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Synthesis of a New Vitamin D_3 Hapten and Its Protein Conjugates

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Abstract—The synthesis of 25-(1-carboxymethoxy)-imino-3(*S*)-hydroxy-9,10-seco-27-norcholesta-5(*Z*),7(*E*),10(19)-triene, a new hapten of vitamin D_3, has been carried out in ten steps from ergosterol. The hapten was conjugated to horseradish peroxidase and bovine serum albumin. Antibodies to 25-hydroxyvitamin D_3 were obtained using the synthesized conjugates. An immunochemical system was developed for the quantitative determination of 25-hydroxyvitamin D_3.

Keywords: 25-hydroxyvitamin D_3, synthesis of new hapten and its conjugates with horseradish peroxidase and bovine serum albumin, immunoenzyme analysis

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INTRODUCTION

Group D vitamins and their metabolites are involved in the regulation of calcium and phosphorus homeostasis in the body. The lack of vitamin D in the human body can cause various diseases usually associated with disorders of calcium metabolism, including secondary hyperparathyroidism, rickets in children, and osteomalacia in adults [1]. For this reason, it is very important to control the content of vitamins of this group in the blood serum. The most universal and reliable indicator is the concentration of the main metabolite of group D vitamins, 25-hydroxyvitamin D_3, in the blood serum [2]. Enzyme-linked immunosorbent assay becomes more and more popular for the detection of low concentrations of active substances and their metabolites. Immunoenzyme test systems for the determination of metabolites of group D vitamins are characterized by the high cost of compounds and antibodies required for their development, hence interest in the synthesis of the necessary components. Therefore, it is of practical interest to synthesize the required components. Immunogens based on metabolites of group D vitamins with a linker in the steroid skeleton at positions C3, C11, and C16 have been described [3–5]. In this study, we synthesized a new hapten containing a linker in the side chain of the secosteroid molecule.

RESULTS AND DISCUSSION

The synthesis was carried out via 25-keto derivative (**IX**) obtained from ergosterol (**I**) (Scheme 1). The hydroxyl group of ergosterol was initially protected by converting ergosterol to acetate (**II**). The conjugated diene system was protected by 4-phenylurazole. The side chain of Diels-Alder adduct (**III**) was modified by ozonolysis of the Δ^{22}-double bond, after which the ozonide was reduced with sodium borohydride to 22-hydroxy derivative (**IV**). The latter was then converted to 22-iodide (**V**) by reacting with iodine in the presence of imidazole and triphenylphosphine.

The formation of compound (**IV**) is confirmed by the presence of signals of the phenyl ring protons in the ^{1}H NMR spectrum, the absence of olefin proton signals characteristic of the Δ^{22} derivatives, and the presence of two single-proton signals at C22 as a doublet of doublets. A band of stretching vibrations of the hydroxyl group disappears from the IR spectrum upon transition to iodine derivative (**V**). The signals at C22 in the ^{1}H NMR spectrum of the latter are also shifted towards a stronger field.

Abbreviations: 25OHD_3, 25-hydroxyvitamin D_3; BSA, bovine serum albumin; HRP, horseradish peroxidase; and PE, petroleum ether.

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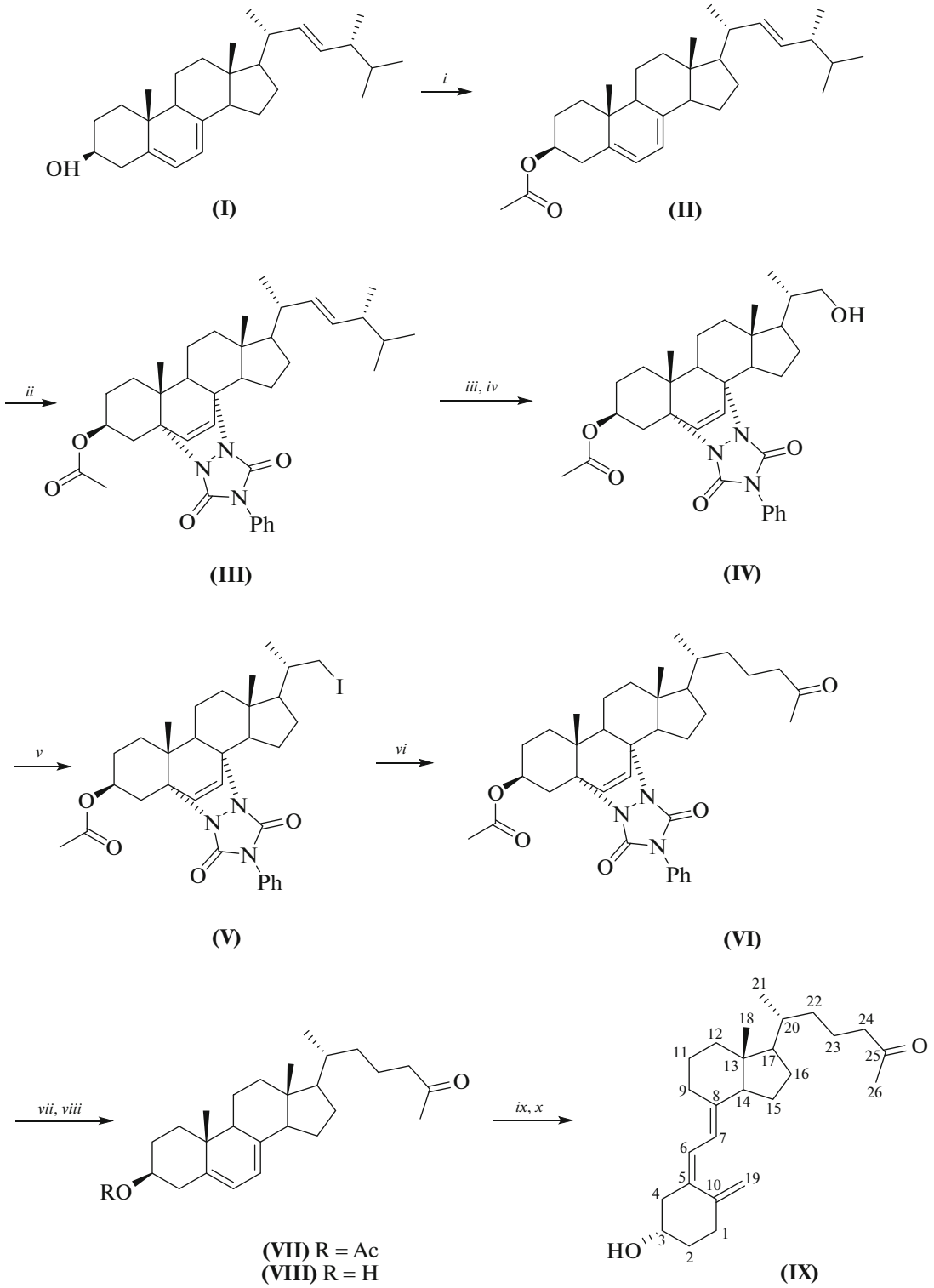


