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Synthesis of C-11 linked active ester derivatives of vitamin D_3 and their conjugations to 42-residue helix–loop–helix peptides

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ABSTRACT

Derivatives of vitamin D_3 carrying an 8-carbon linker at C-11 terminating in an active ester were synthesized from commercial vitamin D_3 using a disassembly–reassembly strategy. Vitamin D_3 was cleaved at the C6–C7 double bond and the 'upper' fragment was converted, via a series of reactions, to derivatives substituted at C-11 with an 8-carbon linker terminating in an ethyl ester. Reassembly with modified 'lower' fragments using Horner–Wittig olefination followed by linker ester hydrolysis and re-esterification with *p*-nitrophenol gave C-11 substituted *p*-nitrophenyl esters. These vitamin D derivatives were conjugated to 42-amino acid helix–loop–helix peptides by reaction of their *p*-nitrophenyl esters with lysyl side-chain amino groups on the peptides. The vitamin D—peptide conjugates, being potential specific binder candidates for vitamin D-binding protein, were characterized by mass spectroscopy and CD measurements.

1. Introduction

The vitamin D family of molecules is associated with the regulation of calcium and phosphorus metabolism in higher organisms, and various vitamin D derivatives have been shown to affect disease conditions, such as rickets, osteoporosis, breast cancer, prostate cancer, psoriasis, and Alzheimer's disease. The influences of vitamin D derivatives on these and other diseases have spurred a wide range of research efforts where a large number of vitamin D derivatives have been synthesized and biologically evaluated (for a recent comprehensive review of the synthetic work, see Okamura and Zhue[1]).

Biological evaluations of vitamin D derivatives have involved both in vivo and in vitro studies, the latter often investigating the ability of various vitamin D derivatives to bind to receptor proteins. Two extensively studied such receptor proteins are the vitamin D receptor (VDR) and the vitamin D-binding protein (DBP). The latter is the major carrier of vitamin D and its metabolites in serum, and as such it is an important regulator of vitamin D levels in the body. Considering the importance of vitamin D in health and disease, it is not surprising that abnormal serum DBP levels have been correlated to disease conditions.[2–4] We are interested in the possibility to use DBP levels in body fluids as biomarker for Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and Parkinson's disease. Accurate and fast measurement of DBP is then an essential requirement, and we have therefore set out to design peptide conjugates[5] of vitamin D derivatives that could potentially, by way of their increased binding strength and multifunctionality, be important tools in such measurements. We have shown previously[6] that the conjugation of benzenesulfonamide with an affinity, K_D, of 1.5 μM for human Carbonic anhydrase II (HCAII) to a 42-residue polypeptide via an aliphatic spacer, gave rise to a polypeptide conjugate binder for HCAII with a dissociation constant of 20 nM. The small molecule ligand and the polypeptide both contributed to binding in a cooperative manner, the polypeptide mainly through hydrophobic interactions between HCAII and the hydrophobic faces of the amphiphilic helices of the helix–loop–helix motif. We hypothesized that amphiphilic helices bind well to proteins through hydrophobic interactions, whereas the polar sides of the helices make them soluble in aq solution. The versatility of polypeptides makes them highly suitable for further functionalization, e.g., for the incorporation of fluorophores.[7,8] They are therefore very well suited for bioanalytical applications, such as the identification and quantification of proteins related to disease.[9]

We here report synthesis of the vitamin D derivatives **1**, **2**, and **3**, carrying an active ester-containing linker attached to the C-11 of the vitamin D skeleton. To our knowledge, this is the first example of vitamin D derivatives with a C11-attached linker. We also report conjugation of the synthesized vitamin D derivatives to 42-residue polypeptides, and characterization of the conjugates by MALDI-TOF

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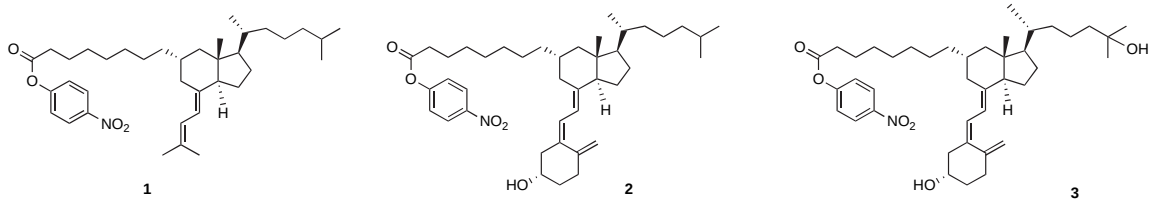
mass spectroscopy and CD spectroscopy. The evaluation of the peptide conjugates as specific binders of DBP and other proteins will be reported in future communications.



1 2 3

2. Results and discussion

2.1. Design and synthesis of C-11 linked active ester derivatives of vitamin D_3

The design of a useful vitamin D—peptide conjugate requires a derivative of vitamin D that binds strongly and specifically to DBP. Also, this derivative should carry a linking moiety protruding from a region of the molecule not involved in the binding to DBP. The linking moiety should terminate in a functionality, which is reactive toward a peptide attachment point, such as a lysyl side-chain amino group. Inspection of the crystal structure of the DBP–ligand complex[10] reveals that C-11 of the vitamin D_3 molecule is not strongly involved in DBP binding, and attachment here of a linking moiety should therefore not affect vitamin D_3 binding to any greater extent. Indeed, vitamin D derivatives with simple C-11 substituents have been synthesized[11–14] and shown[11,12] to bind DBP similarly to unsubstituted controls. Calculations using PyMOL software gave at hand, that an 8-carbon chain extending from C-11 extrudes far enough from the DBP—vitamin D_3 surface that attachment of a peptide ligand at the end of the chain should not interfere with the binding, but rather strengthen it by providing additional peptide–protein interactions with the DBP surface. We therefore decided to synthesize the C-11 substituted active ester vitamin D_3 derivatives **1**, **2**, and **3**. To accomplish this, we used vitamin D_3 as starting material and followed a strategy of disassembly–reassembly previously used in vitamin D syntheses.[1] The strategy is sketched below (Fig. 1) for compound **2**. Briefly, commercial vitamin D_3 was cleaved at the C6–C7 double bond and the 'upper' fragment was converted to the C-11 substituted ethyl ester **13** by a series of reactions, the crucial one (attachment of the C-11 substituent), being a conjugate cuprate addition. Reassembly of the 'upper' and 'lower' fragments using Horner–Wittig olefination followed by ester hydrolysis and re-esterification gave the C-11 substituted *p*-nitrophenyl ester **2**.

The initial vitamin D_3 cleavage steps (giving, after modification, compounds **4** and **16**) have been previously described in detail in the literature,[1] and the following account of synthetic details therefore applies only to the functionalization–reassembly parts of our syntheses of **1**, **2**, and **3** (see Schemes 1–6).

The bromide **10**[15] was considered a suitable first intermediate for C-11 chain extension. It was synthesized starting from the known compound **6**[16], oxidation of which (with PDC) gave the aldehyde **7**[17], which in turn was converted to an acetal **8** by refluxing with 1,2-ethanediol, toluene, and a catalytic amount of *p*-TSA. Cleavage of the benzyl protecting group in **8** to give **9**, followed by treatment with PPh_3 and CBr_4 finally gave bromide **10**.[15]

The Grundmann ketone **4**[18] afforded the α,β-unsaturated ketone **5**[19] under Saegusa conditions.[13,14] Reaction of **5** with the cuprate prepared from bromide **10** by treatment with *t*-BuLi and the complex CuI/*n*-Bu_3P, gave a single C-11 substituted stereoisomer **11** in moderate yield. The alpha-C-11 structure of compound **11** was evident from its ^1H NMR spectrum, which showed one large (13.3 Hz) and one small (4.6 Hz) vicinal coupling constant for H11–H9a/H9b, and also a strong NOE interaction between H11 (multiplet at 2.03 ppm) and the H18 methyl group (singlet at 0.63 ppm). It's not surprising that only the α-stereoisomer was obtained since the copper reagent attacks molecule **5** from the less hindered α-face.

Several different conditions were tried to release the aldehyde from compound **11** (Scheme 2). Strongly acidic deprotection conditions (1 N HCl aq/THF/40 °C), gave, after subsequent Wittig 2-carbon extension (using (ethoxycarbonylmethylidene)-triphenylphosphorane[20,21]), not the desired product **12** but instead the 14-position isomer **12a** in 88% yield. However, milder deprotection conditions (DDQ/MeCN/H_2O/rt/24 h or PPTs/acetone/H_2O/reflux/15 h) gave the desired product **12** in good (76–82%) yield.

To avoid possible side-reactions involving the double bond in **12**, hydrogenation (Pd/C) of **12** was performed to give the expected compound **13** (Scheme 3). A trial Horner–Wittig reaction of **13** with the simple lithium phosphinoxy carboanion prepared from **14**[22] afforded compound **15**. Hydrolysis followed by re-esterification with *p*-nitrophenol gave the active ester **1**, a truncated analog of VD_3 suitable for protein conjugation.

A similar strategy was used to synthesize the vitamin D_3 derivative **2**. Coupling of the phosphine oxide **16**[23] with ketone **13** using *n*-BuLi at −78 °C gave the ethyl ester **17** (Scheme 4).

