrous THF (500 μL) at −78 °C in darkness was added dropwise ^{n}BuLi (34 μL, 1.6 M in hexanes, 0.054 mmol) resulting in a deep red color solution. After 1 h at this temperature, ketone **20** (4 mg, 0.011 mmol) dissolved in THF (100 μL) was added slowly and the reaction mixture was stirred for 3 h at −78 °C and then warmed to −40 °C and stirred for 2 h. The reaction was quenched by the addition of H_2O (500 μL) and the mixture was then poured into a separatory funnel and extracted with EtOAc. The residue was purified by column chromatography (eluent 7.5 EtOAc/hexane) to give **21**. R_f: 0.6 (25% EtOAc/hexane); ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.06 (s, 3H, Si*Me*), 0.07 (s, 3H, Si*Me*), 0.09 (s, 3H, Si*Me*), 0.12 (s, 12H, TMS), 0.56 (s, 3H, Me_{18}), 0.90 (s, 9H, SiCMe_3), 1.22 (s, 6H, Me_{26}+Me_{27}), 0.77–2.89 (several m), 3.31 (m, 2H, $H_{2'}$), 3.72 (m, 2H, $H_{4'}$), 4.84 (s, 1H, H_{19}), 4.88 (m, 1H, H_3), 5.06 (s, 1H, H_{19}), 5.28 (m, 1H, *NH*), 6.06 and 6.20 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm.

4.6. 3-*O*-(*tert*-Butyldimethylsilyl)-25-ethoxymethyloxyvitamin D_3 (23)

To a solution of phosphine oxide **13** (173 mg, 0.382 mmol) in anhydrous THF (1.5 mL) at −78 °C in darkness was added dropwise ^{n}BuLi (213 μL, 1.6 M in hexanes, 0.341 mmol) resulting in a deep red color solution. After 1 h at this temperature, ketone **22** (68 mg, 0.201 mmol) dissolved in THF (2.2 mL) was added slowly and the reaction mixture was stirred for 3 h at −78 °C and then warmed to −40 °C and stirred for 2 h. The reaction was quenched by the addition of H_2O (4 mL) and the mixture was then poured into a separatory funnel and extracted with Et_2O. The residue was purified by column chromatography (gradient eluent 1.5–40% EtOAc/hexane) to give **23** in 94% yield and unreacted phosphine oxide (53 mg). R_f: 0.7 (25% EtOAc/hexane); IR (NaCl): ν 2935, 2854, 1471, 1440, 1379 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.067 (s, 3H, Si*Me*), 0.073 (s, 3H, Si*Me*), 0.55 (s, 3H, Me_{18}), 0.89 (s, 9H, SiCMe_3), 0.93 (d, 3H, Me_{21}, J = 6.3 Hz), 1.20 (t, 3H, $Me_{3''}$, J = 7.0 Hz), 1.22 (s, 6H, Me_{26}+Me_{27}), 0.98-2.50 (several m), 2.83 (d, 1H, J = 11.6 Hz), 3.61 (q, 2H, $H_{2''}$, J = 7.1 Hz), 3.82 (m, 1H, H_3), 4.75 (s, 2H, $H_{1''}$), 4.78 (s, 1H, H_{19}), 5.01 (s, 1H, H_{19}), 6.01 and 6.17 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ −4.5 (CH_3), −4.4 (CH_3), 12.2 (CH_3), 15.3 (CH_3), 18.3 (C), 19.0 (CH_3), 20.7 (CH_2), 22.4 (CH_2), 23.6 (CH_2), 26.0 (CH_3), 26.5 (CH_3), 26.6 (CH_3), 27.9 (CH_2), 29.1 (CH_2), 32.9 (CH_2), 36.3 (CH), 36.5 (CH_2), 36.6 (CH_2), 40.7 (CH_2), 42.4 (CH_2), 45.9 (C), 47.0 (CH_2), 56.5 (CH), 56.7 (CH), 63.1 (CH_2), 70.7 (CH), 76.3 (C), 89.6 (CH_2), 112.3 (CH_2), 118.0 (CH), 121.5 (CH), 136.4 (C), 141.6 (C), 145.5 (C) ppm; MS (ESI^+, m/z): 595 [$(M+Na)^+$, 100%].

