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Photoactivable Analogs for Labeling 25-Hydroxyvitamin D_3 Serum Binding Protein and for 1,25-Dihydroxyvitamin D_3 Intestinal Receptor Protein

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3-Azidobenzoates and 3-azidonitrobenzoates of 25-hydroxyvitamin D_3 as well as 3-deoxy-3-azido-25-hydroxyvitamin D_3 and 3-deoxy-3-azido-1,25-dihydroxyvitamin D_3 were prepared as photoaffinity labels for vitamin D serum binding protein and 1,25-dihydroxyvitamin D_3 intestinal receptor protein. The compounds prepared were easily activated by short- or long-wavelength uv light, as monitored by uv and ir spectrometry. The efficacy of the compounds to compete with 25-hydroxyvitamin D_3 or 1,25-dihydroxyvitamin D_3 for the binding site of serum binding protein and receptor, respectively, was studied to evaluate the vitamin D label with the highest affinity for the protein. The presence of an azidobenzoate or azidonitrobenzoate substituent at the C-3 position of 25-OH-D_3 significantly decreased (10^4- to 10^6-fold) the binding activity. However, the labels containing the azido substituent attached directly to the vitamin D skeleton at the C-3 position showed a high affinity, only 20- to 150-fold lower than that of the parent compounds with their respective proteins. Therefore, 3-deoxy-3-azidovitamins present potential ligands for photolabeling of vitamin D proteins and for studying the structures of the protein active sites.

Photoaffinity labeling (*1–3*) has in recent years become one of the most powerful techniques for studying the properties and function of proteins involved in many carrier-mediated transport processes. The method, taking advantage of the specific ligand–receptor interaction, has been used lately for identification and partial characterization of binding proteins of several classes of steroids including bile salts (*4, 5*), glucocorticoids (*6*), progesterones (*7*), and some fat-soluble vitamins like vitamin A–alcohol (*8*).

To further characterize the vitamin D binding proteins (*9–11*), a search was undertaken for effective photoactivable derivatives of vitamin D_3 which, upon irradiation with uv light, may be covalently attached to the protein. When used in radiolabeled form these compounds may serve as markers for studying the protein active sites.

The binding of vitamin D to protein is known to involve all hydroxy groups of 25-hydroxyvitamin D_3 (25-OH-D_3) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), with hydroxyls at C-1 and C-25 being crucial in this process (*12–14*). The C-3 hydroxyl appeared to be less important, as its removal or derivatization results only in a slight reduction of binding affinity (*15, 16*). For that reason the C-3

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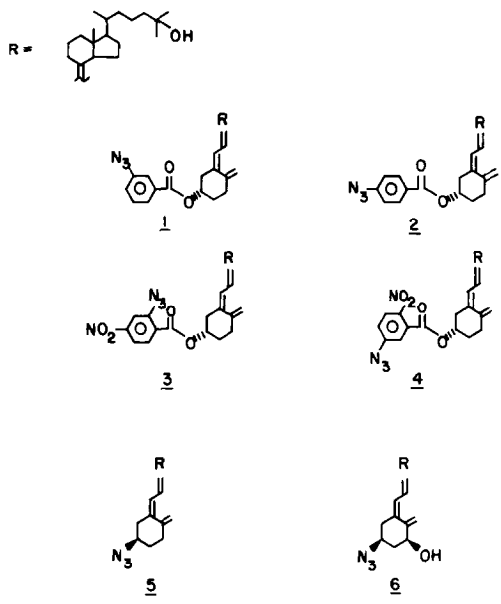


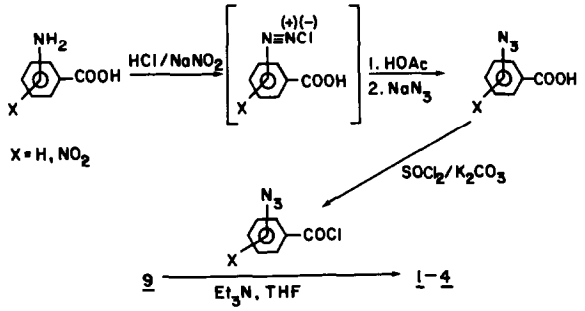
SCHEME I

hydroxyl was chosen in our studies as a place for introduction of a photoactivable group.

In this paper we describe the preparation of azidobenzoic (AzBz) and azidonitrobenzoic (AzNBz) esters of 25-OH-D_3 (**1–4**) (Scheme I) as well as vitamin D analogs containing a photoactivable azido substituent attached directly to the C-3 position (**5,6**). Esters **1** and **2** as well as azidovitamins **5** and **6** were designed for labeling of vitamin D proteins with short-wavelength uv light, and esters **3** and **4** for labeling with long-wavelength uv light. The time-dependent photoactivation of compounds **1–6** was monitored by uv and ir spectroscopic methods. The relative binding affinities of compounds **1–6** for serum binding protein and intestinal receptor protein were studied in order to evaluate the most effective vitamin D label having the highest binding affinity for vitamin D protein.