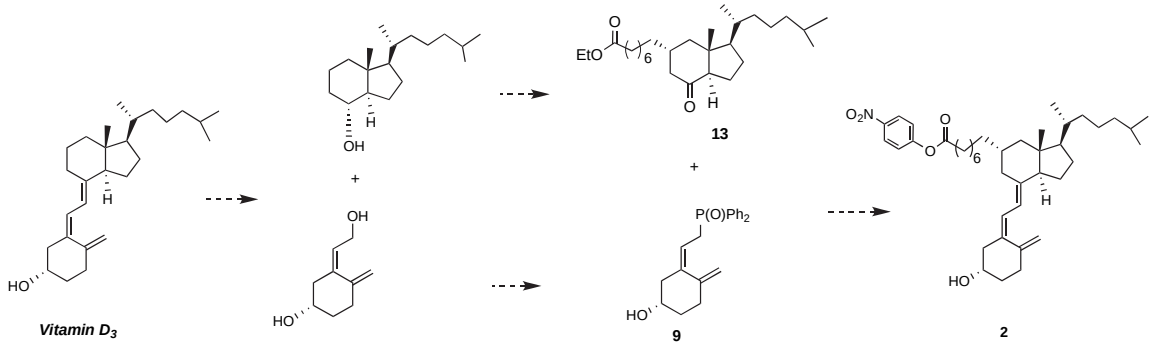
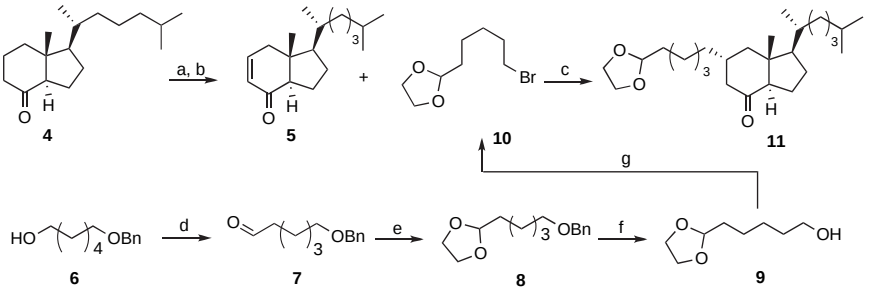
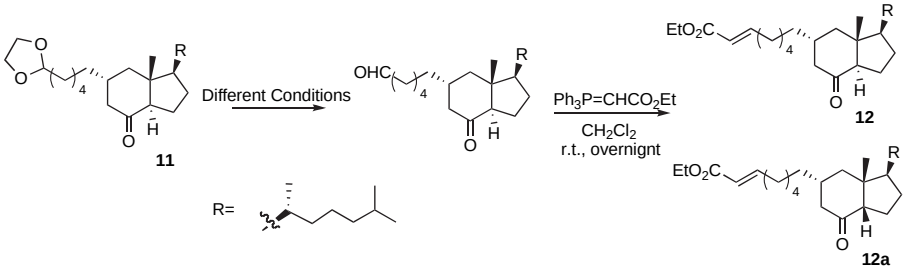


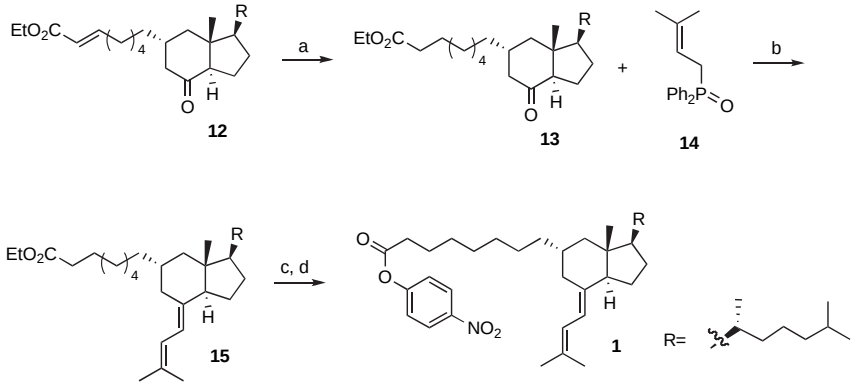
Figure 1. Synthetic strategy for preparation of compound **2**.



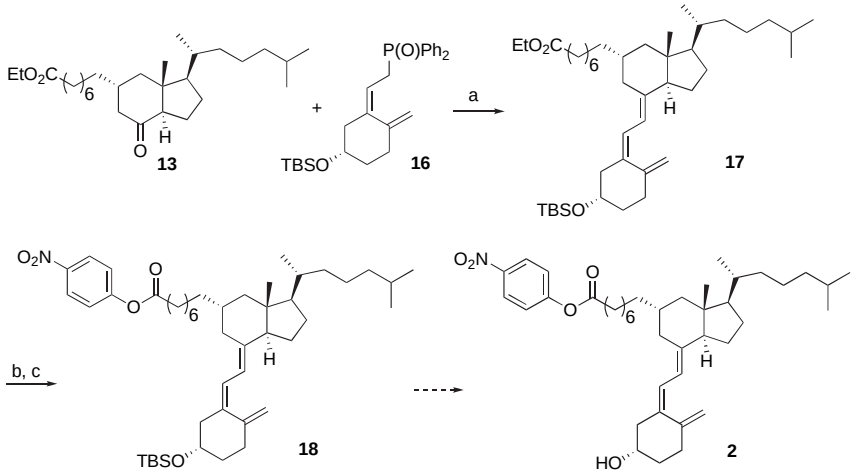
Scheme 1. (a) (i) LDA/THF/−78 °C/2.5 h; (ii) TMSCl/−78 °C to 0 °C; (b) $Pd(OAc)_2$/THF/CH_3CN/rt/overnight, 79% from **4**; (c) (i) **10**/*t*-BuLi/−85 °C/Et_2O/1.5 h; (ii) CuI *n*-Bu_3P/−85 °C to −50 °C/1 h; (iii) **5**/-78 °C/30 min, 69%; (d) PDC/CH_2Cl_2/rt/overnight, 85%; (e) 1,2-ethanediol/*p*-TSA/toluene/reflux/16 h, 98%; (f) Pd/C/H_2/MeOH/rt/9 h, 91%; (g) PPh_3/CBr_4/CH_2Cl_2/0 °C/1 h, 82%.



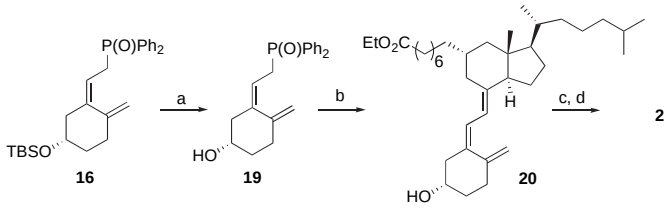
Scheme 2. Chain extension of **11** and attempted acetal removal under different conditions.



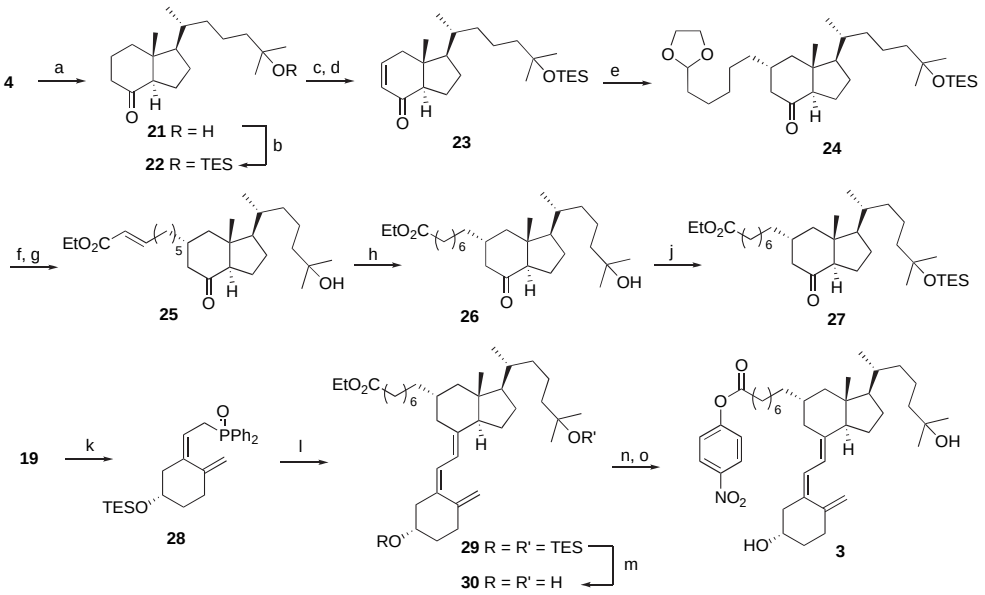
Scheme 3. (a) Pd/C/H_2/MeOH/2 h, 88%; (b) (i) **14**/*n*-BuLi/−78 °C/40 min; (ii) **13**/−78 °C to 0 °C/5 h, 50%; (c) LiOH·H_2O/THF/H_2O/rt/36 h; (d) *p*-nitrophenol/DCC/DMAP/CH_2Cl_2/rt/24 h, 78%.



Scheme 4. (a) (i) **16**/*n*-BuLi/−78 °C/40 min; (ii) **13**/−78 °C/2 h, 82%; (b) LiOH·H_2O/THF/H_2O/rt/72 h; (c) *p*-nitrophenol/DCC/DMAP/CH_2Cl_2/rt/overnight, 78%.



Scheme 5. (a) TBAF/THF/5 h, 97%; (b) (i) *n*-BuLi/−78 °C/1 h; (ii) **13**/−78 °C/2 h, 75%; (c) LiOH·H_2O/THF/H_2O/rt/36 h; (d) *p*-nitrophenol/DCC/CH_2Cl_2/rt/overnight, 71%.



Scheme 6. (a) $NaIO_4$/$RuCl_3$/CCl_4/CH_3CN/phosphate buffer/45 °C/2–4 d, 43%–55%; (b) TESCl/imidazole/DMF/rt/overnight, 97%; (c) (i) LDA/THF/−78 °C/1 h; (ii) TMSCl/−78 °C to 0 °C; (d) $Pd(OAc)_2$/THF/CH_3CN/rt/overnight, 75% from **22**; (e) (i) **10**/*t*-BuLi/−85 °C/Et_2O/1.5 h; (ii) CuI·*n*-Bu_3P/−85 °C to −50 °C/1 h; (iii) **23**/−78 °C/2 h, 73%; (f) PPTs/acetone/H_2O/reflux/17 h; (g) $Ph_3P{=}HCO_2Et$/CH_2Cl_2/rt/overnight, 83% (from **24**); (h) Pd/C/H_2/MeOH/2 h, 100%; (j) TESCl/imidazole/DMF/rt/6 h, 97%; (k) TESCl/imidazole/DMF/rt/overnight, 69%; (l) (i) **28**/*n*-BuLi/−78 °C/40 min; (ii) **27**/−78 °C/2 h, 84%; (m) PPTs/acetone/H_2O/reflux/3 h, 100%; (n) LiOH·H_2O/THF/H_2O/rt/44 h; (o) *p*-nitrophenol/DCC/DMAP/CH_2Cl_2/rt/2.5 h., 76% (from **30**).

Hydrolysis followed by re-esterification with *p*-nitrophenol gave the active ester **18**. However, attempted removal of the TBS group from **18** gave unexpectedly low yields (~25%) despite several attempts using different conditions.

Therefore, cleavage of the TBS group was instead effected earlier, at the stage of the phosphine oxide **16**, which gave **19** in good yield (97%) on treatment with TBAF in THF (Scheme 5). Conversion of **19** into the dianion by treatment with 2 equiv of *n*-BuLi −78 °C and subsequent Wittig–Horner reaction with the ketone **13** gave the desired triene **20** in 75% yield. Hydrolysis followed by re-esterification with *p*-nitrophenol gave the final active ester **2**, a VD_3 derivative suitable for protein conjugation.