4.7. 25-Ethoxymethyloxyvitamin D_3 (24)

TBAF (1.2 mL, 1 M in THF, 1.151 mmol) was added dropwise to a solution of silyl ether **23** (264 mg, 0.460 mmol) in anhydrous THF (9.2 mL) at 0 °C in darkness. The reaction mixture was followed by TLC (25% EtOAc/hexane) until complete consumption of starting material (5–7 h). Then, solvent was evaporated and the residue purified by column chromatography (gradient eluent 10–15% EtOAc/hexane) to give **24** in 91% yield. R_f: 0.4 (25% EtOAc/hexane); IR (NaCl): ν 3383, 2937, 2874, 1645, 1472, 1442, 1380 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, Me_{18}), 0.93 (d, 3H, Me_{21}, J = 6.3 Hz), 1.20 (t, 3H, $Me_{3''}$, J = 7.1 Hz), 1.21 (s, 6H, Me_{26}+Me_{27}), 0.87–2.50 (several m), 2.57 (dd, 1H, J = 13.0, 3.5 Hz), 2.82 (dd, 1H, J = 11.5, 3.5 Hz), 3.61 (q, 2H, $H_{2''}$, J = 7.1 Hz), 3.94 (m, 1H, H_3), 4.75 (s, 2H, $H_{1''}$), 4.82 (d, 1H, H_{19}, J = 2.5 Hz), 5.05 (d, 1H, H_{19}, J = 2.4 Hz), 6.03 and 6.23 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.7 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 26.5 (CH_3), 26.6 (CH_3), 27.8 (CH_2), 29.2 (CH_2), 32.1 (CH_2), 35.3 (CH_2), 36.3 (CH), 36.6 (CH_2), 40.7 (CH_2), 42.4 (CH_2), 46.0 (CH_2), 46.1 (C), 56.5 (CH), 56.7 (CH), 63.1 (CH_2), 69.4 (CH), 76.4 (C), 89.6 (CH_2), 112.6 (CH_2), 117.6 (CH), 122.6 (CH), 135.2 (C), 142.4 (C), 145.2 (C) ppm; MS (ESI^+, m/z): 481 [$(M+Na)^+$, 100%].

4.8. 3-*O*-(Vinyloxy)carbonyl-25-ethoxymethyloxyvitamin D_3 (25)

To a solution of **24** (57 mg, 0.124 mmol) in pyridine (300 μL) at 0 °C was added dropwise vinyl chloroformate (30 μL, 0.312 mmol). The reaction was stirred overnight. The crude was diluted with EtOAc and filtered over Celite. Solvents were evaporated under vacuum and the residue was filtered through a short column of silica gel (EtOAc as eluent) to afford **25** (92% yield), which was sufficiently pure for direct use in the next step. Further purification can be performed by column chromatography (4% EtOAc/hexane). R_f: 0.5 (10% EtOAc/hexane); IR (NaCl): ν 2946, 2873, 1758, 1650,

1469 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.55 (s, 3H, *Me*$_{18}$), 0.94 (d, 3H, *Me*$_{21}$, J = 6.2 Hz), 1.01–2.80 (several m), 1.21 (t, 3H, *Me*$_{3''}$, J = 7.0 Hz), 1.22 (s, 6H, *Me*$_{26}$+*Me*$_{27}$), 3.62 (q, 2H, $H_{2''}$, J = 7.1 Hz), 4.57 (dd, 1H, $H_{3'}$-*cis*, J = 6.2, 2.0 Hz), 4.76 (s, 2H, $H_{1''}$), 4.87 (s, 1H, H_{19}), 4.88 (m, 1H, H_3), 4.91 (dd, 1H, $H_{3'}$-*trans*, J = 13.9, 2.0 Hz), 5.08 (s, 1H, H_{19}), 6.03 and 6.25 (2d, 2H, H_6+H_7, J = 11.1 Hz), 7.10 (dd, 1H, $H_{2'}$, J = 13.9, 6.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.7 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 26.5 (CH_3), 26.6 (CH_3), 27.8 (CH_2), 29.2 (CH_2), 31.9 (CH_2), 32.0 (CH_2), 36.3 (CH), 36.6 (CH_2), 40.7 (CH_2), 42.0 (CH_2), 42.4 (CH_2), 46.1 (C), 56.5 (CH), 56.7 (CH), 63.1 (CH_2), 76.3 (CH), 76.7 (C), 89.6 (CH_2), 97.7 (CH_2), 113.2 (CH_2), 117.6 (CH), 123.1 (CH), 133.5 (C), 142.7 (CH), 143.0 (C), 144.2 (C), 152.3 (C) ppm; MS (ESI^+, m/z): 551 [$(M+Na)^+$, 100%]; HRMS (ESI^+, m/z): calcd for $C_{33}H_{52}NaO_5$ $(M+Na)^+$: 551.3707, found: 551.3710.