SYNTHESES

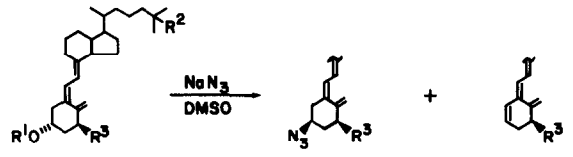
Esters **1–4** were prepared by regioselective C-3 acylation of alcohol **9** with respective azido- or azidonitrobenzoic acid chlorides (Scheme II). These acylation reagents were obtained from commercially available amino- or aminonitrobenzoic acids by replacement of diazonium salts with sodium azide. Azido- or azidonitrobenzoic acids were next converted to acid chlorides and used without isolation for immediate acylation of alcohol **9** (Scheme II). In this way the photoactivable azido- or azidonitrobenzoic group was attached to the parent vitamin **9** at the last step of the procedure, which makes the method especially valuable when starting from radiolabeled 25-OH-[26,27-^{3}H]D_3. The presence of unde-



SCHEME II

rivatized 25-hydroxyl in esters **1–4** was confirmed by ^{1}H NMR and mass spectrometry (MS). The peak at *m/e* 59 (*19, 20*) originating from the C-25 side-chain fragment was observed in all spectra. The characteristic MS fragmentation pattern of esters **1–4** comprised the loss of an acid from the parent molecule followed by the abstraction of the side chain. A downfield shift in the ^{1}H NMR spectra of 3 protons compared with the respective value for alcohol **9**, as well as an unchanged chemical shift in the signal of 26 or 27 protons, provided additional proof of the structure. As anticipated, esters **1** and **2** showed a maximum uv absorption at short wavelengths and ester **3** at long wavelengths. Unexpectedly, ester **4** showed a uv maximum much below 300 nm. Nevertheless, the compound was successfully activated with long-wavelength uv light (Fig. 3).

(3*R*)-3-Deoxy-3-azidovitamins **5**, **6**, and **13** (Scheme III) were prepared by nucleophilic substitution of respective tosylates **8**, **10**, and **12** (*21, 22*) with azido ion according to the S_N2 mechanism with inversion of the absolute configuration at the C-3 center. An unexpected regioselectivity was observed in the course of tosylation of 1,25-$(OH)_2D_3$ (**11**). 3β-Tosylated compound **12** was exclusively formed and no trace of 1α-tosylated by-product could be detected by HPLC chromatography. Similarly, no reaction was observed in our attempt to 1α-tosylate 3β-acetoxy-1α,25-$(OH)_2D_3$, in contrast to the known easy 1α-acetylation of 1,25-$(OH)_2D_3$ (*23*). The selectivity of tosylation may be rationalized in terms of strong steric hindrance of the 1α-hydroxyl by the 19-methylene group. 3-Azido-



	R^1	R^2	R^3
7	H	H	H
8	Ts	H	H
9	H	OH	H
10	Ts	OH	H
11	H	OH	OH
12	Ts	OH	OH

	R^2	R^3
13	H	H
5	OH	H
6	OH	OH

	R^2	R^3
14	H	H
15	OH	H
16	OH	OH

SCHEME III

vitamin **13**, Az-25-OH-D_3 **5**, and Az-1,25-$(OH)_2D_3$ **6** were separated from elimination by-products **14**, **15**, and **16** by preparative HPLC. Control experiments confirmed the formation of tetraenes **14**, **15**, and **16** directly from tosylates **8**, **10**, and **12** under reaction conditions applied for the preparation of azidovitamins. An interesting observation was that the HPLC mobility of azidovitamin **5** was lower than that of respective tetraene **15** (Fig. 1), while the presence of the 1α-hydroxyl caused a higher mobility of azidovitamin **6** compared to that of tetraene **16** (Fig. 2). 3-Deoxy-3-azidovitamins D_3 and D_2 were reported (*24*) as products of the treatment of vitamin D with hydrazoic acid in the presence of triphenylphosphine and an azodicarboxylate system. Azidovitamins thus obtained and purified by silica gel chromatography were described as very unstable compounds, even when stored under an inert atmosphere at lowered temperature. The compounds were immediately reduced to 3-deoxy-3-aminovitamins and characterized as acetamino derivatives (*24*). In our hands, however, HPLC-purified azides **5** and **6** were stable for several months when stored at −18°C and also stable for at least 24 h under the conditions of binding affinity assays, as determined by measuring the uv absorbance ratio OD_{265}/OD_{230} and by HPLC chromatography monitored at 265 nm. Incidentally, we observed a fast decomposition of azidovitamin **13** when the sample was only partially purified by silica Sep-Pak filtration, even when stored at lower temperature.

Electron impact fragmentation of azidovitamins **5**, **6**, and **13** showed the loss of N_2 as well as HN_3 from the parent compound. Molecular peaks of relatively medium intensity were observed in the mass spectra of all azidovitamins prepared. Stereochemical purity of these compounds at the C-3 chiral center was confirmed by the presence of an isolated multiplet of 3 protons at 3.6–3.7 ppm (*24*) in the ^1H NMR spectra of azidovitamins **5** and **13**. A strong absorption band at 2100 cm^{-1} ($\nu_{as}N_3$) accompanied by weak absorption at 1250 cm^{-1} ($\nu_{sym}N_3$) provided additional proof for the structure of azides **5**, **6**, and **13**.