In consideration of the fact that the 25-hydroxy derivative of vitamin D_3 binds more strongly to DBP than vitamin D_3 itself[24–27] we decided to also prepare the 25-hydroxylated vitamin D_3 derivative **3**. The synthesis (Scheme 6) started again from Grundmann ketone **4**,[18] which was selectively hydroxylated at C-25 by ruthenium-catalyzed oxidation[28,29] to give **21**. After TES ether protection of the C-25 hydroxy group, the resulting ketone **22**[30] was converted to corresponding enone **23**.[31,13,14] Michael addition with the curprate generated from bromide **10** using *t*-BuLi/CuI·*n*-Bu_3P[31] gave compound **24** (again only the alpha C-11 isomer). Hydrolysis of the acetal and TES ether groups of **24** in acetone/H_2O catalyzed by PPTs and treatment as before of the intermediate aldehyde with (ethoxycarbonylmethylidene)-triphenylphosphorane[20,21] gave ester **25**. Subsequent hydrogenation (Pd/C) followed by TES-imidazole/DMF re-protection of the C-25 hydroxyl group afforded the expected 'upper' fragment **27**.

Considering the low yield in the final TBS deprotection step during the synthesis of **2**, we now introduced the more acid-sensitive TES group to protect the hydroxy group in phosphine oxide **19**, giving phosphine oxide **28**. The compounds **27** and **28** underwent Horner–Wittig reaction yielding triene **29**. To our satisfaction, the TES groups in **29** were smoothly cleaved by acid to give **30** in high yield. The ethyl ester in **30** was then finally saponified and converted to the target VD_3 active ester **3**, suitable for protein conjugation.

2.2. Peptide design, synthesis, and conjugation

The design and synthesis of 42-residue helix–loop–helix peptides and their conjugations to small molecules containing active esters has been described in detail previously.[32–34] In short, the peptide primary sequences were designed to fold into two amphiphilic helices connected by a short loop. The amino acids were selected based on their propensity for helix or loop formation. Residues capable of salt bridge formation, N- and C-terminal capping groups, a conjugation point (a lysyl ε-amino group), and a fluorophore group (for protein binding monitoring) were introduced in selected positions. In this paper, an example conjugation of **2** to the peptide **4-D15L8** (Fig. 2a, this peptide has a dansyl fluorophore in position 15 and a free lysyl ε-amino group in position 8) is described in detail. Several other analogous peptides were conjugated in a similar way to either **1**, **2**, and **3** to form a library of peptide conjugates. The evaluation of the binding properties of

(a) Ac-N-A-A-D-X-E-A-K-I-R-H-L-R-E-K-X-A-A-R-G-P-R-D-C-A-Q-X-A-E-Q-L-A-R-R-F-E-R-F-A-R-K-G-NH2
8 15

(b)

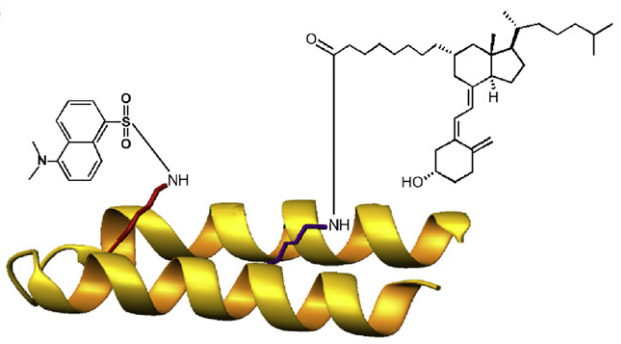


Figure 2. a) The amino acid sequence of peptide **4-D15L8**, where lysine residues in bold represent addressable sites for modification. The one letter code for amino acids is used: A=Ala, C=Cys, D=Asp, E=Glu, F=Phe, G=Gly, H=His, I=Ile, K=Lys, L=Leu, N=Asn, P=Pro, Q=Gln, R=Arg, V=Val, X=Nle. (b) Cartoon of peptide **4-D15L8** conjugated with **2**. The tertiary structure shown is based on the crystal structure of the four-helix-bundle rop protein (PDB entry code: 1ROP) [36]. The vitamin D residue from **2** and the dansyl residue were attached at Lys-8 (purple) and Lys-15 (red), respectively. The peptide is folded only when dimerized, but for reasons of clarity the monomeric form is shown.

these conjugates toward VDB and other proteins will be reported in a coming publication.

The peptide **4-D15L8** was synthesized on solid phase using Fmoc chemistry and a dansyl fluorophore group was introduced after selective deprotection of the ε-amino group of Lys15. The peptide was then cleaved from the resin by 95% trifluoroacetic acid, purified by reversed-phase HPLC and identified by MALDI-TOF mass spectrometry. After attempting several different conjugation conditions, the derivative **2** was reacted (twofold excess) with **4-D15L8** in DMSO containing 5% DIPEA, giving the desired conjugate (Fig. 2b) in 38% yield after HPLC purification.

The secondary structures of unconjugated **4-D15L8** and **4-D15L8** conjugated with **2** were investigated by CD spectroscopy. Both peptides gave rise to minima at 208 and 222 nm and a maximum at 190 nm characteristic of α-helices. Their mean residue ellipticities at 222 nm were −12,519 and −17,520 deg cm^2 $dmol^{-1}$ (10 μM in H_2O at 298 K), respectively, which are in the same range as those previously reported[35], indicating that both peptides were helical dimers.

3. Conclusion

Three C-11 linked active ester derivatives of vitamin D_3 were designed and synthesized, utilizing a strategy of disassembly–reassembly. Conjugations of these vitamin D_3 derivatives to 42-residue peptides gave a library of conjugates suitable for future binding screening.

4. Experimental section

4.1. General materials and methods

Concentrations were performed with a rotatory evaporator at reduced pressure (bath temperature <40 °C). The ^1H NMR and ^{13}C NMR spectra were recorded for $CDCl_3$ solutions (internal $CHCl_3$, δ_H 7.26, internal $CDCl_3$ δ_C 77.66, 77.34, 77.02 ppm) at 25 °C using Varian Unity 400 or 500 MHz instrument, unless otherwise stated. Assignments were corroborated by appropriate 2-D experiments. For ES-MS spectra, dilute methanol solutions of the analyte were infused, using a syringe pump, directly into the ion source of a SCIEX API 150-EX mass spectrometer. For MALDI-TOF spectra, a dilute aq solution of the sample was mixed (1:1) with a saturated solution of α-cyano-4-hydroxycinnamic acid in 1:1 acetonitril/water, the mixture was spotted onto a source plate, dried, the plate was inserted into the ion source of a Bruker Daltonics Ultraflex II TOF/TOF instrument, and spectra were recorded in the positive ion mode. High resolution MS spectra were run on an Agilent LC/MSD TOF instrument (Agilent Technologies, Santa Clara, CA, USA), with detection in the positive ion mode. Agilent TOF software and Agilent QS software were used to record and analyze mass spectra, respectively. Standard autotune of masses was performed in the TOF-MS instrument before the experimental runs, and typical mass errors of 1–3 ppm were achieved in the calibration.

Thin layer chromatography (TLC) was performed on Silica Gel F_{254} aluminum plates (Merck, Darmstadt, Germany) with detection by UV-light and/or by staining with 8% phosphomolybdic acid in ethanol. Column chromatography was performed in the flash mode (unless otherwise stated) on Matrex silica gel 60 (35–70 μm).

Dry THF and dry Et_2O were distilled from Na/Ph_2CO under N_2. Dry CH_2Cl_2 was distilled over CaH_2 under N_2. All other solvents and reagents were commercially available and used as received without any further purification. Abbreviations: THF=tetrahydrofuran, LDA=lithium diethylamide, TMSCl=trimethylsilyl chloride, *t*-Buli=*tert*-butylithium, PDC=pyridinium dichromate, *p*-TSA=*p*-toluenesulfonic acid, DDQ=dichlorodibensoquinone, PPTs=pyridinium *p*-toluenesulfonate, DCC=dicyclohexyl carbodiimide, DMAP=4-(*N*,*N*-dimethylamino)pyridine, TBS=*tert*-Butyldimetylsilyl, TES=triethylsilyl, TBAF=tetrabutylammonium fluoride, TBTU=*O*-(7-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, DIPEA=diisopropylethylamine, DMSO=dimethyl sulfoxide.

*4.1.1. De-A, B-cholest-9(11)-en-8-one (**5**).* This compound was prepared from compound **4**[18] essentially as described by Okamura et al.[19]

*4.1.2. 6-Benzyloxyhexanal (**7**).* 6-Benzyloxyhexanol (**6**)[16] (3 g, 14.42 mol) was dissolved in dry CH_2Cl_2 (50 mL) and PDC (10.8 g, 28.72 mmol) was added. The reaction mixture was stirred at room temperature overnight, then CH_2Cl_2 (200 mL) was added and the reaction solution was filtered through a Celite pad. The filtrate was concentrated and the residue was purified by column chromatography (10:1 *n*-pentane/EtOAc) to give **7**[17] as colorless oil (2.52 g, 85%). ^1H NMR (400 MHz, $CDCl_3$) δ 9.75 (t, J=2.2 Hz, 1H), 7.34–7.20 (m, 5H), 4.49 (s, 2H), 3.47 (t, J=6.4 Hz, 2H), 2.40–2.48 (m, 2H), 1.72–1.61 (m, 4H), 1.46–1.38 (m, 2H).

*4.1.3. 1,1-Ethylenedioxy-6-benzyloxyhexane (**8**).* A catalytic amount of *p*-TSA (380 mg) was added to a stirred solution of aldehyde **7** (2.038 g, 9.89 mmol) and 1,2-ethanediol (2.76 mL, 49.5 mmol) in toluene (20 mL) and the reaction mixture was refluxed for 16 h with a Dean–Stark water trap. The reaction mixture was then cooled, washed with saturated aq $NaHCO_3$ solution (15 mL), and brine, then dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (10:1 *n*-pentane/EtOAc) to give **8** as colorless oil (2.423 g, 98%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.37–7.28 (m, 5H), 4.86 (t, J=4.8 Hz, 1H), 4.52 (s, 2H), 4.00–3.94 (m, 2H), 3.90–3.84 (m, 2H), 3.49 (t, J=6.4 Hz, 2H), 1.71–1.63 (m, 4H), 1.46–1.45 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.90, 128.60, 127.88, 127.74, 104.82, 73.11, 70.56, 65.09, 34.11, 29.95, 26.41, 24.20; ESIMS m/z 273.2 ($[M+Na]^+$); ESIHRMS calcd for $C_{15}H_{23}O_3$ ($[M+H]^+$) 251.1648, found 251.1842.