4.9. 3-*O*-[*N*-(3-Hydroxypropyl)carbamoyl]-25-ethoxymethyloxy vitamin D_3 (26)

A solution of vinyl carbonate **25** (50 mg, 0.095 mmol) in anhydrous THF (1.2 mL) was treated with 30 μL (0.371 mmol) of 3-amino-1-propanol and the mixture was stirred at 60 °C until TLC (10% EtOAc/hexane) showed complete consumption of starting material (72 h). Then, solvent was evaporated and the residue purified by column chromatography (eluent 40% EtOAc/hexane) to give **26** in 50% yield. R_f: 0.7 (100% EtOAc); IR (NaCl): ν 3382, 2944, 2840, 1698, 1531 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, *Me*$_{18}$), 0.93 (d, 3H, *Me*$_{21}$, J = 6.3 Hz), 0.98–2.45 (several m), 1.20 (t, 3H, *Me*$_{3''}$, J = 7.0 Hz), 1.22 (s, 6H, *Me*$_{26}$+*Me*$_{27}$), 2.58 (dd, 1H, J = 13.6, 3.7 Hz), 2.82 (dd, 1H, J = 10.9, 2.8 Hz), 3.34 (dd, 2H, $H_{2'}$, J = 11.8, 6.2 Hz), 3.61 (q, 2H, $H_{2''}$, J = 7.1 Hz), 3.68 (br s, 2H, $H_{4'}$), 4.76 (s, 2H, $H_{1''}$), 4.84 (d, 1H, H_{19}, J = 2.4 Hz), 4.85–4.99 (m, 2H, *NH*+H_3), 5.06 (d, 1H, H_{19}, J = 2.2 Hz), 6.03 and 6.22 (2d, 2H, H_6+H_7, J = 11.3 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.7(CH_2), 22.4 (CH_2), 23.7 (CH_2), 26.5 (CH_3), 26.6 (CH_3), 27.8 (CH_2), 29.2 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 32.9 (CH_2), 36.3 (CH), 36.5 (CH_2), 37.4 (CH_2), 40.7 (CH_2), 42.3 (CH_2), 42.6 (CH_2), 46.1 (C), 56.5 (CH), 56.7 (CH), 59.4 (CH_2), 63.1 (CH_2), 72.4 (CH), 76.4 (C), 89.6 (CH_2), 112.8 (CH_2), 117.6 (CH), 122.5 (CH), 134.7 (C), 142.5 (C), 144.9 (C), 157.4 (C) ppm; MS (ESI^+, m/z): 582 [$(M+Na)^+$, 100%].

4.10. 3-*O*-[*N*-(3-Aminopropyl)carbamoyl]-25-ethoxymethyloxy vitamin D_3 (27)

A solution of vinyl carbonate **25** (35 mg, 0.066 mmol) in anhydrous THF (0.830 mL) was treated with 15 μL (0.165 mmol) of 1,3-propanediamine and the mixture was stirred at 50 °C overnight. Then, solvent was evaporated and the residue purified by column chromatography (eluent 0.5% NH_4OH/MeOH) to afford **27** in 50% yield. R_f: 0.4 (5% NH_4OH/MeOH); IR (NaCl): ν 3330, 2949, 2869, 1707, 1517 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, *Me*$_{18}$), 0.93 (d, 3H, *Me*$_{21}$, J = 6.3 Hz), 0.98–2.86 (several m), 1.20 (t, 3H, *Me*$_{3''}$, J = 7.0 Hz), 1.21 (s, 6H, *Me*$_{26}$+*Me*$_{27}$), 3.26 (m, 2H, $H_{2'}$), 3.61 (q, 2H, $H_{2''}$, J = 7.1 Hz), 4.75 (s, 2H, $H_{1''}$), 4.83 (d, 1H, H_{19}, J = 2.2 Hz), 4.86 (m, 1H, H_3), 5.05 (s, 1H, H_{19}), 6.03 and 6.22 (2d, 2H, H_6+H_7, J = 11.3 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.7(CH_2), 22.4 (CH_2), 23.7 (CH_2), 26.5 (CH_3), 26.6 (CH_3), 27.8 (CH_2), 29.2 (CH_2), 29.8 (CH_2), 32.5 (CH_2), 36.3 (CH), 36.6 (CH_2), 38.2 (CH_2), 40.7 (CH_2), 42.4 (CH_2), 46.0 (C), 56.5 (CH), 56.8 (CH), 63.1 (CH_2), 72.3 (CH), 76.3 (C), 89.6 (CH_2), 112.8 (CH_2), 117.7 (CH), 122.5 (CH), 134.7 (C), 142.4 (C), 144.8 (C), 156.6 (C) ppm; MS (ESI^+, m/z): 559 [$(M+H)^+$, 100%].