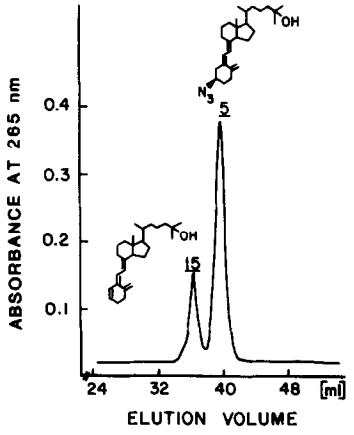


FIG. 1. HPLC separation of Az-25-OH-D_3 (**5**) from 3-deoxy-3-dehydro-25-OH-D_3 (**15**). The silica gel column (6.2 × 250 mm) was developed with 7% ethyl acetate in hexane at a flow rate 4 ml/min.

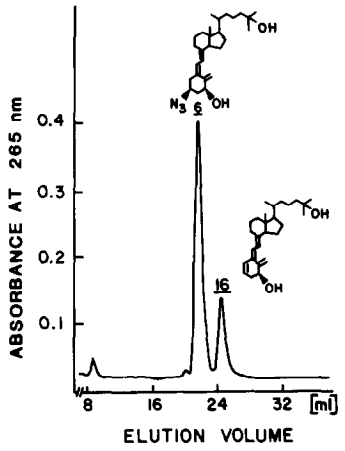


FIG. 2. HPLC separation of Az-1,25-$(OH)_2D_3$ (**6**) from 3-deoxy-3-dehydro-1,25-$(OH)_2D_3$ (**16**). The silica gel column (6.2 mm × 25 cm) was developed with 5% 2-propanol in hexane at a flow rate 4 ml/min.

PHOTOLYSIS

The suitability of vitamin D_3 derivatives synthesized for use as photoaffinity labels was tested by irradiation of alcoholic solutions of compounds **1–6** with the uv light. Some representative examples are shown in Figs. 3 and 4. As short an irradiation time as 1 min was determined to be sufficient to activate more than 50% of ester **2** present in the solution with the use of a medium-energy lamp operating

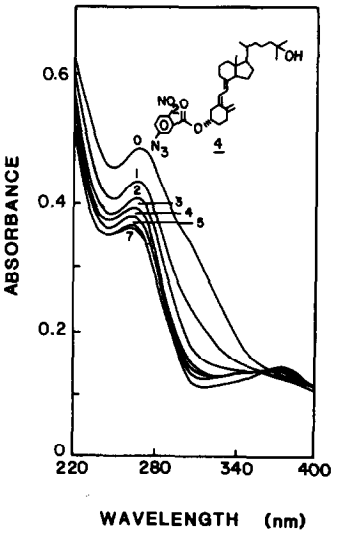


FIG. 3. The effect of photolysis with long-wavelength (366-nm) uv on the absorption spectrum of AzNBz-25-OH-D_3 **4**. A 17 nM ethanolic solution of ester **4** was irradiated with a Mineralight Model UVSL-25 lamp. Following the indicated time periods (min) the uv spectra were taken.

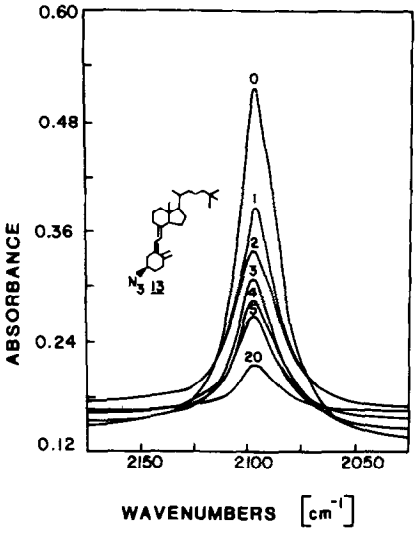


FIG. 4. The effect of photolysis with short-wavelength (254-nm) uv on the absorption maximum at 2097 cm^{-1} ($\nu_{as}N_3$) in the ir spectrum of Az-D_3 (**13**). A film of the compound was irradiated with a Mineralight Model SL 2537 lamp. Following the indicated time (min) a selected part of the ir spectrum was recorded.

at short-wavelength uv. Irradiation for a longer time did not cause further significant changes in the uv profile. Similarly, an irradiation time of 1–2 min was determined to be optimal for the activation of ester **1**. A low-energy uv source with maximum output in long-wavelength uv was strong enough to activate ester **4** within an irradiation time of 3–5 min (Fig. 3).

The uv activation of 3-azidovitamins **5**, **6**, and **13** could not be monitored by uv spectrometry, as the very weak absorption maximum of these alicyclic azides was entirely hidden behind the very strong absorption peak of the vitamin D triene system. Therefore, the activation of azides **5**, **6**, and **13** was followed by monitoring the decrease in absorbance of selected strong $\nu_{as}N_3$ bands in the ir spectra of these compounds. Figure 4 shows a representative example of the activation of azidovitamin **13**, prepared as a model compound, with the use of low-energy short-wavelength uv light. An irradiation time of 3 min was found to be optimal for uv activation of azidovitamins **5** and **6** as well as azidovitamin **13**.

