Bioorganic & Medicinal Chemistry Letters, Vol.3, No.9, pp. 1809-1814, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00
Pergamon Press Ltd

FLUORESCENCE-LABELING REACTION OF VITAMIN D METABOLITES AND ANALOGS WITH FLUORESCENT 1,2,4-TRIAZOLINE-3,5-DIONE (DMEQ-TAD)

Masato Shimizu, Toshie Yamazaki, and Sachiko Yamada*

Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, 2-3-10 Surugadai Kanda, Chiyoda-ku, Tokyo 101, JAPAN

(*Received* 17 *January* 1993)

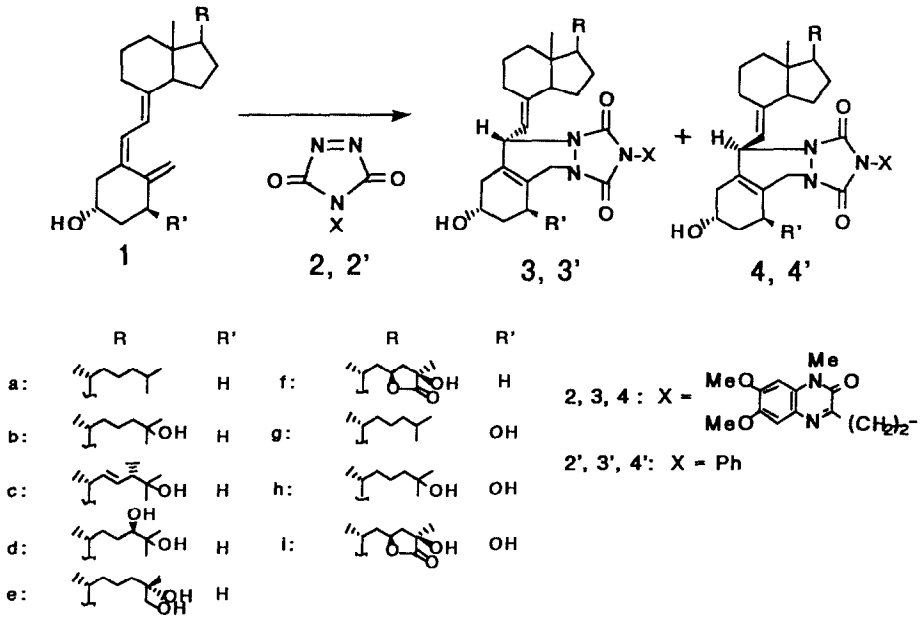
Abstract: To develop a fluorometric method for assaying vitamin D metabolites and synthetic analogs, the reaction of major vitamin D_3 metabolites and synthetic analogs with fluorescent dienophile (DMEQ-TAD) was studied, and the stereochemical structures of the resulting fluorescent adducts were determined on the basis of the CD spectra.

Besides the active vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, **1h**), a number of vitamin D_3 metabolites are known to be present in normal human plasma.[1] However biological importance of those metabolites has not been clearly understood, even though the plasma concentrations of many of them are higher than that of the active metabolite. It is important for clear understanding of the role of vitamin D_3 to know accurately the concentration of all major metabolites in human plasma in normal and various clinical situations. However a general, accurate, and highly sensitive method which can quantify all major vitamin D metabolites has not been known.[2]

HPLC-fluorometry is a sensitive method; theoretically 10^{-15}-10^{-18} mol of materials are detectable depending on the fluorogenic chromophore. However the method has not been successfully applied to the assay of plasma vitamin D metabolites.[3] Limitation of this method arises from the difficulty with which a minute amount (10^{-12}-10^{-15} mol) of substrate must be efficiently fluorescence-labeled by a chemical reaction. The need for a convenient, accurate, and highly sensitive assay method applicable to all vitamin D metabolites has prompted us to develop a novel fluorescence-labeling reagent for the vitamin. Our idea is that if a reagent targets the conjugated triene function of vitamin D the labeling reaction would be specific for vitamin D and regardless of the structure of the vitamin D only one to one product is possible by the labeling. We designed and synthesized dienophile bearing a highly fluorescent chromophore, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD, **2**),[4] and the reagent was successfully used to assay 25-OHD_3 and 24,25-$(OH)_2D_3$ in plasma .[5] This paper reports the reaction of DMEQ-TAD with all major vitamin D metabolites and some of synthetic analogs, and the determination of the stereochemistries of the products.