*4.1.4. 1,1-Ethylenedioxy-6-hydroxyhexane (**9**).* A suspension of compound **8** (3.5 g, 14 mmol) and Pd/C (350 mg, 10 wt %) in MeOH (28 mL) was stirred for 9 h under H_2 atmosphere at room temperature, then the mixture was filtered through a Celite pad and the filtrate was concentrated. The residue was purified by column

chromatography (1.5:1 *n*-pentane/EtOAc) to give **9**[37] as a colorless oil (2.04 g, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.82 (t, J=5.0 Hz, 1H), 3.96–3.90 (m, 2H), 3.87–3.82 (m, 2H), 3.61 (t, J=6.6 Hz, 2H), 1.75 (br s, 1H), 1.67–1.62 (m, 2H), 1.59–1.52 (m, 2H), 1.47–1.37 (m, 4H).

*4.1.5. 1,1-Ethylenedioxy-6-bromohexane (**10**).* A mixture of **9** (995 mg, 6.22 mmol) and PPh_3 (2.45 g, 9.33 mmol) in CH_2Cl_2 (31 mL) was stirred in an ice-water bath while CBr_4 (3.1 g, 9.33 mmol) was added in portions. After completed addition, stirring was continued at 0 °C for 1 h. The mixture was diluted with *n*-pentane (200 mL), filtered through a Celite pad and the filtrate was concentrated. The residue was purified by column chromatography (10:1 *n*-pentane/EtOAc) to give **10**[15] as a colorless oil (1.14 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.85 (t, J=4.6 Hz, 1H), 3.98–3.92 (m, 2H), 3.89–3.83 (m, 2H), 3.41 (t, J=6.8 Hz, 2H), 1.91–1.84 (m. 2H), 1.70–1.65 (m, 2H), 1.49–1.44 (m, 4H).

*4.1.6. 11α-[(6,6-Ethylenedioxy)hex-1-yl]-de-A, B-cholest-8-one (**11**).* A solution of bromide **10** (1.7 g, 7.62 mmol) in dry diethyl ether (16 mL) was added dropwise to a cooled (−85 °C) *t*-BuLi solution (9 mL, 15.24 mmol, 1.7 M in heptane) during 1 h under N_2. The mixture was stirred for 30 min. Separately and simultaneously, *n*-Bu_3P (0.95 mL, 3.81 mmol) was added to a suspension of CuI (725 mg, 3.81 mmol) in dry diethyl ether (21 mL). At −85 °C, the solution of the complex CuI/*n*-Bu_3P complex was transferred by cannula to the initial reaction mixture. The resulting solution was warmed to −50 °C during 1 h, then again cooled to −78 °C and a solution of **5** (500 mg, 1.91 mmol) in dry diethyl ether (10 mL) was added dropwise. After stirred for 30 min at −78 °C, the reaction was quenched by addition of saturated aq NH_4Cl (5 mL), and was then diluted with diethyl ether (50 mL), washed with, successively, saturated aq NH_4Cl, water and brine, and dried ($MgSO_4$) and evaporated. The residue was purified by column chromatography (8:1 *n*-pentane/EtOAc) to give **11** as colorless oil (531 mg, 69%). $[\alpha]_D^{26}$ +9.1 (*c* 0.78, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.83 (t, J=4.8 Hz, 1H), 3.96 (m, 2H), 3.84 (m, 2H), 2.42 (dd, J=7.4, 11.7 Hz, 1H, H-14), 2.35 (dd, J=4.8, 13.4 Hz, 1H, H-9a), 2.16 (dd, J=4.0, 12.9 Hz, 1H, H-12a), 2.03 (m, 1H, H-11), 1.89 (m, 1H), 1.87 (ddd, J=1.0, 12.3, 13.4 Hz, 1H, 9b), 1.70 (m, 1H), 1.67–1.62 (signal cluster, 2H), 1.56–1.45 (signal cluster, 2H), 1.44–1.22 (signal cluster, 14H), 1.20–1.08 (signal cluster, 3H), 1.02 (m, 1H), 0.95 (d, J=6.0 Hz, 3H), 0.87 (d, J=6.6 Hz, 3H), 0.86 (d, J=6.6 Hz, 3H), 0.63 (s, 3H, H-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.8, 104.8, 65.1, 62.1, 56.7, 49.4, 48.0, 46.6, 39.6, 37.6, 36.9, 36.2, 35.7, 34.0, 29.8, 28.2, 28.0, 27.2, 24.2, 24.0, 23.0, 22.8, 19.1, 19.1, 13.5; ESIMS *m*/*z* 429.4 ([M+Na]$^+$); ESIHRMS calcd for $C_{26}H_{47}O_3$ ([M+H]$^+$) 407.3526, found 407.4081.

*4.1.7. 11α-(7-Ethoxycarbonylhept-5-en-1-yl)-de-A,B-cholest-8-one (**12**).* DDQ (8.3 mg, 0.036 mmol) was added to a solution of **3** (73.5 mg, 0.18 mmol) in CH_3CN/H_2O (v/v=9/1, 1 mL). The reaction mixture was stirred at room temperature for 24 h, after which the reaction was quenched by saturated aq Na_2SO_3 (2 mL). The mixture was diluted with diethyl ether (20 mL), washed with saturated aq Na_2SO_3, H_2O and brine, and dried ($MgSO_4$), filtered, and the filtrate was concentrated. The residual oil was dissolved in dry CH_2Cl_2 (2 mL) and (ethoxycarbonylmethylidene)-triphenylphosphorane[20,21] (125 mg, 0.36 mmol) was added. The mixture was stirred at room temperature for 24 h, and then concentrated and the residue was purified by column chromatography (20:1 *n*-pentane/EtOAc) to give **12** as colorless oil (56.1 mg, 72%). $[\alpha]_D^{26}$ +10.4 (*c* 1.43, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (dt, J=15.6, 7.0 Hz, 1H), 5.80 (d, J=15.6 Hz, 1H), 4.18 (q, J=7.2 Hz, 2H), 2.42 (dd, J=11.8, 7.4 Hz, 1H), 2.35 (dd, J=13.6, 4.8 Hz, 1H), 2.22–2.14 (m, 3H), 2.05–2.01 (m, 1H), 1.90–1.83 (m, 2H, including 1.88 (t, J=12.6 Hz, 1H)), 1.77–1.66 (m, 1H), 1.59–1.02 (cluster of signals, 23H), 0.96 (d, J=5.6 Hz, 3H), 0.87 (d, J=6.8 Hz, 3H), 0.86 (d, J=6.8 Hz, 3H), 0.64 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.8, 167.0, 149.4, 121.6, 62.2, 60.4, 56.8, 49.4, 48.0, 46.7, 39.7, 37.6, 36.9, 36.2, 35.7, 32.3, 29.5, 28.2, 28.2, 28.0, 27.1, 24.0, 23.0, 22.8, 19.2, 19.1, 14.5, 13.5; ESIMS *m*/*z* 455.0 ([M+Na]$^+$); ESIHRMS calcd for $C_{28}H_{49}O_3$ ([M+H]$^+$) 433.3683, found 433.3847.

*4.1.8. 11α-(7-Ethoxycarbonylhept-5-en-1-yl)-de-A,B-14-epi-8-cholestanone (**12a**).* Compound **11** (63 mg, 0.16 mmol) was dissolved in THF (0.8 mL) and 1 N HCl aq (0.8 mL) was added. The reaction mixture was stirred vigorously at 40 °C for 3 h, then diluted with diethyl ether and washed with, successively, saturated aq $NaHCO_3$, water, and brine, then dried ($MgSO_4$), filtered, and the filtrate was concentrated. The residual oil was then converted into **12a** (54 mg, 80%) by a similar Wittig-reaction as described above (**12**). $[\alpha]_D^{26}$ +62.7 (*c* 2.52, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.97 (dt, J=15.6, 6.8 Hz, 1H), 5.82 (d, J=16.0 Hz, 1H), 4.19 (q, J=7.2 Hz, 2H), 2.43–2.35 (m, 2H), 2.20 (ddd, J=1.6, 6.8, 14.4 Hz, 2H), 2.10–2.01 (m, 2H), 1.93–1.82 (m, 2H), 1.73 (dt, J=13.6, 2.4 Hz, 1H), 1.68–1.59 (m, 1H), 1.54–0.94 (cluster of signals, 19H), 1.28 (t, J=7.2 Hz, 3H), 1.06 (s, 3H), 0.88 (d, J=6.4 Hz, 3H), 0.86 (d, J=6.4 Hz, 3H), 0.85 (d, J=6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 215.4, 167.0, 149.5, 121.6, 60.4, 60.2, 56.7, 47.7, 46.1, 45.9, 39.7, 36.9, 35.7, 34.3, 33.3, 32.4, 29.5, 28.7, 28.2, 28.2, 26.8, 24.4, 23.0, 22.8, 22.5, 22.2, 19.4, 14.5; ESIMS *m*/*z* 455.0 ([M+Na]$^+$); ESIHRMS calcd for $C_{28}H_{49}O_3$ ([M+H]$^+$) 433.3683, found 433.3610.

*4.1.9. 11α-(7-Ethoxycarbonylhept-1-yl)-de-A,B-cholest-8-one (**13**).* A solution of compound **12** (41 mg, 0.095 mmol) in MeOH (1 mL) was mixed with Pd/C (6 mg, 10 wt %). The suspension was stirred for 2 h under H_2 atmosphere at room temperature, then the suspension was filtered through a Celite pad and the filtrate was concentrated. The residue was purified by column chromatography (17:1 *n*-pentane/EtOAc) to give **13** as colorless oil (36 mg, 88%). $[\alpha]_D^{25}$ +11.1 (*c* 0.75, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.12 (q, J=7.2 Hz, 2H), 2.42 (dd, J=11.6, 7.6 Hz, 1H), 2.34 (dd, J=5.0, 13.4 Hz, 1H), 2.27 (t, J=7.2 Hz, 2H), 2.16 (dd, J=4.2, 13.0 Hz, 1H), 2.07–2.00 (m, 1H), 1.95–1.83 (m, 2H, including 1.86 (t, J=12.6 Hz, 1H)), 1.76–1.00 (cluster of signals, 28H, including 1.24 (t, J=7.2 Hz, 3H)), 0.96 (d, J=5.6 Hz, 3H), 0.87 (d, J=6.4 Hz, 6H), 0.63 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 215.5, 174.1, 60.4, 60.2, 56.7, 47.7, 46.1, 45.9, 39.7, 37.0, 35.7, 34.6, 34.3, 33.3, 29.8, 29.4, 29.3, 28.7, 28.2, 27.0, 25.2, 24.4, 23.0, 22.8, 22.5, 22.2, 19.4, 14.5; ESIMS *m*/*z* 457.0 ([M+Na]$^+$); ESIHRMS calcd for $C_{28}H_{51}O_3$ ([M+H]$^+$) 435.3839, found 435.3892.