i: Ac_2O, Py; *ii*: 4-phenylurazole, $Pb(OAc)_4$, CH_2Cl_2, 0°C; *iii*: O_3, CH_2Cl_2/MeOH, –60°C; *iv*: $NaBH_4$; *v*: I_2, ImH, PPh_3, CH_2Cl_2; *vi*: 3-buten-2-one, Zn-Cu, $NiCl_2$, Py; *vii*: K_2CO_3, DMSO, 140°C; *viii*: NaOH, MeOH; *ix*: *hν*, EtOH; *x*: 78°C

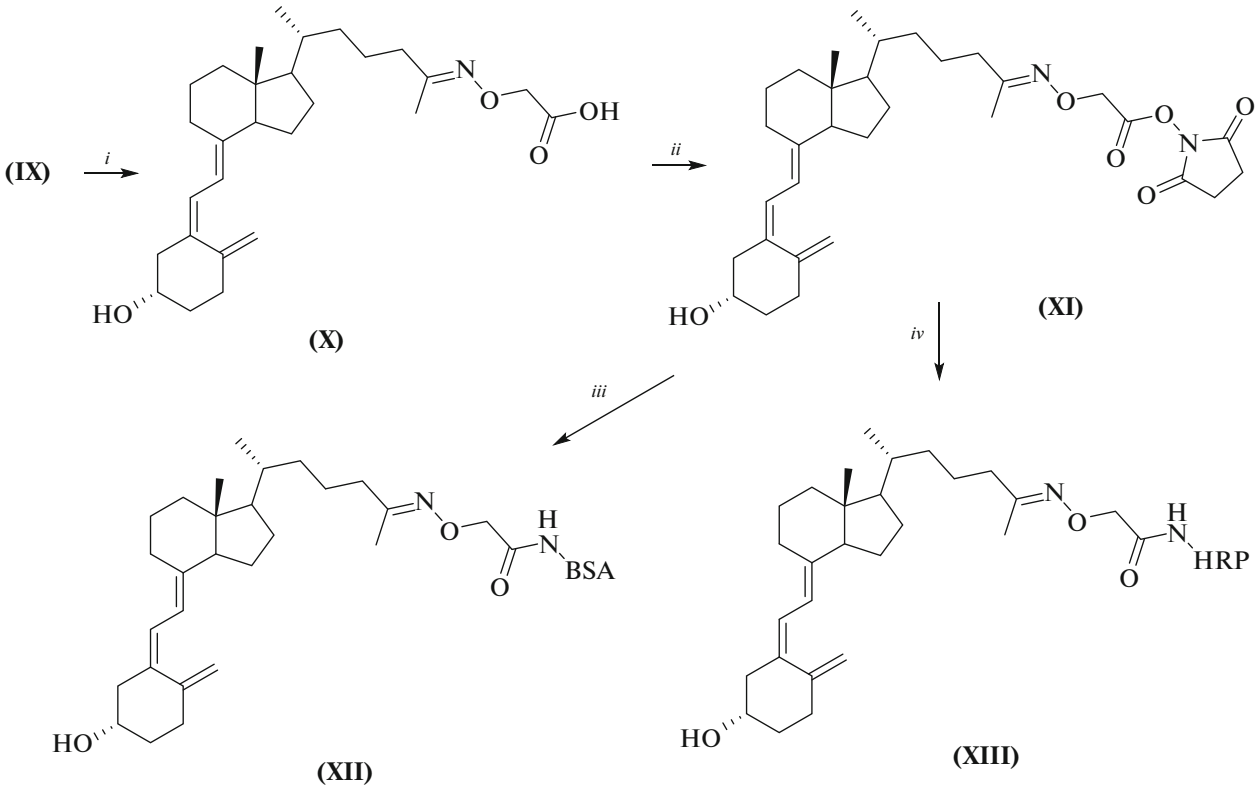
Scheme 1.

To construct the side chain, iodide (**V**) was treated with a complex of nickel with vinyl methyl ketone and pyridine. As a result, 25-keto derivative (**VI**) was isolated with a yield of 69%. The structure of the derivative follows from the IR spectrum (appearance of the stretching band of the keto group) and the ^{1}H NMR spectrum (three-proton singlet of the C26 methyl group). The triazoline protection in compound (**VI**) was removed by heating the compound at 140°C in dimethyl sulfoxide containing potassium carbonate. Resulting 3-acetoxy-5,7-diene (**VII**) was hydrolyzed with a 5% sodium hydroxide solution in methanol to yield 3β-hydroxy derivative (**VIII**). Formation of the latter was confirmed by the presence of olefin proton signals characteristic of 5,7-diene derivatives in the ^{1}H NMR spectrum, the shift of the proton signal at C3 towards a stronger field, and the appearance of the band corresponding to stretching vibrations of the hydroxyl group in the IR spectrum.

The conversion of 5,7-diene (**VIII**) to 9,10-secosteroid was carried out by irradiation with ultraviolet radiation and subsequent thermal isomerization of the cleaved compound by boiling in ethanol [6]. The ^{1}H NMR spectrum of the resulting 25-keto analog of vitamin D_3 (**IX**) contains characteristic signals of olefinic protons at C6 and C7 as doublets and signals of two geminal olefinic protons as broadened singlets.