The reaction of vitamin D_3 (**1a**) and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, **2'**) has been reported to give two C(6)-epimeric cycloadducts (Scheme 1);[6] major (6*S*)-adduct (**3'a**) and minor (6*R*)-adduct (**4'a**), the structures of which were determined by an X-ray analysis.[7] We examined the reaction of DMEQ-TAD (**2**) with vitamin D_3 (**1a**), its major metabolites, 25-OHD_3 (**1b**); 24*R*,25-$(OH)_2D_3$ (**1d**); 25S,26-$(OH)_2D_3$ (**1e**);[8] 25*R*-OHD_3 26,23*S*-lactone (**1f**);[9] 1,25-$(OH)_2D_3$ (**1h**); 1,25*R*-$(OH)_2D_3$ 26,23*S*-lactone (**1i**),[10] and analogs, 25-OHD_2 (**1c**); 1α-OHD_3 (**1g**). The reaction was conducted under a variety of conditions including extreme

conditions in a supposition of labeling of plasma metabolites (Table I). The reaction of the vitamins with DMEQ-TAD proceeded in high yields to give pairs of C(6) epimeric adducts (3 and 4) (Scheme 1).



Scheme 1.

The stereochemistries at C(6) of the DMEQ-TAD adducts (3 and 4) were determined by the CD spectra. A pair of D_3-PTAD adducts (**3'a** and **4'a**) shows distinct property in their CD spectra. (6*S*)-D_3-PTAD adduct (**3'a**) shows strong positive cotton effect at 206 nm, while the 6*R*-isomer (**4'a**) exhibits negative one at 207 nm. This contrasting behavior in the CD spectra can be explained by the exiton chirality theory.[11] According to a force field calculation,[12] the most stable conformations around the C(6) of the PTAD adducts (**3'a** and **4'a**) are as shown in Figure 1. In the adducts (**3'a** and **4'a**), the two homoconjugated vinyl chromophores are arranged to produce a positive and negative chirality, respectively, and these are consistent with the sign of the first cotton effect of the compounds. Similarly contrasting cotton effects have been observed in other vitamin D 6,19-cycloadducts such as vitamin D-singlet oxygen adducts[13] and vitamin D-sulfur dioxide adducts[14] and the relationship between the sign of the cotton effect and the configuration at C(6) was identical with the present examples. Thus it is evident that the configuration of a series of vitamin D 6,19-cycloadducts can be determined by the CD spectra of the homoconjugated diene chromophore. In the DMEQ-TAD adducts (3 and 4), an additional weaker cotton effect due to the DMEQ-TAD moiety was observed at longer wavelength region. But the intense cotton effect of the diene part at the shorter wavelength region was little affected by that chromophore. We assigned the stereochemistry of the DMEQ-TAD adducts on the basis of the sign of the cotton effect around 205 nm: the isomer which showed a positive cotton effect was assigned as 6*S*-epimer and that with a negative one as 6*R*- epimer. The assignments were further supported by their ^{1}H NMR spectra and the behavior on HPLC (Table II).

Table I. Reaction of vitamin D with DMEQ-TAD[a]

entry	comp.	reagent	conditions			products		
			solv.	conc.	temp.	6*S*	6*R*	ratio (*S*: *R*)[b]
1[c]	**1a**	**2'**	CH_2Cl_2	10^{-1} M	0°	**3'a**	**4'a**	3:1
2	**1a**	**2**	CH_2Cl_2	10^{-4} M	rt	**3a**	**4a**	2.0:1
3	"	"	"	"	-78°	"	"	2.1:1
4	"	"	AcOEt	"	rt	"	"	2.8:1
5	"	"	"	"	-78°	"	"	2.8:1
6	**1g**	"	CH_2Cl_2	"	rt	**3g**	**4g**	1:1.1
7	"	"	"	"	-78°	"	"	1:2.0
8	"	"	AcOEt	"	rt	"	"	1:1.4
9	"	"	"	"	-78°	"	"	1:1.6
10	**1b**	"	CH_2Cl_2	10^{-8} M	rt	**3b**	**4b**	2:1
11	"	"	AcOEt	"	"	"	"	3:1
12	**1d**	"	CH_2Cl_2	"	"	**3d**	**4d**	2:1
13	"	"	AcOEt	"	"	"	"	3:1
14	**1h**	"	CH_2Cl_2	"	"	**3h**	**4h**	1:1.1
15	"	"	AcOEt	"	"	"	"	1:2
16	**1b**	"	CH_2Cl_2	prep.[d]	rt	**3b**	**4b**	2:1
17	"	"	DMF	"	"	"	"	3:1
18	**1c**	"	CH_2Cl_2	"	"	**3c**	**4c**	2:1
19	**1d**	"	"	"	"	**3d**	**4d**	2:1
20	"	"	DMF	"	"	"	"	4:1
21	**1e**	"	CH_2Cl_2	"	"	**3e**	**4e**	2:1
22	**1f**	"	"	"	"	**3f**	**4f**	2:1
23	**1h**	"	"	"	"	**3h**	**4h**	1:1.1
24	"	"	DMF	"	"	"	"	1:2.4
25	**1i**	"	CH_2Cl_2	"	"	**3i**	**4i**	1:1.1