PROTEIN BINDING STUDIES

A competitive binding assay was used to test the efficacy of the photoactive analogs to compare either with 1,25-$(OH)_2D_3$ for pig intestinal receptor or with 25-OH-D_3 for pig serum DBP. This assay measures the displacement of tritiated 1,25-$(OH)_2D_3$ or 25-OH-D_3 from its respective protein by increasing concentrations of a vitamin D analog.

The competitive binding curves obtained for the pig intestinal receptor studies with compounds **2**, **3**, **5**, and **6** are shown in Fig. 5 along with the curves for 1,25-

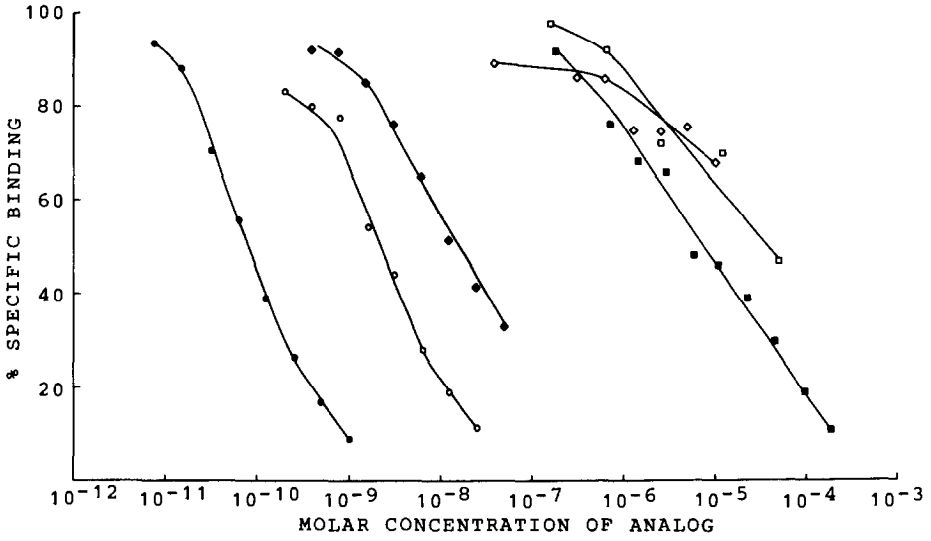


FIG. 5. Competitive binding analysis of vitamin D analogs for 1,25-$(OH)_2$-[26,27-^{3}H]D_3 with pig intestinal receptor: (●) 1,25-$(OH)_2D_3$ **11**, (○) 25-OH-D_3 **9**, (◆) Az-1,25-$(OH)_2D_3$ **6**, (■) AzBz-25-OH-D_3 **2**, (◇) AzNBz-25-OH-D_3 **3**, (□) Az-25-OH-D_3 **5**.

$(OH)_2D_3$ and 25-OH-D_3. A summary of the data is provided in Table 1. The binding affinity of 25-OH-D_3 for receptor was found to be 20-fold lower than that observed for 1,25-$(OH)_2D_3$. The higher affinity of 25-OH-D_3 for pig receptor than previously reported for chick receptor is due to a variation in incubation proce-

TABLE 1

RELATIVE EFFICACY OF PHOTOAFFINITY LABELS TO COMPETE WITH 25-OH-[26,27-^{3}H]D_3 AND WITH 1,25-$(OH)_2$-[26,27-^{3}H]D_3 FOR BINDING TO PIG SERUM BINDING PROTEIN (DBP) AND RECEPTOR PROTEIN, RESPECTIVELY

Compound		Binding to receptor		Binding to serum DBP	
		Molar concentration[a] (50% specific binding)	Relative binding affinity[b]	Molar concentration[a] (50% specific binding)	Relative binding affinity[c]
1,25-$(OH)_2D_3$	**11**	$9.8 \pm 0.5 \times 10^{-11}$	1	$3.0 \pm 1.1 \pm 10^{-8}$	7×10^{-2}
25-OH-D_3	**9**	$2.2 \pm 0.1 \times 10^{-9}$	5×10^{-2}	$2.2 \pm 0.3 \times 10^{-9}$	1
Az-1,25-$(OH)_2D_3$	**6**	$1.5 \pm 0.1 \times 10^{-8}$	7×10^{-3}	$1.0 \pm -.6 \times 10^{-6}$	2×10^{-3}
Az-25-OH-D_3	**5**	$4.6 \pm 1.4 \times 10^{-4}$	2×10^{-7}	$3.9 \pm 1.0 \times 10^{-8}$	6×10^{-2}
AzBz-25-OH-D_3	**2**	$8.1 \pm 1.6 \times 10^{-6}$	1×10^{-5}	$4.4 \pm 2.1 \times 10^{-6}$	5×10^{-4}
AzNBz-25-OH-D_3	**3**	$1.5 \pm 1.0 \times 10^{-5}$	5×10^{-6}	5×10^{-6d}	5×10^{-4}

[a] The concentration of analog that permits 50% of the specific binding of tritiated compound. The data are the averages from two experiments done in duplicate.

[b] Ratio of molar concentration for 50% specific binding of compound **11** to the analog.

[c] Ratio of molar concentration for 50% specific binding of compound **9** to the analog.