The preparation of conjugates of the 25-keto derivative with proteins was performed via carboxymethyloxime (**X**) according to the procedure described previously for the 6-oxosteroids [7]. A mixture of two isomers is formed in this case, as indicated by the presence of two singlets (at δ 1.86 and 1.90 ppm) of approximately the same integral intensity in the ^{1}H NMR spectrum. These singlets correspond to C26 methyl groups. Activated ester (**XI**) was prepared by reacting derivative (**X**) with *N*-hydroxysuccinimide in dioxane in the presence of dicyclohexylcarbodiimide. After purification by silica gel chromatography, it was used in a conjugation reaction with proteins.

Protein conjugate (**XII**) was obtained in the reaction of a dioxane solution of ether (**XI**) with a water-dioxane solution of BSA (1 : 1, pH 8.3) (Scheme 2). After dialysis, the conjugate was used in the immunization of animals. The steroid component content in the conjugate was ~26 moles per 1 mole of protein as determined by MALDI-mass spectrometry.



i: NH_2OCH_2COOH, Py; *ii*: NHS, DCC; *iii*: BSA; *iv*: HRP

Scheme 2.

Labeled antigen (**XIII**), which is required for the enzyme immunoassay, was performed similarly (Scheme 2). Horseradish peroxidase was chosen as a label. For this purpose, a solution of activated ester (**XI**) in dioxane was added to a peroxidase solution in bicarbonate buffer (pH 8.35). Resulting conjugate (**XIII**) was purified by Sephadex G-25 chromatography.

Antisera were prepared according to the methods described in [7, 8]. Several antisera to $25OHD_3$ were selected during testing. The immunochemical analysis was performed using an antiserum with a titer of 1 : 10000, which was indirectly immobilized on standard 96-well plates via secondary antibodies. The immobilized antiserum became the basis for the quantitative determination of $25OHD_3$. Figure 1 shows that the calibration curve is linear in the $25OHD_3$ concentration range of 25–1250 nmol/L. The sensitivity of the determination was 15 nmol/L.

EXPERIMENTAL

The following reagents were used in this study: *N*-hydroxysuccinimide (Aldrich, United States), BSA (Acros Organics, United States), and horseradish peroxidase (Sigma-Aldrich, United States). Animals were immunized using Freund's complete adjuvant from ICN Biochemicals. Sheep antibodies to rabbit IgG were obtained by affinity purification of the corresponding antiserum. Staining was performed using an Enhanced K-Blue TMB (NEOGEN, United States) chromogen–substrate mixture, which is a ready-mixed solution of 3,3',5,5'-tetramethylbenzidine in the substrate buffer with hydrogen peroxide. Staining was stopped with 5% H_2SO_4.

Melting points were measured on a Boetius microheating table. Their values are given without correction. The IR spectra were recorded at 700–3600 cm^{-1} in a film or in KBr tablets using a UR-20 device. ^1H NMR (δ, ppm, SSCC, Hz) and ^{13}C NMR (δ, ppm) spectra were recorded in deuterochloroform using a Bruker Avance DRX-500 instrument (the operating frequencies were 500 and 125 MHz, respectively). The residual solvent peak was used as the internal standard (δ_H 7.26 ppm and δ_C 77.16 ppm for $CDCl_3$). Mass spectra were obtained on an Agilent 1200 mass spectrometer using the positive polarity electrospray ionization mode (ESI Positiv). A chromatograph was equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The temperature program was as follows: 50°C (2 min)—10°C/min until 300°C (23 min). The evaporator temperature was 290°C. Helium was used as the carrier gas at 0.8 mL/min. The injection volume was 1 μL. Optical rotation was determined at 25°C on a Rudolph Research Analytical Autopol I polarimeter (λ = 589 nm). High-resolution mass spectra were obtained by electrospray ionization on a Thermo Fisher Scientific LTQ Orbitrap Velos device. MALDI mass spectrometry was performed on a Microflexs LRF time-of-flight mass spectrometer (Bruker Daltonik GmbH, Germany) with a MALDI source equipped with a nitrogen laser (335 nm) having a frequency of 60 Hz. Ozone was synthesized according to a conventional method in a barrier-type gas discharge using a Groza-10 ozonizer, which produces two gramms of ozone per hour. Dialysis was performed using cellulose tubes (Sigma-Aldrich, United States) with a diameter of 25 mm. Purification and absolutization of solvents were carried out by conventional methods. Analytical TLC was carried out on aluminum plates with a layer of a Kieselgel 60 F_{254} silica gel (Merck). A Kieselgel 60 (Merck) Silica gel was used for column chromatography.

3β-Acetoxy-ergosta-5,7,22-triene (II). A solution of 1 g (2.53 mmol) of ergosterol (**I**) in 4 mL of pyridine was supplied with 2 mL (21 mmol) of acetic anhydride and 30 mg (0.25 mmol) of dimethylaminopyridine. The reaction was carried out overnight at room temperature. The reaction was monitored by TLC (PE–EtOAc, 5 : 1). The reaction mixture was diluted with water (40 mL) and the resulting crystals were filtered off, washed with water, and recrystallized from ethanol. As the result, 791 mg (70%) of 3-acetate (**II**) was obtained as yellowish fine crystals. Mp: 150–152°C (EtOAc–PE). $[\alpha]_D^{25}$ –89° (*c* 0.55, $CHCl_3$). IR (KBr): 2975, 1725, 1520, 1425, 1215, 1045. ^1H NMR: 0.62 (3 H, s, H18), 0.80–0.86 (6 H, m, H26, H27), 0.91 (3 H, d, *J* 6.8, H28), 0.95 (3 H, s, H19), 1.03 (3 H, d, *J* 6.5, H21), 2.04 (3 H, s, $COCH_3$), 2.36 (1H, m, H4), 2.50 (1H, m, H4), 4.71 (1 H, m, H3), 5.14–5.25 (2 H, m, H22, H23), 5.38 (1 H, m, H7), 5.56 (1 H, m, H6). ^{13}C NMR: 12.2 q, 16.3 q, 17.7 q, 19.8 q, 20.1 q, 21.1 t, 21.2 q, 21.5 q, 23.1 t, 28.2 t, 28.4 t, 33.2 d, 36.8 t, 37.2 s, 38.0 t, 39.1 t, 40.5 d, 42.9 s and d, 46.1 d, 54.6 d, 55.8 d, 72.9 d, 116.4 d, 120.3 d, 132.1 d, 135.7 d, 138.7 s, 141.7 s, 170.7 s. HRMS (ESI^+), *m/z* (I_{rel}, %): 379 ([M-AcOH + H]$^+$, 100), 301 (45), 163 (62).