[a] A solution of vitamin D (1) and DMEQ-TAD (2) (1.2 equivalents at $>10^{-4}$ M substrate concentration or 2000-10000 equivalents at 10^{-8} M) in the indicated solvent was stirred at the indicated temperature for 60 min, MeOH was added, the solvent was evaporated, and the residue was purified (SiO_2 column or HPLC) or analyzed (HPLC). The total yields of the adducts are >90% except for entries 22 and 25 (60-70%). [b] The ratio was determined by HPLC. [c] See ref. 6. [d] Preparative scale experiments with 10^{-2}-10^{-3} M substrate concentration.

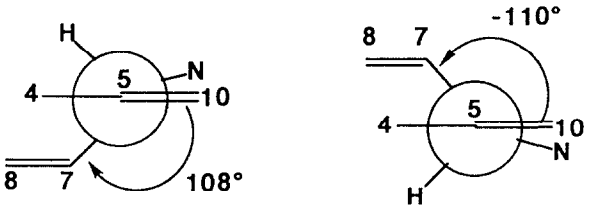


Fig. 1.

Table II. Selected spectral data of vitamin D PTAD and DMEQ-TAD adducts

comp.	CD[a]	^{1}H NMR ($CDCl_3$) δ (*J* = *Hz*)				HPLC[b]
	nm (Δε)	Me(18)	H-4α	H-6	-CH_2-$\dot{C}$=N	
3'a	206 (+46)	0.51	-[c]	4.78, d (9.9)	-	slow
4'a	207 (-55)	0.54	2.42, d (16.1)	4.77, d (9.6)	-	fast
3a	206 (+46), 223 (-11)	0.52	-[c]	4.69, d (9.2)	3.20, m	slow
4a	207 (-140), 247 (+10)	0.51	2.37, d (16.1)	4.67, d (9.9)	3.22, t (6.8)	fast
3b	206 (+46), 224 (-10)	0.53	-[c]	4.70, d (9.9)	3.20, m	slow
4b	206 (-82), 245 (+6)	0.51	2.37, d (16.5)	4.67, d (10.2)	3.22, t (6.6)	fast
3d	206 (+48), 223 (-12)	0.54	-[c]	4.70, d (9.9)	3.20, m	slow
4d	205 (-58), 245 (+4)	0.52	2.38, d (16.5)	4.67, d (9.9)	3.22, t (6.3)	fast
3g	206 (+38), 227 (-9)	0.50	2.22, d (14.5)	4.66, d (9.6)	3.20, m	slow
4g	207 (-74), 243 (+4)	0.49	2.46, d (12.9)	4.70, d (9.6)	3.25, t (6.6)	fast
3h	206 (+44), 225 (-10)	0.52	2.25, d (16.4)	4.67, d (9.6)	3.20, m	slow
4h	205 (-80), 243 (+4)	0.51	2.47, d (14.5)	4.71, d (9.6)	3.22, t (6.6)	fast

[a] CD spectra were measured in MeOH (Ca. 10^{-5} M) with 1 or 0.5 cm cell. [b] TSK gel ODS-80TM, MeOH-H_2O. [c] Superimposed with other methylene protons.

The proportion of the two epimeric adducts (3 and 4) produced by the reaction of vitamin D with DMEQ-TAD depends on the solvent used and the A-ring structure. In CH_2Cl_2 at room temperature, the vitamin D compounds bearing no 1α-hydroxyl group gave two adducts, 6S- and 6R-isomers (3 and 4), in 2:1 ratio. The isomers which were produced by the upper (β) face attack of the reagent were predominant in accord with the reaction with PTAD.[6] The β-face selectivity was higher in more polar solvents like AcOEt (3:1) and DMF (3-4:1). Introduction of an 1α-hydroxyl group hinders the β-face attack improving the production of the 6*R*-isomer (**4**). In these cases, the increment of the solvent polarity favors the formation of the α-face adducts (**4**) (entries 15 and 24).