4.11. 3-*O*-[*N*-(8-Hydroxyoctyl)carbamoyl]-25-ethoxymethyloxyvitamin D_3 (28)

A solution of vinyl carbonate **25** (45 mg, 0.085 mmol) in anhydrous THF (1.0 mL) was treated with 37 mg (0.255 mmol) of 8-amino-1-octanol and the mixture was stirred at 50 °C until TLC (10% EtOAc/hexane) showed complete consumption of starting material (48 h). Then, solvent was evaporated and the residue purified by column chromatography (eluent 30% EtOAc/hexane) to afford **28** in 42% yield. R_f: 0.5 (50% EtOAc/hexane); IR (NaCl): ν 3343, 2931, 2866, 1695, 1539 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, *Me*$_{18}$), 0.92 (d, 3H, *Me*$_{21}$, J = 6.2 Hz), 0.96–2.49 (several m), 1.20 (t, 3H, *Me*$_{3''}$, J = 7.1 Hz), 1.21 (s, 6H, *Me*$_{26}$+*Me*$_{27}$), 2.57 (dd, 1H, J = 13.2, 3.2 Hz), 2.81 (dd, 1H, J = 10.7, 3.3 Hz), 3.15 (m, 2H, $H_{2'}$), 3.61 (q, 2H, $H_{2''}$, J = 7.2 Hz), 3.62 (t, 2H, $H_{9'}$, J = 6.6 Hz), 4.68 (br s, 1H, *NH*), 4.75 (s, 2H, $H_{1''}$), 4.82 (d, 1H, H_{19}, J = 2.4 Hz), 4.86 (m, 1H, H_3), 5.04 (s, 1H, H_{19}), 6.03 and 6.21 (2d, 2H, H_6+H_7, J = 11.3 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.7 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 25.8 (CH_2), 26.5 (CH_3), 26.6 (CH_3), 26.8 (CH_2), 27.8 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 30.1 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 32.9 (CH_2), 36.3 (CH), 36.6 (CH_2), 40.7 (CH_2), 41.0 (CH_2), 42.4 (CH_2), 42.7 (CH_2), 46.0 (C), 56.5 (CH), 56.7 (CH), 63.1 ($2CH_2$), 71.8 (CH), 76.3 (C), 89.6 (CH_2), 112.6 (CH_2), 117.6 (CH), 122.3 (CH), 135.0 (C), 142.3 (C), 145.1 (C), 156.2 (C) ppm; MS (ESI^+, m/z): 652 [$(M+Na)^+$, 100%].

4.12. 3-*O*-[*N*-(8-Aminooctyl)carbamoyl]-25-ethoxymethyloxy vitamin D_3 (29)