[d] Indicates highest concentration used in assay.

dures and species differences. When either azidobenzoate, azidonitrobenzoate, or the azido group was substituted for the 3-hydroxyl on 25-OH-D_3, the binding affinity for the receptor was reduced 3–5 orders of magnitude, as compared to the parent compound. This would indicate that the receptor protein has little tolerance for such substituents at the C-3 position of the ligand. However, Az-1,25-$(OH)_2D_3$ **6**, as compared to 1,25-$(OH)_2D_3$, showed only a 150-fold reduction in binding affinity. Therefore, it appears that the addition of the 1α-hydroxyl to Az-25-OH-D_3 **5** raises its affinity for the receptor by 4 orders of magnitude. This may imply a reduced importance of the 3-hydroxyl in receptor binding when the 1α-hydroxyl is present. It appears that 1,25-$(OH)_2D_3$ will have to be used as the parent compound for photoaffinity labels for receptor if the vitamin is modified at the C-3 position.

The competitive binding curves obtained for compounds **2**, **3**, **5**, and **6** with pig serum DBP are shown in Fig. 6 along with the curves for 1,25-$(OH)_2D_3$ and 25-OH-D_3. Table 1 provides a summary of the data. When either an azidobenzoate or an azidonitrobenzoate group was substituted at the C-3 position, the binding affinity for DBP was reduced 3 orders of magnitude. However, Az-25-OH-D_3 **5** showed only a 20-fold reduction in binding affinity compared to the parent compound. Therefore, it appears that DBP cannot accommodate the benzoate group at the C-3 position. The 10-fold reduction in the binding affinity of Az-1,25-$(OH)_2D_3$ **6** over the parent compound **11** suggests that addition of the 1α-hydroxyl and substitution of the 3-hydroxyl with an azido group have an additive effect on the reduction of binding of 25-OH-D_3 to DBP.

These studies have provided an excellent photoaffinity label for serum DPB, Az-25-OH-D_3 **5**. Preliminary experiments show covalent incorporation of the label

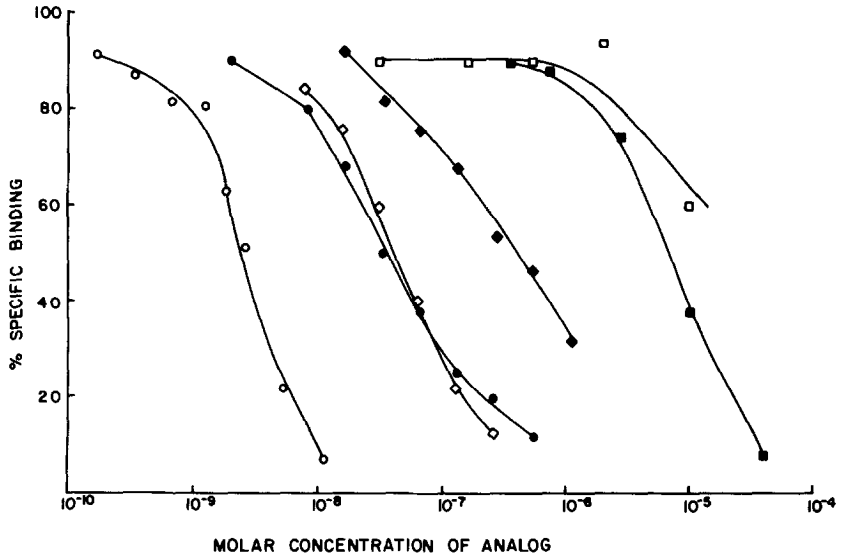


FIG. 6. Competitive binding analysis of vitamin D analogs for 25-OH-[26,27-^{3}H]D_3 with pig serum DBP: (○) 25-OH-D_3 **9**, (●) 1,25-$(OH)_2D_3$ **11**, (◇) Az-25-OH-D_3 **5**, (◆), Az-1,25-$(OH)_2D_3$ **6**, (■) AzBz-25-OH-D_3 **2**, (□) AzNBz-25-OH-D_3 **3**.

into purified human DBP and a complete study is in progress. Though the binding affinity for Az-1,25-$(OH)_2D_3$ **6** with receptor is lower than the affinity for Az-25-OH-D_3 **5** with DBP, it also has strong possibilities as a photoaffinity label. The azidobenzoate derivative of 1,25-$(OH)_2D_3$ is also being prepared for study with receptor, since the affinity of AzBz-25-OH-D_3 **2** was higher than that of Az-25-OH-D_3 **5** for receptor.

EXPERIMENTAL PROCEDURES

Methods

TLC was performed using precoated aluminum silica gel sheets with uv indicator ($60F_{254}$). The following solvent systems were used: (A) chloroform–methanol 1 : 1 (v/v), (B) hexane–ethyl acetate 1 : 1, (C) hexane–ethyl acetate 1 : 4, (D) hexane–ethyl acetate 95 : 5. Spots originated from 2-nitro-5-azidobenzoic acid and its 25-OH-D_3 ester were visualized by irradiation of the plate with long-wavelength (366-nm) uv light. Other compounds were visualized by spraying the plates with 50% $SbCl_3$ in acetic acid or 50% ethanolic sulfuric acid.

Electron impact low- and high-resolution mass spectra were recorded at 110–120°C at 70 eV with an AEI MS-9 spectrometer coupled to a DS-50 data system or with a Kratos MS-25 spectrometer and a DS-55 data system.