*4.1.10. 11α-[7-(4-Nitrophenyloxy)carbonylhept-1-yl]-8-(3″-methylbut-2″-enylidene)-de-A,B-cholestane (**1**).* A solution of **14**[22] (107 mg, 0.40 mmol) in dry THF (1 mL) was added dropwise to a solution of *n*-BuLi (0.26 mL, 0.42 mmol, 1.6 M) in dry THF (1 mL) at −78 °C under N_2. After stirring at −78 °C for 40 min, a solution of compound **10** (115 mg, 0.26 mmol) in dry THF (1 mL) was added by syringe. The reaction mixture was allowed to warm to 0 °C during 5 h, then saturated aq NH_4Cl (3 mL) was added to quench the reaction. The mixture was diluted by diethyl ether (20 mL), washed with saturated aq NH_4Cl and brine, and dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (100:1 *n*-pentane/EtOAc) to give **15** as colorless oil (64 mg, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.15 (d, J=10.8 Hz, 1H), 5.79 (d, J=11.6 Hz, 1H), 4.14 (q, J=7.2 Hz, 2H), 2.85 (dd, J=3.4, 13.4 Hz, 1H), 2.31 (t, J=7.4 Hz, 2H), 2.05–1.89 (m, 3H), 1.83 (s, 3H), 1.77 (s, 3H), 1.66–1.13 (cluster of signals, 30H), 0.95 (d, J=6.0 Hz, 3H), 0.89 (d, J=6.8 Hz, 3H), 0.88 (d, J=6.4 Hz, 3H), 0.56 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 139.8, 132.8, 120.7, 116.7, 60.4, 56.8, 56.6, 48.0, 45.8, 39.8, 38.1, 36.4, 36.0, 35.0, 34.7, 30.1, 29.5, 29.4, 28.3, 28.3, 27.4, 26.6, 25.3, 24.1, 23.1, 22.8, 22.3, 19.2, 18.4, 14.5, 13.1; ESIMS *m*/*z* 509.0 ([M+Na]$^+$).

Solid $LiOH\cdot H_2O$ (18 mg, 0.43 mmol) was added to a solution of **15** (38 mg, 0.078 mmol) in THF/H_2O (v/v=1/1, 2 mL). The reaction

mixture was stirred at room temperature for 36 h, then poured into H_2O (20 mL) and washed with diethyl ether (5 mL). The aqueous layer was concentrated to about 5 mL, the pH value was adjusted to about four and the mixture was extracted with ethyl acetate (30 mL×3). The combined organic extracts were washed with brine (1×10 mL), dried ($MgSO_4$), filtered, and concentrated. The residue (a colorless oil), *p*-nitrophenol (12.6 mg, 0.0906 mmol), DCC (18.7 mg, 0.0906 mmol), and DMAP (1.8 mg, 0.0151 mmol) were dissolved in dry CH_2Cl_2 (1 mL). The reaction mixture was stirred at room temperature for 24 h, then diluted with diethyl ether (20 mL) and filtered. The filtrate was washed by H_2O until the washings were no longer yellow in color, then with brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (50:1 *n*-pentane/EtOAc) to give **1** as colorless oil (35 mg, 78%). $[\alpha]_D^{25}$ +13.8 (*c* 1.05, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, *J*=8.8 Hz, 2H), 7.29 (d, *J*=9.6 Hz, 2H), 6.14 (d, *J*=10.8 Hz, 1H), 5.79 (d, *J*=11.2 Hz, 1H), 2.85 (dd, *J*=3.8, 13.4 Hz, 1H), 2.62 (t, *J*=7.6 Hz, 2H), 2.05–1.90 (m, 3H), 1.81 (s, 3H), 1.77 (s, 3H), 1.61–1.01 (cluster of signals, 27H), 0.95 (d, *J*=6.0 Hz, 3H), 0.88 (d, *J*=6.8 Hz, 3H), 0.88 (d, *J*=6.4 Hz, 3H), 0.56 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 155.7, 145.5, 139.7, 132.8, 125.5, 122.7, 120.7, 116.8, 56.8, 56.6, 48.0, 45.8, 39.8, 38.1, 36.4, 36.4, 36.0, 35.0, 34.6, 30.1, 29.5, 29.3, 28.3, 28.3, 27.4, 26.7, 25.0, 24.1, 23.1, 22.9, 22.3, 19.2, 18.4, 13.1; ESIMS *m*/*z* 601.8 ($[M+Na]^+$); ESIHRMS calcd for $C_{37}H_{56}NO_4$ ($[M-H_2+H]^+$) 578.4211, found 578.4209.

*4.1.11. 3β-(tert-Butyldimethylsilyloxy)-11α-(7-ethoxycarbonylhept-1-yl)-vitamin D$_3$ (**17**).* A solution of **16**[23] (455 mg, 1.01 mmol) in dry THF (1 mL) was added slowly to a solution of *n*-BuLi (0.63 mL, 1.01 mmol, 1.6 M) in dry THF (1 mL) at −78 °C under N_2. After stirring at −78 °C for 40 min, a solution of compound **13** (110 mg, 0.25 mmol) in dry THF (1 mL) was added dropwise. The reaction mixture was stirred at −78 °C for another 2 h, and then warmed to −50 °C during 3 h. Saturated aq NH_4Cl (3 mL) was added to quench the reaction. The mixture was diluted with diethyl ether (25 mL), washed with saturated aq NH_4Cl and brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (50:1 *n*-pentane/EtOAc) to give **17** as colorless oil (138 mg, 82%). $[\alpha]_D^{25}$ +31.2 (*c* 0.64, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.18 (d, *J*=10.8 Hz, 1H), 6.02 (d, *J*=11.2 Hz, 1H), 5.03 (s, 1H), 4.80 (s, 1H), 4.15 (q, *J*=7.2 Hz, 2H), 3.87–3.82 (m, 1H), 2.87 (dd, *J*=13.4, 3.8 Hz, 1H), 2.48 (dd, *J*=13.0, 3.8 Hz, 1H), 2.39 (dt, *J*=13.2, 4.8 Hz, 1H), 2.34–2.26 (m, 3H), 2.14–1.91 (m, 5H), 1.67–1.01 (cluster of signals, 31H, including 1.28 (t, *J*=7.2 Hz, 3H)), 0.95 (d, *J*=6.4 Hz, 3H), 0.91 (s, 9H), 0.89 (d, *J*=6.4 Hz, 6H), 0.56 (s, 3H), 0.10 (s, 3H), 0.09 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 145.7, 141.1, 136.6, 121.6, 118.1, 112.4, 70.8, 60.4, 56.8, 56.6, 47.9, 47.1, 46.0, 39.8, 38.1, 36.6, 36.4, 36.2, 35.0, 34.6, 33.0, 30.1, 29.5, 29.42 28.3, 27.4, 26.1, 25.2, 24.1, 23.1, 22.8, 22.3, 19.2, 18.4, 14.5, 13.1, −4.3, −4.4; ESIMS *m*/*z* 669.5 ($[M+H]^+$); ESIHRMS calcd for $C_{43}H_{77}O_3Si$ ($[M+H]^+$) 669.5643, found 669.5412.

*4.1.12. 3β-(tert-Butyldimethylsilyloxy)-11α-[7-(4-nitrophenyloxy) carbonylhept-1-yl]-vitamin D$_3$ (**18**).* Solid $LiOH\cdot H_2O$ (37 mg, 0.90 mmol) was added to a solution of **17** (60 mg, 0.090 mmol) in THF/H_2O (v/v=1/1, 2 mL). The reaction mixture was stirred at room temperature and monitored by TLC. When the reaction was complete (72 h), the pH value was adjusted to about five with diluted aq HCl (0.1 M). The mixture was extracted with diethyl ether (3×40 mL). The combined organic extracts were washed with brine (1×10 mL), dried ($MgSO_4$), filtered, and concentrated. The residue (a colorless oil), *p*-nitrophenol (15.3 mg, 0.11 mmol), DCC (22.7 mg, 0.11 mmol), and DMAP (2.2 mg, 0.018 mmol) were dissolved in dry CH_2Cl_2 (1 mL). The reaction mixture was stirred at room temperature overnight, then diluted with diethyl ether (20 mL) and filtered. The filtrate was washed by H_2O until the washings were no longer yellow in color, then with brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (50:1 *n*-pentane/EtOAc) to give **18** as a colorless oil (52 mg, 78%). $[\alpha]_D^{25}$ +25.2 (*c* 0.83, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, *J*=9.2 Hz, 2H), 7.30 (d, *J*=9.2 Hz, 2H), 6.18 (d, *J*=10.8 Hz, 1H), 6.03 (d, *J*=11.2 Hz, 1H), 5.03 (s, 1H), 4.80 (s, 1H), 3.87–3.82 (m, 1H), 2.87 (dd, *J*=13.4, 3.8 Hz, 1H), 2.62 (t, *J*=7.6 Hz, 2H), 2.48 (dd, *J*=13.2, 3.6 Hz, 1H), 2.40 (dt, *J*=13.6, 5.0 Hz, 1H), 2.26 (dd, *J*=9.0, 12.6 Hz, 1H), 2.13–2.03 (m, 2H), 2.00–1.89 (m, 3H), 1.83–1.76 (m, 2H), 1.63–1.13 (cluster of signals, 25H), 1.06–1.02 (m, 1H), 0.95 (d, *J*=6.0 Hz, 3H), 0.90 (s, 9H), 0.89 (d, *J*=6.8 Hz, 6H), 0.56 (s, 3H), 0.093 (s, 3H), 0.086 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5, 155.8, 145.6, 145.5, 141.0, 136.7, 125.4, 122.7, 121.6, 118.1, 112.5, 70.8, 56.8, 56.6, 47.9, 47.1, 46.0, 39.8, 38.0, 36.6, 36.4, 36.2, 35.0, 34.6, 33.0, 30.6, 29.4, 29.3, 28.3, 27.4, 26.1, 25.0, 24.1, 23.1, 22.8, 22.3, 19.2, 18.4, 13.1, −4.3, −4.4; ESIMS *m*/*z* 762.5 ($[M+H]^+$); ESIHRMS calcd for $C_{47}H_{76}NO_5Si$ ($[M+H]^+$) 762.5494, found 762.5255.