3β-Acetoxy-5α,8α-(4-phenyl-1,2,4-triazolidine-3,5-dione-1,2-diyl)ergosta-6,22-diene (III). 3-Acetate (**II**) (790 mg, 1.8 mmol) was dissolved in 10 mL of anhydrous CH_2Cl_2 and supplied with 637 mg (3.6 mmol) of 4-phenylurazole. A solution of 1.59 g (3.6 mmol) of lead tetraacetate in 5 mL of absolute CH_2Cl_2 containing 80 μL of CH_3COOH was then added dropwise to the resulting solution for 40 min at 0°C. The resulting mixture was stirred at 0°C for 20 minutes. The reaction was monitored by TLC (PE– EtOAc, 5 : 1). The reaction mixture was diluted

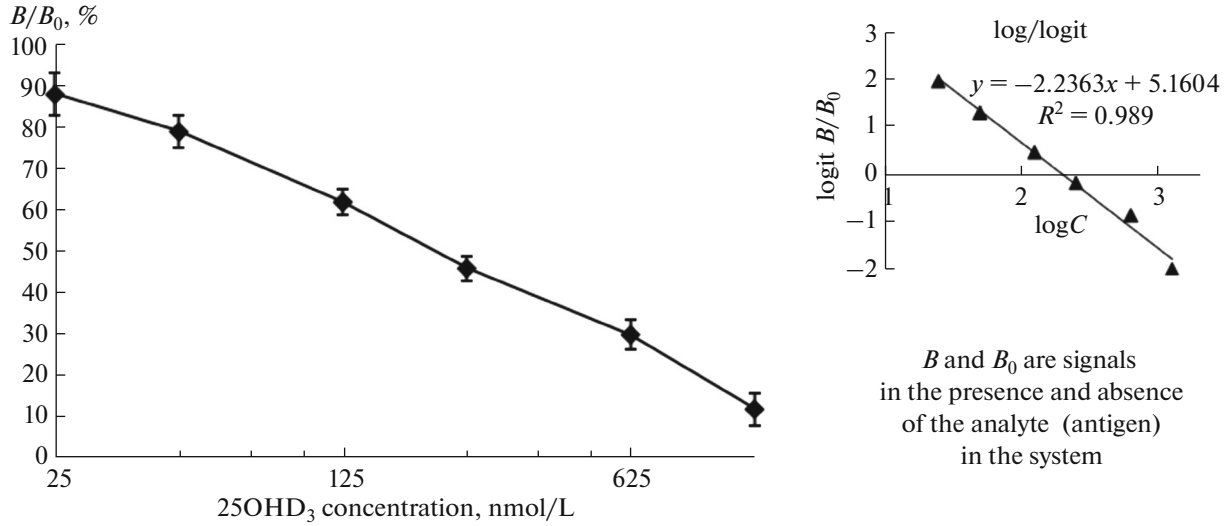


Fig. 1. Dependence of the binding of labeled antigen (**XIII**) with anti-25OHD$_3$ antibodies on the 25OHD$_3$ concentration in the calibration samples.

with water (15 mL) and extracted with $CHCl_3$ (3 × 10 mL). The combined organic extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue was chromatographed on a silica gel column with a PE–EtOAc mixture (15 : 1). As a result, 1.037 g (94%) of Diels-Alder adduct **(III)** was obtained as a yellowish amorphous substance. Mp: 93–96°C (EtOAc–PE). $[\alpha]_{589}$ –103° (*c* 0.52; $CHCl_3$). IR (film): 2950, 2870, 1750, 1735, 1395, 1245. ^{1}H NMR: 0.84–0.79 (9 H, m, H26, H27, H18), 0.89 (3 H, d, *J* 6.8, H28), 0.98 (3 H, s, H19), 1.02 (3 H, d, *J* 6.5, H21), 2.01 (3 H, s, $COCH_3$), 2.49 (1 H, m, H4), 3.22 (1 H, m, H4), 5.13–5.26 (2 H, m, H22, H23), 5.46 (1 H, m, H3), 6.23 (1 H, d, *J* 8.3, H7), 6.41 (1 H, d, *J* 8.3, H6), 7.29 (1 H, m, NPh), 7.36–7.45 (4 H, m, NPh). ^{13}C NMR: 13.3 q, 17.5 q, 17.6 q, 19.7 q, 20.0 q, 21.3 q, 21.4 q, 22.5 t, 23.4 t, 26.0 t, 27.6 t, 31.0 t, 33.1 d, 33.7 t, 38.1 t, 39.6 d, 41.2 s, 42.8 d, 43.9 s, 49.4 d, 52.9 d, 55.2 d, 65.0 s, 65.4 s, 70.6 d, 126.3 2d, 127.8 d, 128.9 2d, 129.3 d, 131.8 s, 132.5 d, 135.2 d, 135.3 d, 146.5 s, 149.1 s, 170.1 s. HRMS (ESI$^+$), *m/z* (I_{rel}, %): 636 ([*M* + Na]$^+$, 18), 614 ([*M* + H]$^+$, 100). Found: *m/z* 614.3959 [*M* + H]$^+$. $C_{38}H_{51}N_3O_4$ + H. Calculated: 614.3958.