A solution of vinyl carbonate **25** (57 mg, 0.108 mmol) in anhydrous THF (1.3 mL) was treated with 63 mg (0.437 mmol) of 1,8-octanediamine and the mixture was stirred at 50 °C until TLC (10% EtOAc/hexane) showed complete consumption of starting material (5 d). Then, solvent was evaporated and the residue purified by column chromatography (eluent 1% NH_4OH/MeOH) to give **29** in 63% yield. R_f: 0.2 (1% NH_4OH/MeOH); IR (NaCl): ν 3349, 2931, 2861, 1716, 1539 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.53 (s, 3H, *Me*$_{18}$), 0.79–2.89 (several m), 0.92 (d, 3H, *Me*$_{21}$, J = 6.2 Hz), 1.19 (t, 3H, *Me*$_{3''}$, J = 6.9 Hz), 1.20 (s, 6H, *Me*$_{26}$+*Me*$_{27}$), 3.13 (m, 2H, $H_{2'}$), 3.33 (t, 1H, *NH*, J = 6.9 Hz), 3.60 (q, 2H, $H_{2''}$, J = 7.0 Hz), 4.74 (s, 2H, $H_{1''}$), 4.82 (d, 1H, H_{19}, J = 2.1 Hz), 4.85 (br s, 1H, H_3), 5.04 (s, 1H, H_{19}), 5.23 (br s, 2H, NH_2), 6.02 and 6.20 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.6 (CH_2), 22.3 (CH_2), 23.7 (CH_2), 26.48 (CH_3), 26,54 (CH_3), 26.75 (CH_2), 26.78 (CH_2), 27.8 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 30.0 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 36.2 (CH), 36.5 (CH_2), 40.6 (CH_2), 41.0 (CH_2), 42.3 (CH_2), 42.7 (CH_2), 46.0 (C), 56.5 (CH), 56.7 (CH), 63.0 (CH_2), 71.8 (CH), 76.3 (C), 89.6 (CH_2), 112.6 (CH_2), 117.6 (CH), 122.3 (CH), 134.9 (C), 142.3 (C), 145.0 (C), 156.2 (C) ppm; MS (ESI^+, m/z): 629 [$(M+H)^+$, 100%].

4.13. 3-*O*-[*N*-(8-Hydroxy-3,6-dioxaoctyl)carbamoyl]-25-ethoxy methyloxyvitamin D_3 (30)

A solution of vinyl carbonate **25** (50 mg, 0.095 mmol) in anhydrous THF (1.2 mL) was treated with 40 μL (0.284 mmol) of 8-amine-3,6-dioxaoctan-1-ol and the mixture was stirred at 50 °C until TLC (10% EtOAc/hexane) showed complete consumption of starting material (3 d). Then, solvent was evaporated and the residue purified by column chromatography (eluent 70% EtOAc/hexane) to afford **30** in 42% yield. R_f: 0.3 (75% EtOAc/hexane); IR (NaCl): ν 3382, 2944, 2873, 1695, 1538 cm^{-1}; ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.54 (s, 3H, *Me*$_{18}$), 0.93 (d, 3H, *Me*$_{21}$, J = 6.4 Hz), 0.80–2.43 (several m), 1.20 (t, 3H, *Me*$_{3''}$, J = 7.1 Hz), 1.21 (s, 6H, *Me*$_{26}$+*Me*$_{27}$), 2.58 (d, 1H, J = 10.5 Hz), 2.82 (d, 1H,

J = 12.0 Hz), 3.38 (m, 2H, $H_{2'}$), 3.51–3.69 (several m, 10H, $H_{3'-6'}$+$H_{2''}$), 3.73 (br s, 1H, $H_{7'}$), 4.75 (s, 2H, $H_{1''}$), 4.83 (s, 1H, H_{19}, J = 1.7 Hz), 4.88 (br s, 1H, H_3), 5.05 (s, 1H, H_{19}), 5.35 (m, 1H, *NH*), 6.04 and 6.22 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.7(CH_2), 22.4 (CH_2), 23.7 (CH_2), 26.5 (CH_3), 26.6 (CH_3), 27.8 (CH_2), 29.2 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 36.3 (CH), 36.6 (CH_2), 40.7 (CH_2), 40.8 (CH_2), 42.4 (CH_2), 42.7 (CH_2), 46.0 (C), 56.5 (CH), 56.7 (CH), 61.9 (CH_2), 63.0 (CH_2), 70.3 (CH_2), 70.4 (CH_2), 70.5 (CH_2), 72.0 (CH), 72.6 (CH_2), 76.3 (C), 89.6 (CH_2), 112.7 (CH_2), 117.6 (CH), 122.4 (CH), 135.0 (C), 142.4 (C), 145.0 (C), 156.3 (C) ppm; MS (ESI$^+$, m/z): 656 [(M+Na)$^+$, 100%].