HPLC was performed using a Waters Associates liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 6 UK universal injector, and a Model 450 variable-wavelength detector. A Zorbax-Sil (Dupont) column (6.2 mm × 25 cm) and the following solvent systems were used: (A) 2% 2-propanol in hexane, (B) 3% 2-propanol in hexane, (C) 5% 2-propanol in hexane, (D) 7% ethyl acetate in hexane, (E) 0.05% ethyl acetate in hexane. Silica gel Sep-Pak (Waters Associates) cartridges were used for prefiltration of HPLC samples.

The uv absorption spectra were recorded in absolute ethanol or in hexane with a Hitachi Model 100-60 spectrometer. The ir spectra were recorded on a Nicolet MX-1 FT-IR spectrophotometer using films of oily substances. ^1H NMR spectra were taken with a Bruker WH-270 FT spectrometer or Varian EM-390, Nicolet NMC-200 instrument using acetone-d_6 solution with Me_4Si as an internal standard.

Photolyses

Photolytic experiments were conducted at room temperature in a 1-cm quartz cuvette positioned at a 3-cm distance from the surface of the uv lamp. Mineralight uv lamps (Ultra-Violet Products, San Gabriel, Calif.) used were Model UV SL-25 with an illumination intensity at a 15-cm distance of 180 $\mu W/cm^2$ at short wavelength (254 nm) and 260 $\mu W/cm^2$ at long wavelength (366 nm), and Model R-52G with an illumination intensity at a 15-cm distance of 1250 $\mu W/cm^2$ at short wavelength (254 nm), and Model SL 2537 with low-intensity illumination at short wavelength.

Synthesis

All operations involving azido compounds were carried out under low-intensity yellow light.

Preparation of azidobenzoic and azidonitrobenzoic acids—standard procedure.[2] A modified procedure of Galardy and co-workers (*25*) and Lewis and co-workers (*18*) was employed. The suspension of aminobenzoic acid (3.4 g, 25 mM) or aminonitrobenzoic acid (4.6 g, 25 mM) in 40 ml of concentrated HCl was cooled in an acetone–dry ice bath to −10°C with stirring. The solution of $NaNO_2$ (3 g, 43 mM) in 15 ml of water was added dropwise to keep the reaction mixture below 0°C. Glacial acetic acid (40 ml) was then added followed by NaN_3 solution (3 g, 46 mM) in 15 ml of water. Stirring of the reaction mixture at 0°C was continued for 1 h and the suspension was diluted with cold water (100 ml). The white precipitate (of azidobenzoic acids) or red-brown solid (of azidonitrobenzoic acids) was separated by filtration and washed with water (ca. 300 ml). Product was dried overnight in air and dissolved in ethyl ether. Some undissolved material was filtered off and the solution evaporated to dryness under reduced pressure to give azidobenzoic or azidonitrobenzoic acids with a yield of 85–95%. No starting material was observed as determined by TLC using solvent system A. Analytical samples were obtained by passing the solution of azidobenzoic or azidonitrobenzoic acid in a mixture of chloroform–methanol 2:1 (v/v) through the Sep-Pak cartridge (*25*) followed by the elution with the same solvents to yield the chromatographically pure acid. The uv spectroscopic data of the acids prepared were as follows:

3-Azidobenzoic acid	λ_{max} 250 nm, ε 8.8×10^3
4-Azidobenzoic acid	λ_{max} 269 nm, ε 1.8×10^4
2-Azido-4-nitrobenzoic acid	λ_{max} 250 nm, ε 1.8×10^4
	λ_{max} 330 nm, ε 4.1×10^3
2-Nitro-5-azidobenzoic acid	λ_{max} 301 nm, ε 9.5×10^3

All acids showed $\nu_{as}N_3$ band in the ir spectra at 2100 cm^{-1}.

*Preparation of azidobenzoic and azidonitrobenzoic esters of 25-hydroxyvitamin D_3 (**1–4**).* Esters **1–4** were obtained by the improved acid chloride method described by Nielsen and co-workers (*27*). Azidobenzoic acid (5×10^{-4} M, 81.5 mg, 0.5 mM) or azidonitrobenzoic acid (104 mg, 0.5 mM) was dissolved with stirring in thionyl chloride (0.5 ml). To this solution anhydrous Na_2CO_3 (0.3 g) was added and the resulting suspension was magnetically stirred at 75°C for 30 min. The mixture was allowed to cool to room temperature and clear solution was decanted. Sodium carbonate was washed with benzene (3 × 1 ml) and solvent was removed under reduced pressure. The excess thionyl chloride was distilled off with benzene (2 × 5 ml). The acid chloride thus obtained was dissolved in 1 ml of anhydrous tetrahydrofuran and added in one portion to a stirred solution of alcohol **9** (20 mg, 50 μM) in 1 ml of anhydrous tetrahydrofuran containing 0.2 ml of redistilled triethylamine. Stirring was continued for an additional 2–4 h until none or very little of the starting alcohol was detected by TLC (solvent system B). The

[2] After the experiments were completed, Fluka Chemical Company introduced 2-nitro-5-azidobenzoic acid. However, none of the spectroscopic data presented here has been previously reported.

mixture was then diluted with ethyl ether (20 ml) and washed with diluted $NaHSO_4$ (10 ml), $NaHCO_3$ (2 × 15 ml), and saturated NaCl (3 × 20 ml) until neutralized. The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. The oily residue was dissolved in 3% 2-propanol in hexane (5 ml) and filtered through a silica Sep-Pak cartridge followed by elution of the cartridge with 30 ml of the same solvent mixture. Esters **1–4** were purified by preparative HPLC using solvent system B at a flow rate of 4 ml/min and solvent system A at a flow rate of 2 ml/min for compounds **1–3** and **4**, respectively. Retention volumes (R_V) of esters **1**, **2**, **3**, and **4** were 11, 11, 14, and 20 ml, respectively (solvent system B).