3β-Acetoxy-5α,8α-(4-phenyl-1,2,4-triazolidine-3,5-dione-1,2-diyl)-23,24-bisnorchol-6-en-22-ol (IV). A current of ozone was passed through a solution of 1.037 g (1.69 mmol) of Diels-Alder adduct (**III**) in 40 mL of CH_2Cl_2 and 15 mL of methanol at –60°C. The ozonolysis was monitored by TLC (PE– EtOAc, 5 : 1) by the disappearance of starting compound (**III**). The reaction time was about 50 min. The reaction mixture was flushed with argon and slowly heated to room temperature while adding 225 mg (5.92 mmol) of $NaBH_4$ in small portions. The reduction was carried out at room temperature for 2 hours, after which the reaction mixture was washed with a solution of 0.5M HCl (25 mL), and the organic phase was separated and washed with a saturated NaCl solution (3 × 20 mL). The combined organic extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue was chromatographed on a silica gel column. After elution with a PE–EtOAc mixture (3 : 1), 629 mg (68%) of 22-hydroxy derivative (**IV**) was obtained as a colorless amorphous substance. Mp 128–130°C (EtOAc–PE). $[\alpha]_{589}$ –79° (*c* 0.42; $CHCl_3$). IR (KBr): 3450, 2955, 2860, 1750, 1735, 1405, 1245. ^{1}H NMR: 0.82 (3 H, s, H18), 0.98 (3 H, s, H19), 1.06 (3 H, d, *J* 6.5, H21), 2.01 (3 H, s, $COCH_3$), 2.56 (1 H, dd, *J* 13.9, 4.8, H4), 3.22 (1 H, dd, *J* 13.9, 4.8, H4), 3.35 (1 H, dd, *J* 10.4, 7.1, H22), 3.64 (1 H, dd, *J* 10.4, 3.1, H22), 5.46 (1 H, m, H3), 6.24 (1 H, d, *J* 8.3, H7), 6.41 (1 H, d, *J* 8.3, H6), 7.17 (1 H, m, NPh), 7.25 (1 H, m, NPh), 7.41 (3 H, m, NPh). ^{13}C NMR: 12.9 q, 16.9 q, 17.4 q, 21.2 q, 22.3 t, 23.3 t, 25.8 t, 26.9 t, 30.8 t, 33.5 t, 37.9 t, 38.2 d, 41.0 s, 43.9 s, 48.9 d, 51.6 d, 52.6 d, 64.8 s, 65.2 s, 67.4 t, 70.4 d, 125.2 d, 126.1 d, 127.7 d, 128.1 d, 128.7 d, 128.9 d, 131.6 s, 135.2 d, 146.4 s, 148.9 s, 169.9 s. HRMS (ESI$^+$), *m/z* (I_{rel}, %): 570 ([*M* + Na]$^+$, 16), 548 ([*M* + H]$^+$, 100). Found: *m/z* 548.3126 [*M* + H]$^+$. $C_{32}H_{41}N_3O_4$ + H. Calculated: 548.3124.

3β-Acetoxy-22-iodo-5α,8α-(4-phenyl-1,2,4-triazolidine-3,5-dione-1,2-diyl)-23,24-bisnorchol-6-ene (V). A solution of 313 mg (4.60 mmol) of imidazole

and 603 mg (2.30 mmol) of triphenylphosphine recrystallized from hexane in 20 mL of absolute CH_2Cl_2 was cooled to 0°C and supplied with 593 mg (2.30 mmol) of iodine in 20 mL of anhydrous CH_2Cl_2. A solution of 629 mg (1.15 mmol) of 22-ol (**IV**) in 5 mL of anhydrous CH_2Cl_2 was added at 0°C after 15 minutes of stirring. The resulting solution was stirred under an argon atmosphere for 30 minutes at 0°C and then for 1.5 hours at room temperature. The reaction was monitored by TLC (PE–EtOAc, 2 : 1). The resulting mixture was filtered, and the residue was washed with CH_2Cl_2 on a filter (3 × 4 mL). The combined filtrate was washed successively with a 2% $Na_2S_2O_3$ solution (3 × 10 mL, until iodine decolorization), 0.1 M HCl (10 mL), and a saturated NaCl solution (2 × 10 mL). The organic phase was dried with Na_2SO_4, the solvent was removed in vacuo, and the residue was chromatographed on a silica gel column. After elution with a PE–EtOAc mixture (19 : 1), 688 mg (91%) of 22-iodo derivative (**V**) was obtained as a yellowish amorphous substance. Mp 130–134°C (EtOAc–PE). $[\alpha]_{589}$ −73° (*c* 0.30; $CHCl_3$). IR (KBr): 2945, 1755, 1735, 1405, 1240. ^{1}H NMR: 0.83 (3 H, s, H18), 0.98 (3 H, s, H19), 1.05 (3 H, d, *J* 6.5, H21), 2.02 (3 H, s, $COCH_3$), 2.59 (1 H, dd, *J* 10.3, 4.4, H4), 3.15 (1 H, dd, *J* 9.5, 5.6, H22), 3.23 (1 H, dd, *J* 10.3, 4.4, H4), 3.33 (1 H, dd, *J* 9.5, 5.6, H22), 5.45 (1 H, m, H3), 6.25 (1 H, d, *J* 8.3, H7), 6.40 (1 H, d, *J* 8.3, H6), 7.30 (1 H, m, NPh), 7.41 (4 H, m, NPh). ^{13}C NMR: 13.6 q, 17.4 q, 19.9 t, 20.9 q, 21.2 q, 22.2 t, 23.1 t, 25.8 t, 26.9 t, 30.8 t, 33.6 t, 36.5 d, 37.8 t, 41.0 s, 43.8 s, 48.9 d, 52.6 d, 54.4 d, 64.7 s, 65.2 s, 70.3 d, 126.1 2d, 127.7 d, 128.7 2d, 128.8 d, 131.6 s, 135.3 d, 146.5 s, 148.9 s, 169.9 s. HRMS (ESI^+), *m/z* (I_{rel}, %): 1337 $[2M + Na]^+$ (15), 658 $[M + H]^+$ (100). Found: *m/z* 658.2146 $[M + H]^+$. $C_{32}H_{40}IN_3O_4$ + H. Calculated: 658.2142.