*4.1.13. (S)-(Z)-[2-(5-Hydroxy-2-methylene-cyclohexylidene)ethyl]diphenylphosphine oxide (**19**).* Bu_4NF in THF (0.33 mL, 0.33 mmol, 1 M) was added to a stirred solution of **16**[23] (100 mg, 0.22 mmol) in THF (1.1 mL). After being stirred at room temperature for 5 h, the reaction mixture was diluted with CH_2Cl_2, washed with H_2O and brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (20:1 CH_2Cl_2/MeOH) to give **19** as a white wax (72 mg, 97%). $[\alpha]_D^{23}$ +9.1 (*c* 1.61, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72–7.67 (m, 4H), 7.52–7.42 (m, 6H), 5.35 (q, *J*=7.2 Hz, 1H), 4.93 (s, 1H), 4.70 (s, 1H), 3.72–3.66 (m, 1H), 2.73 (br s, 1H), 3.29 (dd, *J*=7.6, 14.0 Hz, 2H), 2.46 (br d, *J*=12.8 Hz, 1H), 2.23–2.15 (m, 2H), 1.82–1.75 (m, 2H), 1.61–1.54 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.5 (d, *J*=3.0 Hz, P split), 142.7 (d, *J*=12.1 Hz, P split), 133.3 (d, *J*=40.2 Hz, P split), 132.3 (d, *J*=40.9 Hz, P split), 132.1 (d, *J*=2.3 Hz, P split), 132.1 (d, *J*=3.0 Hz, P split), 131.3 (d, *J*=9.1 Hz, P split), 131.1 (d, *J*=9.1 Hz, P split), 128.9 (d, *J*=6.8 Hz), 128.8 (d, *J*=6.1 Hz, P split), 114.0 (d, *J*=9.1 Hz, P split), 111.8, 69.1 (d, *J*=2.2 Hz, P split), 46.2 (d, *J*=2.3 Hz, P split), 35.3, 32.2, 31.2 (d, *J*=69.1 Hz, P split); ESIMS *m*/*z* 339.1 ($[M+H]^+$); ESIHRMS calcd for $C_{21}H_{24}O_2P$ ($[M+H]^+$) 339.1515, found 339.1417.

*4.1.14. 11α-(7-Ethoxycarbonylhept-1-yl)-vitamin D$_3$ (**20**).* A solution of **19** (60 mg, 0.177 mmol) in dry THF (1.2 mL) was added slowly to a solution of *n*-BuLi (0.23 mL, 0.363 mmol, 1.6 M) in dry THF (1 mL) at −78 °C under N_2. After stirring at −78 °C for 1 h, a solution of compound **13** (40 mg, 0.092 mmol) in dry THF (1 mL) was added dropwise. The reaction mixture was stirred at −78 °C for another 2 h, then saturated aq NH_4Cl (2 mL) was added to quench the reaction. The mixture was diluted with diethyl ether (25 mL), washed with saturated aq NH_4Cl and brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (5:1 *n*-pentane/EtOAc) to give **20** as colorless oil (38 mg, 75%). $[\alpha]_D^{26}$ +9.7 (*c* 0.62, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.27 (d, *J*=10.8 Hz, 1H), 6.04 (d, *J*=11.6 Hz, 1H), 5.07 (s, 1H), 4.84 (s, 1H), 4.15 (q, *J*=7.1 Hz, 2H), 3.98–3.95 (m, 1H), 2.88 (dd, *J*=13.4, 4.2 Hz, 1H), 2.61 (dd, *J*=13.2, 4.0 Hz, 1H), 2.44–2.39 (m, 1H), 2.34–2.30 (m, 3H, including 2.32 (t, *J*=7.4 Hz, 2H)), 2.23–2.18 (m, 1H), 2.04 (dd, *J*=3.8, 13.0 Hz, 1H), 2.00–1.89 (m, 3H), 1.73–1.13 (cluster of signals, 31H, including 1.28 (t, *J*=7.2 Hz, 3H)), 1.04–0.99 (m, 1H), 0.95 (d, *J*=6.4 Hz, 3H), 0.89 (d, *J*=6.8 Hz, 3H), 0.88 (d, *J*=6.4 Hz, 3H), 0.56 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 145.4, 141.9, 135.3, 122.6, 117.7, 112.6, 69.4, 60.4, 56.9, 56.6, 48.0, 46.2, 46.1, 39.8, 38.0, 36.4, 36.2, 35.4, 35.1, 34.6, 32.2, 30.0, 29.5, 29.4, 28.3, 28.2, 27.4, 25.2, 24.1, 23.1, 22.8, 22.3, 19.2, 14.5, 13.0; ESIMS *m*/*z* 555.5 ($[M+H]^+$); ESIHRMS calcd for $C_{37}H_{63}O_3$ ($[M+H]^+$) 555.4778, found 555.4787.

*4.1.15. 11α-[7-(4-Nitrophenyloxy)carbonylhept-1-yl]-vitamin D$_3$ (**2**).* Solid $LiOH\cdot H_2O$ (16 mg, 0.38 mmol) was added to a solution of **20** (20 mg, 0.036 mmol) in THF/H_2O (v/v=1/1, 1 mL). The reaction

mixture was stirred at room temperature and monitored by TLC. When the reaction was complete (36 h), the pH value was adjusted to about five with diluted aq HCl (0.1 M). The mixture was extracted with ethyl acetate (3×10 mL), and the combined organic extracts were washed with brine (1×5 mL), dried ($MgSO_4$), filtered, and concentrated. The residue (a colorless oil), *p*-nitrophenol (10 mg, 0.072 mmol), and DCC (15 mg, 0.072 mmol) were dissolved in dry CH_2Cl_2 (1 mL). The reaction mixture was stirred at room temperature overnight, then diluted with diethyl ether (20 mL) and filtered. The filtrate was washed with H_2O until the washings were no longer yellow in color, then with brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (5:1 *n*-pentane/EtOAc) to give **2** as colorless oil (16.5 mg, 71%). $[\alpha]_D^{25}$ −2.9 (*c* 1.09, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, *J*=9.2 Hz, 2H), 7.30 (d, *J*=9.2 Hz, 2H), 6.26 (d, *J*=11.2 Hz, 1H), 6.03 (d, *J*=11.2 Hz, 1H), 5.07 (s, 1H), 4.84 (s, 1H), 3.98–3.96 (m, 1H), 2.89 (dd, *J*=13.2, 4.0 Hz, 1H), 2.65–2.58 (m, 3H, including 2.63 (t, *J*=7.4 Hz, 2H)), 2.46–2.39 (m, 1H), 2.31 (dd, *J*=7.4, 13.0 Hz, 1H), 2.20 (ddd, *J*=5.0, 8.2, 13.4 Hz, 1H), 2.04 (dd, *J*=3.4, 12.6 Hz, 1H), 2.00–1.89 (m, 3H), 1.83–1.13 (cluster of signals, 25H), 1.06–0.98 (m, 1H), 0.95 (d, *J*=6.4 Hz, 3H), 0.89 (d, *J*=6.4 Hz, 3H), 0.88 (d, *J*=6.8 Hz, 6H), 0.56 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 155.8, 145.5, 145.3, 141.8, 135.4, 125.4, 122.6, 122.6, 117.7, 112.7, 69.4, 56.9, 56.7, 47.9, 46.2, 46.1, 39.8, 38.0, 36.4, 36.2, 35.4, 35.1, 34.6, 32.2, 30.0, 29.5, 29.3, 28.3, 28.2, 27.4, 25.0, 24.1, 23.1, 22.8, 22.3, 19.2, 13.1; ESIMS *m*/*z* 648.5 ($[M+H]^+$); ESIHRMS calcd for $C_{41}H_{62}NO_5$ ($[M+H]^+$) 648.4629, found 648.4643.

*4.1.16. 25-Triethylsilyloxy-de-A, B-cholest-9(11)-en-8-one (**23**).* This compound was prepared from compound **4**[18] essentially as previously described.[13,14,28–31]

*4.1.17. 11α-[(6,6-Ethylenedioxy)hex-1-yl]-25-triethylsilyloxy-de-A, B-cholest-8-one (**24**).* A solution of bromide **10** (350 mg, 1.50 mmol) in dry diethyl ether (3.2 mL) was added slowly to a cooled (-85 °C) *t*-BuLi solution (1.8 mL, 3.0 mmol, 1.7 M in heptane) under N_2 during 1 h. The mixture was stirred for 30 min. Separately and simultaneously, *n*-Bu_3P (0.19 mL, 0.75 mmol) was added to a suspension of CuI (143 mg, 0.75 mmol) in dry diethyl ether (4.3 mL). At −85 °C, the solution of CuI/*n*-Bu_3P complex was transferred by cannula to the initial reaction mixture. The resulting solution was warmed to −50 °C during 1 h, then again cooled to −78 °C and a solution of **23** (147 mg, 0.38 mmol) in dry diethyl ether (5.3 mL) was added dropwise. After stirred for 2 h at −78 °C, the reaction was quenched by addition of methanol (0.5 mL), and was then diluted with diethyl ether (20 mL), washed with, successively, saturated aq NH_4Cl, water and brine, and dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography (3:1 *n*-pentane/diethyl ether) to give **24** as colorless oil (146 mg, 73%). $[\alpha]_D^{24}$ +9.5 (*c* 2.08, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$) δ 4.83 (t, *J*=4.8 Hz, 1H), 3.96 (m, 2H), 3.84 (m, 2H), 2.43 (dd, *J*=7.4, 11.6 Hz, 1H, H-14), 2.36 (dd, *J*=4.6, 13.4 Hz, 1H, H-9a), 2.15 (dd, *J*=3.8, 12.9 Hz, 1H), 2.03 (m, 1H, H-11), 1.90 (m, 1H), 1.89 (ddd, 0.9, 12.3, 13.3 Hz, 1H, H-9b), 1.70 (m, 1H), 1.64 (m, 2H), 1.53–1.21 (cluster of signals, 19H), 1.18 (s, 6H), 0.94 (m, 12H), 0.63 (s, 3H, H-18), 0.56 (q, *J*=7.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.7, 104.8, 73.6, 65.1, 62.2, 56.9, 49.5, 48.0, 46.7, 45.7, 37.7, 37.0, 36.6, 35.7, 34.1, 30.3, 30.1, 29.9, 28.0, 27.2, 24.2, 21.0, 19.2, 19.1, 13.5, 7.4, 7.1; ESIMS *m*/*z* 559.4 ($[M+Na]^+$); ESIHRMS calcd for $C_{32}H_{61}O_4Si$ ($[M+H]^+$) 537.4340, found 537.4277.