(**1**) uv (EtOH) λ_{max} 254 nm, ε 2.1 × 10^4; MS, *m/e* 545 M^+ (4), 382 (24), 253 (5), 59 (32).

(**2**) uv (EtOH) λ_{max} 271 nm, ε 2.6 × 10^4; MS, *m/e* 545 M^+ (1), 382 (17), 253 (5), 59 (27).

(**3**) uv (EtOH) λ_{max} 255, ε 2.0 × 10^4 and λ_{max} 340 nm, ε 1.6 × 10^3; MS, *m/e* 590 M^+ (18), 382 (100), 253 (24), 59 (51).

(**4**) uv (EtOH) λ_{max} 268 nm, ε 2.3 × 10^4; MS, *m/e* 590 M^+ (100), 382 (91), 253 (23), 59 (38).

*Tosylation of hydroxyvitamins D_3 (**8**, **10**, and **12**).* To a stirred solution of 2.5 nM vitamin D (**7**, **9**, or **11**) in 0.2 ml of dry pyridine was added 1.3 mg (7 nM) of freshly recrystallized *p*-toluenesulfonyl chloride. The reaction mixture was stirred at 4°C until no starting material was detected (20–24 h). The mixture was then poured over saturated $NaHCO_3$ and stirred with ice for 30–60 min to decompose the excess tosyl chloride. The suspension was extracted with ethyl ether and combined organic extracts were washed with diluted HCl, $NaHCO_3$, H_2O, saturated $CuSO_4$, and saturated NaCl and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was dissolved in 3% 2-propanol in hexane and filtered through a silica Sep-Pak cartridge. Analytical samples of tosylates **10** and **12** were obtained by preparative HPLC.

(**8**) TLC, system A, R_f 0.68; uv (EtOH) λ_{max} 265 nm.

(**10**) TLC, system A, R_f 0.48; HPLC, system A, R_V = 28 ml; uv (EtOH) λ_{max} 265 nm.

(**12**) TLC, system B, R_f 0.40; HPLC, system C, R_V = 82 ml, uv (EtOH) λ_{max} 265 nm; MS, *m/e* 298 $[M\text{-}TsOH]^+$ (1), 380 $[M\text{-}TsOH\text{-}H_2O]^+$ (15), 251 (7), 155 (21), 59 (83), 43 (100).

*(3R)-3-Deoxy-3-azidovitamin D_3 (**5**, **6**, and **13**).* A warm solution of 1.3 mg (20 nM) of sodium azide in 0.2 ml of DMSO was added with stirring to a solution of 20 nM hydroxyvitamin D_3 tosylate (**8**, **10**, and **12**) in 0.1 ml of DMSO. The resulting solution was stirred at 35–45°C for 5–15 h until no starting material was detected by TLC. The reaction mixture was diluted with water and the products were extracted with Et_2O. The organic phase was washed with H_2O and saturated NaCl and dried over $MgSO_4$. The solvent was evaporated under reduced pressure, and the residue was redissolved in 3% 2-propanol in hexane and purified by silica Sep-Pak filtration. Azidovitamins **5**, **6**, and **13** were separated from elimination products **14**, **15**, and **16**, respectively, by silica gel HPLC.

(**13**) TLC, system C, R_f 0.55; HPLC, system E, R_V = 21 ml; MS, *m/e*, 409 M^+ (19), 381 $[M\text{-}N_2]^+$ (23), 366 (63), 253 (19), 118 (45), 41 (100); ir [cm^{-1}], 2097.74 ($\nu_{as}N_3$), 1255.75 ($\nu_{sym}N_3$); uv (hexane) λ_{max} 265 nm; NMR δ 0.57 (3H, s, 18-H_3), 0.87 (6H, d, J = 7.0 Hz, 26,27-H_3), 3.7 (1H, m, 3-H), 4.82 (1H, m, 19(*Z*)-H), 5.09 (1H, m, 19(*E*)-H), 6.05 (1H, d, J = 11 Hz, 7-H), 6.22 (1H, d, J = 11 Hz, 6-H).

(**14**) TLC, system C, R_f 0.66; HPLC, system E, R_V = 11 ml; uv (hexane), λ_{max} 305, ir [cm^{-1}], 1625 ($\nu_{c=c}$).