3β-Acetoxy-5α,8α-(4-phenyl-1,2,4-triazolidine-3,5-dione-1,2-diyl)-27-norcholesta-6-en-25-one (VI). To form the Ni^0 complex with pyridine and methyl vinyl ketone, 10 mL of degassed anhydrous pyridine were supplied with 1.234 g (5.25 mmol) $NiCl_2 \cdot 6H_2O$, 1.323 g (21 mmol) zinc-copper pair, and 0.43 mL (5.30 mmol) of methyl vinyl ketone. The mixture was heated to 60°C. After the appearance of a dark red coloration indicative of the formation of the abovementioned Ni^0 complex, the temperature was lowered to room temperature. The solution of the complex (~4.5 eq.) was gradually added to a solution of 688 mg (1.05 mmol) of 22-iodide (**V**) in 4 mL of degassed anhydrous pyridine under an argon atmosphere. The reaction was carried out under argon for 1 hour at room temperature. The reaction was monitored by TLC (PE–EtOAc, 10 : 1). The resulting reaction mixture was diluted with EtOAc (10 mL) and filtered through celites. The celites were then thoroughly washed with EtOAc. The resulting filtrate was washed with 1 M HCl to an acidic pH of the aqueous phase (~5 × 20 mL) with a saturated NaCl solution (2 × 20 mL) and dried with Na_2SO_4. The solvent was removed in vacuo. The residue was chromatographed on a silica gel column. Elution with PE–EtOAc (15 : 1) gave 435 mg (69%) of 25-keto derivative (**VI**) as an oily product. $[\alpha]_{589}$ −50° (*c* 0.42; $CHCl_3$). IR (film): 2920, 1750, 1735, 1705, 1399, 1245. ^{1}H NMR: 0.79 (3 H, s, H18), 0.94 (3 H, d, *J* 6.5, H21), 0.98 (3 H, s, H19), 2.00 (3 H, s, $COCH_3$), 2.12 (3 H, s, H26), 5.45 (1 H, m, H3), 6.23 (1 H, d, *J* 8.3, H7), 6.41 (1 H, d, *J* 8.3, H6), 7.29 (1 H, m, NPh), 7.40 (4 H, m, NPh). ^{13}C NMR: 12.9 q, 17.4 q, 18.8 q, 20.4 t, 21.2 q, 22.3 t, 23.2 t, 25.9 t, 27.4 t, 29.6 t, 29.8 q, 33.6 t, 35.1 t, 35.2 d, 38.1 t, 41.0 s, 43.9 s, 44.1 t, 49.2 d, 52.7 d, 54.9 d, 64.9 s, 65.3 s, 70.4 d, 126.2 2d, 127.7 d, 128.8 2d, 129.1 d, 131.7 s, 135.2 d, 146.5 s, 149.0 s, 170.0 s, 209.2 s. HRMS (ESI^+), *m/z* (I_{rel}, %): 602 $[M + H]^+$ (28), 425 (13), 365 (100). Found: *m/z* 602.3596 $[M + H]^+$. $C_{36}H_{47}N_3O_4$ + H. Calculated: 602.3594.

3β-Acetoxy-27-norcholesta-5,7-dien-25-one (VII). A solution of 435 mg (1.02 mmol) of Diels-Alder adduct (**VI**) in 30 mL of DMSO was supplied with 1.408 g (10.20 mmol) of anhydrous potassium carbonate, and the reaction mixture was heated at 140°C for 1.5 hours. The reaction was monitored by TLC (PE–EtOAc, 2 : 1). The excess of potassium carbonate was carefully neutralized with a solution of 0.15 M HCl (60 mL) and diluted with EtOAc (60 mL). The organic phase was separated, and the aqueous phase was extracted with EtOAc (2 × 25 mL). The combined organic extracts were washed with water and dried with Na_2SO_4. The solvent was removed in vacuo. The residue was chromatographed on a silica gel column. Elution with PE–EtOAc (25 : 1) gave 313 mg (72%) of 25-keto derivative (**VII**) as a white amorphous powder. Mp 114–117°C (EtOAc–PE). $[\alpha]_{589}$ −71° (*c* 0.28; $CHCl_3$). IR (KBr): 2945, 2870, 1730, 1710, 1370, 1255. ^{1}H NMR: 0.60 (3 H, s, H18), 0.94 (3 H, s, H19), 0.95 (3 H, d, *J* 6.5, H21), 2.03 (3 H, s, $COCH_3$), 2.13 (3 H, s, H26), 4.69 (1 H, m, H3), 5.37 (1 H, m, H7), 5.56 (1 H, m, H-6). ^{13}C NMR: 11.7 q, 16.1 q, 18.7 q, 20.4 t, 20.9 t, 21.3 q, 22.9 t, 28.0 t, 29.6 t, 30.8 q, 35.3 t, 35.9 d, 36.6 t, 37.0 s, 37.8 t, 39.0 t, 42.8 s, 44.2 t, 45.9 d, 54.4 d, 55.5 d, 72.7 d, 116.3 d, 120.1 d, 138.5 s, 141.4 s, 170.5 s, 209.3 s. HRMS (ESI^+), *m/z* (I_{rel}, %): 444 $[M + H_2O]^+$ (12), 427 $[M + H]^+$ (9), 367 ($[M$-AcOH + $H]^+$, 100). Found: *m/z* 427.3215 $[M + H]^+$. $C_{28}H_{42}O_3$ + H. Calculated: 427.3212.