It is rather surprising that the reaction of an extremely low concentration of vitamin D with large excess of the reagent (entries 10-15) proceeded quite efficiently (>90%), since TADs also act as an enophile, a mild oxidant, and a Michael acceptor.[15] The fact that the fluorescence-labeling reaction produces two isomers seems to be disadvantageous for the sensitivity of the assay. But it turns out to be rather advantageous in identifying vitamin D metabolites among millions of compounds in biological fluid. DMEQ-TAD labeled vitamin D metabolites can be detected as characteristic twin peaks. So they can be identified by the retention times and the ratio of the two peaks. The reagent may also be useful in scrutinizing novel vitamin D compounds in biological fluids.

REFERENCES AND NOTES

1. a) Seamark, D. A.; Trafford, D. J. H.; Makin, H. L. J. *J. Steroid Biochem.* **1981**, 14, 111-123. b) Shepard, R. M.; Horst, R. L.; Hamstra, A. J.; DeLuca, H. F. *Biochem. J.* **1979**, 182, 55-69. c) Belsey, R. E.; DeLuca, H. F.; Potts, J. T. *J. Clin. Endocrinol. Metab.* **1971**, 3, 554-557. d) Taylor, C. M.; Hughes, S. E.; de Silva, P. *Biochem. Biophys. Res. Commun.* **1976**, 70, 1243-1249. e) Horst, R. L.; Shepard, R. M.; Jorgensen, N. A.; DeLuca, H. F. *J. Lab. Clin. Med.* **1979**, 93, 277-285.
2. There are five main methods used to quantify vitamin D metabolites. i) Radio receptor assay:[2a,b,1a,c] the only reliable method to assay $1,25\text{-}(OH)_2D_3$. ii) Competitive binding method using serum vitamin D binding protein:[1b,c] the method generally used to assay other major metabolites such as $25\text{-}OHD_3$, $24R,25\text{-}(OH)_2D_3$, and $25,26\text{-}(OH)_2D_3$. These saturation analyses are sensitive enough to assay all major metabolites but they especially the latter have drawback in the accuracy since any compounds that compete with the radio labeled standard may be quantified as the compound under assay. iii) Radio-immunoassay (RIA).[2c,d] A highly specific RIA method for vitamin D metabolites has not been established, because of the difficulties with which an antibody specific for one particular metabolite is prepared. iv) HPLC-UV method.[1b] v) Gas chromatography mass spectrometric method.[2e-h] These physicochemical methods are accurate, but the sensitivity of the methods is not high enough to quantify most of the metabolites. a) Reinhardt, T. A.; Horst, R. L.; Orf, J. W.; Hollis, B.W. *J. Clin. Endocrinol. Metab.* **1984**, 58, 91-98. b) Reinhardt, T. A.; Hollis, B. W. In *Methods in Enzymology*; Chytil, F.; McCormick, D. B. Eds.; Academic Press, San Diego, 1986, Vol. 123, pp. 176-185. c) Peacock, M.; Taylor, G. A.; Brown, W. *Clin. Chim. Acta.* **1980**, 101, 93-101. d) Gray, T. K.; McAdoo, T. *Clin. Chem.* **1983**, 29, 196-200. e) Coldwell, R. D.; Porteous, C. E.; Trafford, D. J. H.; Makin, H. L. J. *Steroids* **1987**, 49, 155-196. f) Coldwell, R. D.; Trafford, D. J. H.; Makin, H. L. J.; Varley, M. J.; Kirk, D. N. *Clin. Chem.* **1984**, 30, 1193-1198. g) Lisboa, B. P.; Halket, J. M. In *Recent Development in Chromatography and Electrophoresis*; Frigerio, A.; Renoz, L. Eds.; Elsevier, Amsterdam, 1979, pp. 141-162. h) Coldwell, R. D.; Trafford, D. J. H.; Varley, M. J.; Kirk, D. N.; Makin, H. L. J. *Steroids*, **1990**, 55, 418-432.
3. The only HPLC-FL method known for vitamin D metabolites uses dimethoxylquinoxalinone acyl azide which reacts after thermally converted to the corresponding isocyanate. This method has serious drawbacks: the fluorescence-labeling conditions (80 °C, 80 min) are so vigorous as to cause isomerization; since the reagent targets hydroxyl groups complex mixtures are expected in the labeling of polyhydroxylated metabolites. Iwata, T.; Yamaguchi, M.; Hanazono, H.; Imazato, Y.; Nakamura, M.; Ohkura, Y. *Anal. Sciences* **1990**, 6, 361-366.
4. a) Shimizu, M.; Takahashi, T.; Uratsuka, S.; Yamada, S. *J. Chem. Soc., Chem. Commun.* **1990**, 1416-1417. b) Shimizu, M.; Kamachi, S.; Nishii, Y.; Yamada, S. *Anal. Biochem.* **1991**, 194, 77-81.