(**5**) TLC, system A, R_f 0.60; HPLC, system D, R_V = 39 ml; uv (EtOH), λ_{max} 267 nm, 2.0 × 10^4; MS, *m/e* 425 M^+ (13), 397 $[M\text{-}N_2]^+$ (20), 382 $[M\text{-}HN_3]^+$ (13), 161 (19), 132 (44), 59 (89), 28 (100); ir [cm^{-1}], 2098.70 ($\nu_{as}N_3$), 1256.71 ($\nu_{sym}N_3$); NMR δ 0.56 (3H, s, 18-CH_3), 0.94 (3H, d, J = 6 Hz, 21-H_3), 1.12 (6H, s, 26,27-H_3), 3.6 (1H, m, 3-H), 5.02 (1H, m, 19(*Z*)-H), 5.14 (1H, m, 19(*E*)-H), 6.07 (1H, d, J = 11 Hz, 7H), 6.26 (1H, d, J = 11 Hz, 6H).

(**15**) TLC, system A, R_f 0.60; HPLC, system D, R_V = 37 ml; uv (EtOH) λ_{max} 301 nm, ε 2.2 × 10^4; MS, *m/e*, 382 M^+ (14), 364 (30), 251 (37), 59 (34), 41 (100).

(**6**) HPLC, system C, R_V = 22; uv (hexane), λ_{max} 265 nm; MS, *m/e* (intensity relative to *m/e* 380), 441 M^+ (14), 423 $[M\text{-}H_2O]^+$ (51), 413 $[M\text{-}N_2]^+$ (45), 398 $[M\text{-}HN_3]$ (20), 380 $[M\text{-}HN_3\text{-}H_2O]^+$ (100).

(**16**) HPLC, system B, R_V = 25 ml; uv (hexane), λ_{max} 308 nm.

Competitive Binding Assay

Chemicals. 25-OH-D_3 was a gift from the Upjohn Company (Kalamazoo, Mich.) and 1,25-$(OH)_2D_3$ was a gift from the Hoffmann–La Roche Company (Nutley, N.J.). 25-OH-[26,27-^{3}H]D_3 (160 Ci/mmol) (28) and 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol) (*29*) were from DuPont/New England Nuclear (Boston, Mass.). The concentration was determined by uv and a purity of greater than 90% was determined by HPLC.

Preparation of crude nuclear extract for receptor binding studies. Pig intestinal crude nuclear extract was prepared by a modified procedure used for chicken intestine (*9*). The small intestine from a 40- to 60-lb pig was washed with ice-cold TED buffer (50 mM Tris–HCl, 1.5 mM EDTA, 5 mM DTT, pH 7.4). The mucosa was scraped from the serosa and washed 3 times with 3 vol of TED buffer. A 30% tissue homogenate in this buffer was prepared with a Polytron homogenizer (Brinkman Instruments, Westburg, N.J.). The "crude nuclear pellet" was prepared by centrifuging this homogenate for 10 min at 4000*g*. The pellet was washed 3 times by resuspension and centrifugation with TED buffer. The crude nuclear extract was prepared by homogenization of the pellet in TED buffer containing 300 mM KCl (K_{300}) and 10 mM $MgCl_2$, followed by centrifugation for 90 min at 16,000*g*. The supernatant fraction containing receptor had a protein concentration of 2.3 mg/ml as determined by the method of Bradford (*30*) using crystalline bovine serum albumin as a standard. The crude nuclear extract was stored at −70°C.

To ensure the removal of any contaminating serum DBP from the receptor preparation, the extract was precipitated by the addition of solid ultrapure $(NH_4)_2SO_4$ (Schwarz/Mann, Cambridge, Mass.) to 40% saturation. After 1 h on

ice, the suspension was centrifuged at 9000*g* for 10 min. The pellet was resuspended in one-tenth of the initial volume with TED + K_{300}. The concentrated extract was shown to have no DBP by sucrose gradient analysis employing both 1,25-$(OH)_2$[26,27-^{3}H]D_3 and 25-OH-[26,27-^{3}H]D_3 as ligands. Stored at −70°C in aliquots, the extract retained a constant level of binding activity.

Assay. The same general procedure was used for competition studies with both receptor and DBP. For the receptor studies 0.07 nM 1,25-$(OH)_2$-[26,27-^{3}H]D_3 was used as the competing sterol and a freshly prepared 150- to 200-fold dilution in TED + K_{300} of concentrated crude nuclear extract was the source of receptor. For the DBP studies, 0.05 nM 25-OH-[26,27-^{3}H]D_3 was used as the competing sterol and a 1/5000 dilution of pig serum in TE with 150 nM NaCl was the source of DBP.

The tritiated sterol and various concentrations of the vitamin D compound were added in 10 μl of absolute ethanol to glass tubes (1.2 × 7.5 cm) followed by 0.25 ml of protein solution. The tubes were vortexed and incubated on ice overnight. The amount of tritiated sterol bound to the protein was determined by hydroxylapatite (HAP) assay (*31*). To each tube was added 0.25 ml of HAP (a 50% suspension in TED + K_{300}) and the samples were incubated on ice for 20 min. The HAP was pelleted by centrifugation and the pellets were washed with 2 × 1 ml of TE + 0.5% Triton X-100. The pellets were transferred to mini scintilation vials with 2 × 0.25 ml of 95% ethanol, and 3 ml of Scint A (United Technologies) was added to each vial. The radioactivity was determined by liquid scintillation counting in a Prias PLD Tri-Carb minivial counter (Packard, Downers Grove, Ill.).

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