3β-Hydroxy-27-norcholesta-5,7-dien-25-one (VIII). A solution of 313 mg (0.73 mmol) 3-acetate (**VII**) in 10 mL of a 5% solution of NaOH in methanol was boiled for 10 minutes. The reaction was monitored by TLC (PE–EtOAc, 3 : 2). A solution of 1 M HCl was then added to a neutral medium (~13 mL) and the resulting solution was diluted with water. The precipitate was filtered off and washed with water. By chromatography on a silica gel column with PE–EtOAc (15 : 1), 261 mg (93%) of 25-keto derivative (**VIII**) was obtained as a white amorphous powder. Mp 92–94°C (EtOAc–PE). $[\alpha]_{589}$ −83° (*c* 0.40; $CHCl_3$). UV (λ_{max}, nm (ε), MeOH): 271 (8670), 281 (9220), 293 (6575). IR (KBr): 2930, 2850, 1710, 1030. ^{1}H NMR: 0.60 (3 H, s, H18), 0.93 (3 H, s, H19), 0.95 (3 H, d, *J* 6.5, H21), 2.13 (3 H, s, H26), 3.63 (1 H, m, H3), 5.37 (1 H, m, H7), 5.56 (1 H, m, H6). ^{13}C NMR: 11.7 q, 16.2 q, 18.7 q, 20.3 t, 21.0 t, 22.9 t, 28.0 t, 29.8 q, 31.9 t, 35.3 t, 35.9 d, 36.9 s, 38.3 t, 39.1 t, 40.7 t, 42.8 s, 44.2 t, 46.1 d, 54.4 d, 55.4 d, 70.3 d, 116.3 d, 119.5 d, 139.8 s, 141.2 s, 209.3 s. HRMS (ESI$^+$), *m/z* (I_{rel}, %): 407 $[M + Na]^+$ (14), 385 $[M + H]^+$ (45), 367 $[M–H_2O + H]^+$ (100). Found: *m/z* 385.3105 $[M + H]^+$. $C_{26}H_{40}O_2 + H$. Calculated: 385.3107.

3(*S*)-Hydroxy-9,10-seco-27-norcholesta-5(*Z*),7(*E*),10(19)-trien-25-one (IX). Compound (**VIII**) (261 mg, 0.68 mmol) was irradiated in a 20-mg portion to open the cycle B. A solution of 20 mg (0.05 mmol) of 5,7-diene (**VIII**) in 10 mL of ethanol was introduced into a quartz jacketed flask cooled by a stream of cold water. The irradiation was carried out with a mercury lamp (30 V, 254 nm) in a current of argon for 40 min until the conversion degree of 5,7-diene reached ~50%. The reaction was monitored by TLC (PE–EtOAc, 17 : 1). As the result, 65 mg (25%) of 25-keto derivative of vitamin D_3 (**IX**) was obtained as an oily product. $[\alpha]_{589}$ +59° (*c* 0.27; $CHCl_3$). UV (λ_{max}, nm (ε), MeOH): 267 (18230). IR (film): 3435, 2940, 2870, 1715, 1370. ^{1}H NMR: 0.54 (3 H, s, H18), 0.93 (3 H, d, *J* 6.4, H21), 2.13 (3 H, s, H26), 3.95 (1 H, m, H3), 4.82 (1 H, br.s., H19*Z*), 5.05 (1 H, br.s., H19*E*), 6.03 (1 H, d, *J* 11.1, H7), 6.23 (1 H, d, *J* 11.1, H6). ^{13}C NMR: 11.9 q, 18.7 q, 20.4 t, 22.2 t, 23.5 t, 27.6 t, 29.4 t, 30.3 q, 30.6 t, 35.2 t, 35.4 t, 36.0 d, 40.5 t, 44.3 t, 45.8 s, 45.9 t, 56.2 d, 56.3 d, 69.2 d, 112.4 t, 117.5 d, 122.4 d, 135.1 s, 142.1 s, 145.1 s, 209.4 s. HRMS (ESI$^+$), *m/z* (I_{rel}, %): 408 $[M + Na + H]^+$ (14), 385 $[M + H]^+$ (63), 367 $[M–H_2O + H]^+$ (100). Found: *m/z* 385.3104 $[M + H]^+$. $C_{26}H_{40}O_2 + H$. Calculated: 385.3107.

25-(1-Carboxymethoxy)imino-3(*S*)-hydroxy-9,10-seco-27-norcholesta-5(*Z*),7(*E*),10 (19)-triene (X). Carboxymethoxylamine hydrochloride (87 mg, 0.68 mmol) was added to a solution of 65 mg (0.17 mmol) 25-keto derivative (**IX**) in 4 mL of absolute pyridine. The mixture was stirred at room temperature for 1 hour. The reaction was monitored by TLC (PE–EtOAc, 3 : 2). Pyridine was then evaporated, whereas the residue was dissolved in EtOAc and washed thoroughly with water. The organic phase was dried with Na_2SO_4, and the solvent was removed in vacuo. After elution with a chloroform-methanol (20 : 1 → 5 : 1) mixture, 66 mg (85%) of the oxime (**X**) was obtained as an oily product. $[\alpha]_{589}$ + 26° (*c* 0.27; $CHCl_3$). UV (λ_{max}, nm (ε), MeOH): 270 (18260). IR (film): 3420, 2955, 2875, 1740, 1630, 1310, 1050. ^{1}H NMR: 0.53 (3 H, s, H18), 0.92 (3 H, d, *J* 6.4, H21), 1.86 and 1.90 (3 H, s., H26), 3.95 (1 H, m, H3), 4.55 and 4.57 (2 H, s, $NOCH_2$), 4.81 (1 H, br.s., H19*Z*), 5.04 (1 H, br.s., H19*E*), 6.02 (1 H, d, *J* 11, H7), 6.22 (1 H, d, *J* 11, H6). ^{13}C NMR: 11.9 q, 14.3 q, 18.8 and 18.6 q, 22.2 t, 22.8 t, 23.5 t, 27.6 t, 28.9 t, 31.9 t, 35.1 t, 35.4 t, 35.9 d, 36.0 t, 40.7 and 40.5 t, 45.8 t, 46.2 s, 56.2 d, 56.3 d, 69.2 d, 70.0 t, 112.4 t, 117.5 d, 122.4 d, 135.1 s, 142.2 s, 145.1 s, 161.1 s, 173.3 s. HRMS (ESI$^+$), *m/z* (I_{rel}, %): 480 $[M + Na]^+$ (27), 458 $[M + H]^+$ (71), 440 $[M–H_2O + H]^+$ (100). Found: *m/z* 458.3272 $[M + H]^+$. $C_{28}H_{43}NO_4 + H$. Calculated: 458.3270.