4.14. 3-*O*-[*N*-(8-Amino-3,6-dioxaoctyl)carbamoyl]-25-ethoxy methyloxyvitamin D_3 (31)

A solution of vinyl carbonate **25** (50 mg, 0.095 mmol) in anhydrous THF (1.2 mL) was treated with 41 μL (0.284 mmol) of 1,8-diamine-3,6-dioxaoctane and the mixture was stirred at 50 °C until TLC (10% EtOAc/hexane) showed complete consumption of starting material (3 d). Then, solvent was evaporated and the residue purified by column chromatography (eluent 1% NH_4OH/MeOH) to give **31** in 40% yield. R_f: 0.1 (50% MeOH/EtOAc); IR (NaCl): ν 3382, 2941, 2872, 1715 cm^{-1}; ^{1}H NMR (400.13 MHz, $CDCl_3$): δ 0.53 (s, 3H, Me_{18}), 0.92 (d, 3H, Me_{21}, J = 6.3 Hz), 0.80–2.48 (several m), 1.19 (td, 3H, $Me_{3''}$, J = 7.1, 1.1 Hz), 1.20 (s, 6H, Me_{26}+Me_{27}), 2.57 (d, 1H, J = 12.7 Hz), 2.81 (d, 1H, J = 13.7 Hz), 2.86 (dd, 2H, $H_{7'}$, J = 10.3, 5.1 Hz), 3.36 (m, 2H, $H_{2'}$), 3.42–3.66 (several m, 10H, $H_{3'-6'}$+$H_{2''}$), 4.74 (s, 2H, $H_{1''}$), 4.82 (s, 1H, H_{19}), 4.87 (br s, 1H, H_3), 5.04 (s, 1H, H_{19}), 5.48 (m, 1H, *NH*), 6.03 and 6.21 (2d, 2H, H_6+H_7, J = 11.2 Hz) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 12.1 (CH_3), 15.3 (CH_3), 18.9 (CH_3), 20.7 (CH_2), 22.4 (CH_2), 23.7 (CH_2), 26.5 (CH_3), 26.6 (CH_3), 27.8 (CH_2), 29.2 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 36.2 (CH), 36.5 (CH_2), 40.7 (CH_2), 40.8 (CH_2), 41.8 (CH_2), 42.3 (CH_2), 42.7 (CH_2), 46.0 (C), 56.5 (CH), 56.7 (CH), 63.0 (CH_2), 70.2 (CH_2), 70.3 (CH_2), 70.4 (CH_2), 71.9 (CH), 73.5 (CH_2), 76.3 (C), 89.6 (CH_2), 112.7 (CH_2), 117.6 (CH), 122.4 (CH), 134.9 (C), 142.3 (C), 145.0 (C), 156.3 (C) ppm; MS (ESI$^+$, m/z): 633 [(M+H)$^+$, 100%].

4.15. Protocol of an assay to measure vitamin D

Anti-vitamin D binding protein antibody (17355, AbD Serotec) is diluted to 1 μg/mL in carbonate buffer (16 mM sodium carbonate anhydrous, 35 mM sodium hydrogen carbonate, pH 9.5). A microtiter plate is coated with the antibody solution at 100 μL/well. The plate is incubated overnight at 2–8 °C. The contents of the wells are decanted and 100 μL/well of blocking buffer containing 5% bovine serum albumin (BSA) diluted in 0.01 M phosphate buffered saline (PBS) is added. The plate is incubated for 1 h at 18–25 °C. Patients samples (serum) are prepared by adding hydroxyvitamin D analogue (50 μg/mL) and vortexed before being incubated for 1 h at 18–25 °C. Streptavidin is added to the biotinylated analogue at this or subsequent stage. 100 μL of the sample-analogue mixture is added to each of the wells and incubated at 18–25 °C for 1 h. The contents of the wells are decanted and they are washed by dispensing 250 μL/well of 1% Triton X phosphate buffered saline. This is repeated a further two times, after the final wash step the plate is blotted dry. 100 μL/well of anti-DBP antibody (23484, AbCam) conjugated to horseradish peroxidase (HRP) (diluted 1/2000 in 20 mM HEPES, 0.1% *p*-hydroxyphenylacetic acid, 0.15% Proclin, 1% BSA) is added. The plate is incubated at 18–25 °C for 1 h. The contents of the wells are decanted and they are washed by dispensing 250 μL/well of 1% Triton X phosphate buffered saline. This is repeated a further two times, after the final wash step the plate is blotted dry. 100 μL/well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate is added and developed for 15 min before the reaction is stopped with the addition of 100 μL/well of 0.25 M sulfuric acid. The plate is read at 450 nm. A dose–response is seen with absorbance increasing with increasing vitamin D concentration, as measured by a reference method (IDS 25-OH-D EIA assay).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.013.

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