*4.1.18. 11α-(7-Ethoxycarbonylhept-5-en-1-yl)-25-hydroxy-de-A,B-cholest-8-one (**25**).* Compound **24** (125 mg, 0.23 mmol) was dissolved in acetone/H_2O (v/v 9:1, 3 mL). After added PPTs (12 mg, 0.047 mmol), the reaction mixture was heated to reflux for 17 h, then cooled down, diluted with diethyl ether, washed in turn with saturated aq $NaHCO_3$, water and brine, dried ($MgSO_4$), and concentrated. The residual oil was dissolved in dry CH_2Cl_2 (2 mL) and (ethoxycarbonylmethylidene)-triphenylphosphorane (160 mg, 0.46 mmol) was added. The resulting mixture was stirred at room temperature overnight, and then concentrated and the residue was purified by column chromatography (1:1 *n*-pentane/EtOAc) to give **25** as colorless oil (86 mg, 83%). $[\alpha]_D^{24}$ +14.0 (*c* 1.96, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 6.94 (dt, *J*=15.6, 7.0 Hz, 1H), 5.80 (d, *J*=15.6 Hz, 1H), 4.17 (q, *J*=7.2 Hz, 2H), 2.42 (dd, *J*=11.6, 7.2 Hz, 1H), 2.34 (dd, *J*=13.4, 5.0 Hz, 1H), 2.21–2.13 (m, 3H), 2.08–2.00 (m, 1H), 1.94–1.83 (m, 2H, including 1.86 (t, *J*=12.8 Hz, 1H)), 1.75–1.65 (m, 1H), 1.45–1.23 (cluster of signals, 22H, including 1.27 (t, *J*=7.2 Hz, 3H)), 1.21 (s, 6H), 1.10–1.04 (m, 1H), 0.97 (d, *J*=5.6 Hz, 3H), 0.63 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.6, 166.9, 149.4, 121.6, 71.2, 62.2, 60.4, 56.8, 49.5, 48.0, 46.7, 44.6, 37.6, 36.9, 36.5, 35.7, 32.3, 29.7, 29.5, 28.2, 28.0, 27.1, 21.0, 19.2, 19.1, 14.5, 13.5; ESIMS *m*/*z* 471.2 ($[M+Na]^+$); ESIHRMS calcd for $C_{28}H_{49}O_4$ ($[M+H]^+$) 449.3632, found 449.3576.

*4.1.19. 11α-(7-Ethoxycarbonylhept-1-yl)-25-hydroxy-de-A,B-cholest-8-one (**26**).* To a solution of compound **25** (50 mg, 0.11 mmol) in MeOH (1 mL) was added Pd/C (5 mg, 10 wt %). The resulting suspension was stirred for 2 h under H_2 atmosphere at room temperature, then the suspension was filtered through a Celite pad and the filtrate was concentrated. The residue was purified by column chromatography (1.5:1 *n*-pentane/EtOAc) to give **26** as colorless oil (50 mg, 100%). $[\alpha]_D^{25}$ +12.3 (*c* 1.34, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 4.11 (q, *J*=7.1 Hz, 2H), 2.42 (dd, *J*=11.8, 7.4 Hz, 1H), 2.34 (dd, *J*=4.8, 13.6 Hz, 1H), 2.27 (t, *J*=7.6 Hz, 2H), 2.15 (dd, *J*=4.0, 12.8 Hz, 1H), 2.08–1.98 (m, 1H), 1.94–1.83 (m, 2H, including 1.86 (t, *J*=12.8 Hz, 1H)), 1.76–1.66 (m, 1H), 1.64–1.57 (m, 2H), 1.45–1.22 (cluster of signals, 24H, including 1.24 (t, *J*=7.2 Hz, 3H)), 1.20 (s, 6H), 1.09–1.02 (m, 1H), 0.96 (d, *J*=5.2 Hz, 3H), 0.62 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.7, 174.0, 71.2, 62.2, 60.4, 56.8, 49.5, 48.0, 46.7, 44.6, 37.8, 36.9, 36.5, 35.7, 34.6, 29.8, 29.7, 29.5, 29.4, 29.3, 28.0, 27.3, 25.2, 21.0, 19.2, 19.1, 14.5, 13.5; ESIMS *m*/*z* 473.4 ($[M+Na]^+$); ESIHRMS calcd for $C_{28}H_{51}O_4$ ($[M+H]^+$) 451.3789, found 451.3740.

*4.1.20. 11α-(7-Ethoxycarbonylhept-1-yl)-25- triethylsilyloxy-de-A,B-cholest-8-one (**27**).* TESCl (0.03 mL, 0.18 mmol) was added to a solution of compound **26** (53 mg, 0.12 mmol) and imidazole (24.5 mg, 0.36 mmol) in dry DMF (1 mL) under N_2 atmosphere. The reaction solution was stirred at room temperature for 6 h. The resulting mixture was quenched by 1 mL H_2O, diluted by 50 mL diethyl ether and washed by water (1 mL×4), brine (1 mL×1) and dried over $MgSO_4$. After concentrated in vacuum, the residue was purified by column chromatography (10:1 *n*-pentane/EtOAc) to give **27** as colorless oil (64 mg, 97%). $[\alpha]_D^{25}$ +8.6 (*c* 0.95, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 4.12 (q, *J*=7.2 Hz, 2H), 2.41 (dd, *J*=11.6, 7.2 Hz, 1H), 2.33 (dd, *J*=5.0, 13.4 Hz, 1H), 2.26 (t, *J*=7.4 Hz, 2H), 2.14 (dd, *J*=3.8, 13.0 Hz, 1H), 2.04–1.98 (m, 1H), 1.94–1.81 (m, 2H, including 1.84 (t, *J*=12.8 Hz, 1H)), 1.74–1.20 (cluster of signals, 26H, including 1.22 (t, *J*=7.0 Hz, 3H)), 1.18 (s, 6H), 1.06–1.99 (m, 1H), 0.97–0.93 (m, 12H, including 0.95 (t, *J*=8.0 Hz, 9H)), 0.61 (s, 3H), 0.57 (q, *J*=8.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.8, 174.1, 73.6, 62.2, 60.4, 56.9, 49.5, 48.0, 46.7, 45.7, 37.8, 37.0, 36.6, 35.7, 34.6, 30.3, 30.0, 29.8, 29.4, 29.3, 28.0, 27.3, 25.2, 21.0, 19.2, 19.1, 14.5, 13.5, 7.4, 7.1; ESIMS *m*/*z* 587.4 ($[M+Na]^+$); ESIHRMS calcd for $C_{34}H_{65}O_4Si$ ($[M+H]^+$) 565.4653, found 565.4581.

*4.1.21. (S)-(Z)-[2-(5-Triethylsilyloxy-2-methylene-cyclohexylidene) ethyl]diphenylphosphine oxide (**28**).* TESCl (0.05 mL, 0.30 mmol) was added to a stirred solution of **19** (67 mg, 0.20 mmol) and imidazole (41 mg, 0.60 mmol) in dry DMF (1 mL). After being stirred at room temperature overnight, the reaction mixture was concentrated in vacuum and the residue was purified by column chromatography (1:2 *n*-pentane/EtOAc) to give **28** as colorless oil

(62 mg, 69%). $[\alpha]_D^{24}$ +44.9 (*c* 2.47, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 7.76–7.70 (m, 4H), 7.55–7.45 (m, 6H), 5.38 (q, *J*=7.3 Hz, 1H), 4.94 (s, 1H), 4.71 (s, 1H), 3.72–3.49 (m, 1H), 3.43–3.34 (m, 1H), 3.22 (ddt, *J*=6.4, 2.0, 16.8 Hz, 1H), 2.39 (br d, *J*=12.4 Hz, 1H), 2.24 (dt, *J*=4.4, 13.2 Hz, 1H), 2.16–2.10 (m, 1H), 1.82–1.78 (m, 1H), 1.70–1.63 (m, 1H), 1.54–1.44 (m, 1H), 0.93 (t, *J*=8.0 Hz, 9H), 0.56 (q, *J*=8.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.2 (d, *J*=2.3 Hz, P split), 142.9 (d, *J*=12.1 Hz, P split), 133.5 (d, *J*=62.2 Hz, P split), 132.6 (d, *J*=62.9 Hz, P split), 132.0 (d, *J*=3.0 Hz, P split), 131.9 (d, *J*=2.3 Hz, P split), 131.5 (d, *J*=9.1 Hz, P split), 131.2 (d, *J*=9.1 Hz, P split), 128.8 (d, *J*=11.4 Hz, P split), 128.6 (d, *J*=11.3 Hz, P split), 113.5 (d, *J*=8.4 Hz, P split), 111.9, 70.6 (d, *J*=2.3 Hz, P split), 47.1 (d, *J*=2.2 Hz, P split), 36.6, 32.8, 31.4 (d, *J*=69.8 Hz, P split), 7.1, 5.1; ESIMS *m*/*z* 475.2 ($[M+Na]^+$); ESIHRMS calcd for $C_{27}H_{38}O_2PSi$ ($[M+H]^+$) 453.2380, found 453.2308.

*4.1.22. 3β-Triethylsilyloxy-11α-(7-ethoxycarbonylhept-1-yl)-25-triethylsilyloxy-vitamin D_3 (**29**).* A solution of **28** (61.8 mg, 0.14 mmol) in dry THF (1 mL) was added to a solution of *n*-BuLi (0.09 mL, 0.14 mmol, 1.6 M) in dry THF (1 mL) at −78 °C under N_2 atmosphere. After stirring at −78 °C for 40 min, a solution of compound **27** (20 mg, 0.035 mmol) in dry THF (1 mL) was added dropwise. The reaction mixture was stirring at −78 °C for another 2 h. MeOH (0.2 mL) was added to quench the reaction. The mixture was diluted with diethyl ether (25 mL), washed with saturated aq NH_4Cl and brine and dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (50:1 *n*-pentane/EtOAc) to give **29** as colorless oil (23.5 mg, 84%). $[\alpha]_D^{25}$ +14.7 (*c* 1.18, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 6.19 (d, *J*=11.2 Hz, 1H), 6.03 (d, *J*=11.2 Hz, 1H), 5.03 (s, 1H), 4.80 (s, 1H), 4.15 (q, *J*=7.2 Hz, 2H), 3.86–3.81 (m, 1H), 2.87 (dd, *J*=13.6, 3.6 Hz, 1H), 2.50 (dd, *J*=12.8, 3.6 Hz, 1H), 2.39 (dt, *J*=14.0, 4.4 Hz, 1H), 2.33–2.25 (m, 3H, including 2.31 (t, *J*=7.6 Hz, 2H)), 2.14–1.92 (m, 5H), 1.67–1.26 (m, 30H) (including 1.28 (t, *J*=7.2 Hz, 3H)), 1.21 (s, 6H), 0.99 (t, *J*=8.2 Hz, 9H), 0.97 (t, *J*=8.2 Hz, 9H), 0.95 (d, *J*=3.2 Hz, 3H), 0.63 (q, *J*=8.0 Hz, 6H), 0.58 (q, *J*=8.0 Hz, 6H), 0.57 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.1, 145.6, 141.1, 136.6, 121.6, 118.1, 112.5, 73.7, 70.7, 60.4, 56.9, 56.7, 47.9, 47.2, 46.0, 45.8, 38.1, 36.8, 36.7, 36.4, 36.3, 35.0, 34.7, 33.1, 30.3, 30.1, 30.1, 29.5, 29.4, 28.3, 27.4, 25.3, 22.3, 21.1, 19.2, 14.5, 13.1, 7.4, 7.1, 7.1, 5.2; ESIMS *m*/*z* 821.6 ($[M+Na]^+$); ESIHRMS calcd for $C_{49}H_{91}O_4Si_2$ ($[M+H]^+$) 799.6457, found 799.6360.