25-((*N*-Oxysuccinimidyl)acetyl)oximino-3(*S*)-hydroxy-9,10-seco-27-norcholesta-5(*Z*),7(*E*),10(19)-triene (XI). A solution of 64 mg (0.14 mmol) of carboxymethyloxime (**X**) and 21 mg (0.18 mmol) of N-hydroxysuccinimide in 2 mL of anhydrous dioxane was cooled to 5°C and supplied with a solution of 37 mg (0.18 mmol) of dicyclohexylcarbodiimide in 1 mL of anhydrous dioxane. The reaction mixture was stirred for 30 minutes at 5°C and 20 hours at room temperature. The reaction was monitored by TLC (PE–EtOAc, 2 : 1). The precipitated dicyclohexylcarbourea was filtered off and the filtrate was evaporated. The resulting residue was dissolved in EtOAc, washed with water, dried with Na_2SO_4, and evaporated. The residue was chromatographed on a silica gel column. After elution with a chloroform–methanol mixture (15 : 1), 71 mg (91%) of *N*-hydroxysuccinimide ester (**XI**) was obtained as oil, which was then used without further purification.

Synthesis of Immunogenic Conjugate (XII)

A solution of 60 mg (108.30 μmol) of activated ester (**XI**) in 6 mL of dioxane was supplied with 240 mg (3.61 μmol) BSA in 6 mL of a 0.1 M solution of $NaHCO_3$ (pH 8.3) and stirred for 48 hours at room temperature. The reaction mixture was dialyzed through a semipermeable membrane against distilled water for 36 hours at a temperature of 20°C (water was changed after 12 hours) and then against a 1% suspension of activated carbon in water for 20 hours. The

reaction mixture was then freeze-dried and frozen at −20°C. The content of hapten in the conjugate was about 26 moles of hapten per mole of protein as determined by MALDI-mass spectrometry.

Synthesis of Labeled Antigen (XIII)

A solution of 6 mg HRP in 600 μL of a 0.1 M aqueous $NaHCO_3$ solution (pH 8.3) was supplied with a solution of 2 mg (3.61 μmol) of activated ester (**XI**) in 300 μL dioxane, stirred at room temperature for 4 hours, and purified on a Sephadex G-25 column. The eluent was distilled water. A yellow-brown fraction was collected, diluted with glycerol (1 : 1), and stored at −18°C. The conjugate was obtained with a yield close to quantitative.

Immunization. A group of seven rabbits was immunized with BSA conjugate (**XII**). Each rabbit was injected subcutaneously into 15 regions on the back with 1-mg portions of the conjugate dissolved in 0.5 mL of 0.05 M phosphate buffer containing 0.1 M NaCl (pH 7.4) and emulsified in equal volume of complete Freund's adjuvant. Intervals between injections were 3–4 weeks. Immunization was continued for six months with periodic sampling of blood from the ear vein of the animals. The resulting serum samples were tested for the ability to bind peroxidase-labeled antigens. Their titers (working dilution) were determined in a solid phase immunoassay system. An antiserum with a titer of 1 : 10000 was used in further work.

Immobilization of antibodies. Microplates for ELISA were prepared as follows. Each well of the plate was supplied with secondary goat antibodies to rabbit immunoglobulins G (2 μg/mL, 150 μL per well) in a twofold-diluted coating buffer 1 (0.05 M phosphate buffer, pH 7.4 containing 0.9% NaCl). The coating buffer was removed after incubation at 4°C for 18–20 h without washing the wells. The wells were then supplied with anti-25OHD_3 antiserum (the titer was 1 : 10000, 150 μL per well) in coating buffer 2 (0.025 M phosphate buffer, pH 7.4, containing 0.1% BSA and 0.02% TweenTM 20) and incubated at 4°C for 18–20 h. After the removal of the coating solution, the plates were supplied with 200 μL of a stabilizing buffer solution (0.025 phosphate buffer, pH 7.4 containing 0.1% BSA, 2% sucrose, and 5% sorbitol) and incubated for 18–20 h at 4°C. After removing the stabilizing solution, the prepared plates with immobilized anti-25OHD3 antibodies were dried at room temperature for 24 hours. The prepared plates with immobilized antibodies were stored in a moisture-free tightly closed plastic bag at a temperature of 4–8°C.

ELISA. Calibration samples were prepared by serial dilutions of the initial alcoholic solution with a known concentration (10^{-4} mol/L) of 25OHD_3 with a buffer solution (0.05 M Tris, pH 7.4, containing 0.9% NaCl, 0.1% BSA, and 0.02% Tween™ 20). The steroid concentration in the calibration samples was 0, 25, 50, 125, 250, 625, and 1250 nmol/L. A solution of the enzyme conjugate was also prepared using the buffer solution. The conjugate concentration was selected in such a way that the optical density (A_{450}) in the well containing the calibration sample with "zero" steroid content was at least 2.0 opt. units.

Polystyrene wells of the plate with immobilized anti-25OHD_3 antibodies were supplied with 50 μL of the calibration samples or the analyzed samples in duplicate and then with 100 μL of a buffer solution containing the steroid conjugate with HRP. The plate was incubated in a thermostat for 1 hour at 37°C, after which the content of wells was removed, and the wells were washed with a washing solution (1% NaCl containing 0.02% Tween™ 20) (4 × 150 μL). All the washed wells were supplied with 150 μL of a chromogen-substrate mixture and incubated at 37°C for 15 min. The reaction was stopped by adding 50 μL of a stop-solution solution to all the wells. The optical density of the solution in all the wells was measured on an F300TP universal photometer (RUPP Vityaz, Belarus) at a wavelength of 450 nm.

The mean arithmetic values of the optical density were calculated for each calibration sample. The steroid concentration in the calibration samples (nmol/L) was plotted against $B/B_0 \times 100$, where B and B_0 are the optical densities of the product in the enzymatic reaction performed in the presence and absence of free 25OHD_3, respectively. The sigmoidal calibration curve was linearized using a log–logit transformation, i.e. the dependence of logit B/B_0 calculated using formula (1) was plotted against the decimal logarithm of the steroid concentration in calibration samples (log C) [9, 10]:

$$\text{logit}\, B/B_0 = \ln((B/B_0)/(100 - B/B_0)). \quad (1)$$

The sensitivity of the method was determined as the concentration that corresponded to the B_0-2SD value (SD is the standard deviation ($n = 8$) of the optical density of the enzymatic reaction product in the wells with zero calibration sample).

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