5. Shimizu, M.; Gao, Y.; Aso, T.; Nakatsu, K.; Yamada, S. *Anal. Biochem.* **1992**, 204, 258-264.
6. a) Aberhart, D. J.; Hsu, A. C-T. *J. Org. Chem.* **1976**, 41, 2098-2102. b) Reischl, W.; Zbiral, E. *Liebigs Ann. Chem.* **1978**, 745-756.
7. a) Kratky, C.; Reischl, W.; Altmann, E.; Zbiral, E. *Monatsh. Chem.* **1982**, 113, 439-448. b) Reischl, W.; Kratky, C.; Sheldrick, G. M.; Zbiral, E. *Monatsh. Chem.* **1989**, 120, 1165-1173.
8. Suda, T.; DeLuca, H. F.; Schnoes, H. K.; Tanaka, Y.; Holick, M. F. *Biochemistry*, **1970**, 9, 4776-4780. Ikekawa, N.; Koizumi, N.; Ohshima, E.; Ishizuka, S.; Takeshita, T.; Tanaka, Y.; DeLuca, H. F. *Proc. Natl. Acad. Sci. USA* **1983**, 80, 5286-5288.
9. Wichmann, J. K.; DeLuca, H. F.; Schnoes, H. K.; Horst, R. L.; Shepard, R. M.; Jorgensen, N. A. *Biochemistry*, **1979**, 18, 4775-4780. Yamada, S.; Nakayama, K.; Takayama, H. *Tetrahedron Lett.* **1981**, 22, 2591-2594. Yamada, S.; Nakayama, K.; Takayama, H. *Chem. Pharm. Bull.* **1981**, 29, 2393-3198.
10. Ohnuma, N.; Bannai, K.; Yamaguchi, H.; Hashimoto, Y.; Norman, A. W. *Arch. Biochem. Biophys.* **1980**, 204, 387-391. Tanaka, Y.; Wichmann, J. K.; Paaren, H. E.; Schnoes, H. K.; DeLuca, H. F. *Proc. Natl. Acad. Sci. USA.***1980**, 77, 6411-6414. Yamamoto, K.; Shimizu, M.; Yamada, S.; Iwata, S.; Hoshino, O. *J. Org. Chem.* **1992**, 57, 33-39.
11. Crabbe, P. *ORD and CD in Chemistry and Biochemistry;* Academic Press: New York/London, 1972. Harada, N.; Nakanishi, K. *Acc. Chem. Res.* **1972**, 5, 257-263. Harada, N.; Chen, S.-m. L.; Nakanishi, K. *J. Am. Chem. Soc.* **1975**, 97, 5345-5352.
12. MMX force field calculation (Gajewski, J. J.; Gilbert, K. E.; McKelvey, J. *Adv. Mol. Model.* **1990**, 2, 65-92) was carried out using PCMODEL (Serena Software, Bloomington).
13. Yamada, S.; Nakayama, K.; Takayama, H. *Tetrahedron Lett.* **1978**, 4895-4898. Yamada, S.; Nakayama, K.; Takayama, H.; Itai, A.; Iitaka, Y. *Chem. Pharm. Bull.* **1979**, 27, 1949-1950.Yamada, S.; Nakayama, K.; Takayama, H.; Itai, A.; Iitaka, Y. *J. Org. Chem.* **1983**, 48, 3477-3483.
14. Yamada, S.; Takayama, H. *Chemistry Lett.* **1979**, 583-586. Yamada, S.; Suzuki, T.; Takayama, H. *Tetrahedron Lett.* **1981**, 22, 3085-3088. Yamada, S.; Takayama, H. Miyamoto, K.; Matsunaga, I.; Nawata, Y. *J. Org. Chem.* **1983**, 48, 3483-3488.
15. Pirkle, W. H.; Stickler, J. C. *J. Chem. Soc., Chem. Commun.* **1967**, 760-761. Wilson, R. M.; Hengge, A. C.; Ataei, A.; Chantarasiri, N. *J. Org. Chem.* **1990**, 193-197. Squillacot, M.; Mooney, M.; Felippis, J. D. *J. Am. Chem. Soc.* **1990**, 112, 5364-5365. Clennan, E. L.; Koola, J. J.; Oolman, K. A. *Tetrahedron Lett.* **1990**, 31, 6759-6762. Orfanopoulos, M.; Smonou, I.; Foote, C. S. *J. Am. Chem. Soc.* **1990**, 112, 3607-3614. Cookson, R. C.; Stevens, I. D. R.; Watts, C. T. *J. Chem. Soc., Chem. Commun.* **1966**, 744. Dao, L. H.; Mackay, D. *J. Chem. Soc., Chem. Commun.* **1976**, 326-327.