*4.1.23. 11α-(7-Ethoxycarbonylhept-1-yl)-25-hydroxy-vitamin D_3 (**30**).* PPTs (2.3 mg, 0.0092 mmol) was added to a solution of compound **29** (20.5 mg, 0.0257 mmol) in acetone/H_2O (v/v 9:1, 3 mL). The reaction mixture was refluxed for 3 h, then cooled down, diluted with ethyl acetate, washed in turn with saturated aq $NaHCO_3$, water and brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (2:1 *n*-pentane/EtOAc) to give **30** as colorless oil (14.5 mg, 100%). $[\alpha]_D^{24}$ −3.4 (*c* 0.71, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 6.24 (d, *J*=11.2 Hz, 1H), 6.02 (d, *J*=10.8 Hz, 1H), 5.04 (s, 1H), 4.81 (s, 1H), 4.12 (q, *J*=6.9 Hz, 2H), 3.96–3.93 (m, 1H), 2.86 (dd, *J*=13.6, 4.0 Hz, 1H), 2.76 (dd, *J*=12.8, 3.6 Hz, 1H), 2.43–2.37 (m, 1H), 2.31–2.27 (m, 3H, including 2.29 (t, *J*=7.6 Hz, 2H)), 2.17 (ddd, *J*=4.6, 8.6, 13.4 Hz, 1H), 2.07–1.90 (m, 6H), 1.69–1.19 (cluster of signals, 36H, including 1.25 (t, *J*=7.2 Hz, 3H), and 1.21 (s, 6H)), 0.96 (d, *J*=6.4 Hz, 3H), 0.54 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.1, 145.4, 141.7, 135.4, 122.6, 117.7, 112.6, 71.4, 69.5, 60.4, 56.9, 56.6, 48.0, 46.2, 46.1, 44.7, 38.0, 36.7, 36.4, 36.2, 35.4, 35.1, 34.7, 32.2, 30.0, 29.5, 29.5, 29.4, 28.2, 27.4, 25.2, 22.3, 21.1, 19.2, 14.5, 13.1; ESIMS *m*/*z* 593.4 ($[M+Na]^+$); ESIHRMS calcd for $C_{37}H_{63}O_4$ ($[M+H]^+$) 571.4760, found 571.4669.

*4.1.24. 11α-[7-(4-Nitrophenyloxy)carbonylhept-1-yl]-25-hydroxy-vitamin D_3 (**3**).* Solid LiOH·H_2O (12.6 mg, 0.3 mmol) was added to a solution of **30** (14.7 mg, 0.026 mmol) in THF/H_2O (v/v 1:1, 0.5 mL). The reaction mixture was stirred at room temperature and monitored by TLC. When the reaction was complete (44 h), the pH value was adjusted to about two with diluted aq HCl (0.5 M). The mixture was extracted with ethyl acetate (4×20 mL), and the combined organic extracts were washed with brine (1×5 mL), dried ($MgSO_4$), filtered, and concentrated. The residue (a colorless oil), *p*-nitrophenol (9 mg, 0.065 mmol), DCC (8 mg, 0.039 mmol), and DMAP (0.16 mg, 0.0013 mmol) were dissolved in dry CH_2Cl_2 (1 mL). The reaction mixture was stirred at room temperature for 2.5 h, then diluted with diethyl ether (2 mL) and filtered. The filtrate was washed by H_2O until the washings were no longer yellow in color, then with brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (2:1 *n*-pentane/EtOAc) to give **3** as colorless oil (12.6 mg, 76%). $[\alpha]_D^{23}$ −5.0 (*c* 0.42, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, *J*=8.8 Hz, 2H), 7.30 (d, *J*=9.6 Hz, 2H), 6.26 (d, *J*=10.8 Hz, 1H), 6.05 (d, *J*=11.6 Hz, 1H), 5.07 (s, 1H), 4.84 (s, 1H), 3.99–3.94 (m, 1H), 2.89 (dd, *J*=13.4, 3.4 Hz, 1H), 2.65–2.58 (m, 3H, including 2.63 (t, *J*=7.4 Hz, 2H)), 2.44–2.39 (m, 1H), 2.31 (dd, *J*=7.4, 12.6 Hz, 1H), 2.23–2.16 (m, 1H), 2.06–1.06 (cluster of signals, 39H, including 1.24 (s, 6H)), 0.97 (d, *J*=6.4 Hz, 3H), 0.56 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5, 155.8, 147.6, 145.3, 141.7, 135.5, 125.4, 122.6, 122.5, 117.8, 112.7, 71.3, 69.4, 56.9, 56.6, 48.0, 46.2, 46.1, 44.7, 38.0, 36.7, 36.4, 36.2, 35.4, 35.1, 34.6, 32.2, 30.0, 29.6, 29.5, 29.5, 29.3, 28.2, 27.4, 25.0, 22.3, 21.1, 19.2, 13.1; ESIMS *m*/*z* 686.4 ($[M+Na]^+$); ESIHRMS calcd for $C_{41}H_{62}NO_6$ ($[M+H]^+$) 664.4578, found 664.4502.

4.2. Peptide synthesis, purification, and conjugation

The peptide **4-D15L8** was synthesized on a Pioneer automated peptide solid-phase synthesiser (Applied Biosystems) using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The synthesis was performed on a 0.2-mmol scale on an Fmoc-Gly-polyethylene glycol-polystyrene resin (Applied Biosystems) with a substitution degree of 0.18 mmol/g. The side-chains of the amino acids (Calbiochem–Novabiochem AG) were protected by piperidine-stable groups: *tert*-butyl (Asp, Glu), *tert*-butoxycarbonyl (Lys8), allyloxycarbonyl (Lys15), *N*-trifluoroacetyl (Lys41), *S*-acetamidomethyl (Cys), trityl (His, Asn, Gln) and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg). The allyloxycarbonyl group protection of Lys15 allowed for pre-cleavage incorporation of a dansyl group, and the *N*-trifluoroacetamidophenyl protection of Lys41 and acetamidomethyl protection of Cys24 were selected to allow for future post-cleavage conjugations. The Fmoc protecting groups were removed from the amino termini by treatment with 20% piperidine in DMF. A fourfold excess of amino acid was used in each coupling and amino acids were activated with a mixture of TBTU (0.5 M in DMF) and DIPEA (1 M in DMF). A standard amino acid coupling time of 60 min was used, except in the cases of Gln and His (90 min) and Asn and Arg (120 min). The N terminus of the peptide was capped with 0.3 M acetic anhydride in DMF. After completed synthesis, the resin was rinsed with dichloromethane and dried in vacuum.

Before cleaving the peptide from the resin, Lys15 was deprotected over 3 h at room temperature under nitrogen by using [Pd(PPh_3)$_4$] (3 equiv) in a mixture of trichloromethane, acetic acid, and *N*-methylmorpholine (17:2:1 v/v; 12 mL per g of polymer). The resin was washed sequentially (×3) with 20 mM diethyldithiocarbamic acid in DMF, 30 mM DIPEA in DMF, then with DMF and dichloromethane, and finally desiccated. Coupling of dansyl chloride (4 equiv) to the liberated lysine residue was performed in DMF with gentle stirring at room temperature for 3 h in the presence of DIPEA (8 equiv). The resin was washed with DMF and dichloromethane and then desiccated. The crude peptide was cleaved from the resin by treatment with a mixture of TFA, triisopropylsilane, and water (95:2.5:2.5 v/v; 15 mL per g of

polymer) for 2 h at room temperature. After filtration, TFA was evaporated and the peptide was precipitated by addition of cold diethyl ether, centrifuged, washed in diethyl ether and lyophilized.

The crude product was purified by reversed-phase HPLC on a semipreparative Hypersil C-18 Gold column (150×20 mm, pore size 175 Å, particle size 5 Å), eluted with a shallow 35–55% acetonitrile gradient in water and 0.1% TFA as additive at a flow rate of 10 mL/min. The purified peptide was identified by MALDI-TOF mass spectrometry, concentrated and lyophilized.

The ligand was conjugated to the peptide by adding a solution of the active ester **2** (2 equiv) in DMSO (40 mM), to a solution of the peptide **4-D15L8** (0.568 μmol) in DMSO (2 mM) in the presence of DIPEA (30 equiv). The reaction was monitored by MALDI-TOF mass spectrometry. After one day at room temperature, the reaction mixture was purified by reversed-phase HPLC (conditions as above). Elution with a 46–81% acetonitrile gradient gave a pool of pure fractions, as indicated by the peak cluster centered around m/z=5789 in the MALDI-TOF mass spectrum (the calculated M+H value is 5786 for $C_{261}H_{424}N_{77}O_{64}S_2F_3$), which was partially concentrated and then lyoplilized. The yield of conjugation was 38% (by weight).

4.3. Structural analysis by circular dichroism spectroscopy

Circular dichroism spectra were recorded on a JASCO J-810 spectrometer by using 10-mm cuvettes and measuring in the interval 190–260 nm at room temperature. Spectra of 10 μM peptide in water were obtained by collecting data at 0.5 nm intervals with an integration time of 1 s. Each spectrum was collected as an average of three scans and the data were corrected by subtraction of a spectrum of a blank sample containing only buffer. The helical content was determined as the mean residue ellipticity at 222 nm, $[\Theta]_{222}$, and was calculated from Eq. 1, where Θ_{222}^{obs} is the observed ellipticity at 222 nm (deg), mrw is the mean residue weight (g/mol), c is the peptide concentration (g/mL) and l is the optical path length of the cell (cm).

$$[\Theta]_{222} = \frac{\Theta_{222}^{obs} mrw}{10lc} \quad (1)$$

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.04.051.

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