Letters to the Editor

Dienophilic Reagent for Precolumn Derivatization of 7-Dehydrocholesterol in High Performance Liquid Chromatography¹**Kazutake SHIMADA and Tomoyuki OE***Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan***Keywords** High performance liquid chromatography, ultraviolet detection, fluorescence detection, precolumn derivatization reagent, dienophile, 4-(1-pyrenyl)-1,2,4-triazoline-3,5-dione, 7-dehydrocholesterol

Over the last decades, interest in the metabolism and physiological action of vitamin D₃ (VD₃) has increased exponentially. This compound is metabolized to 25-hydroxy-VD₃ in the liver and then to 1,25-dihydroxy-VD₃ in the kidney to produce biological activity.² It is well known that the 7-dehydrocholesterol (7-DHC) present in the skin is converted into VD₃ via pre-VD₃.³ Much interest has recently been focused on the correlation between age and the 7-DHC concentration level in human skin.⁴ Iwata *et al.*^{5,6} converted 7-DHC into the fluorescent derivative by a reaction with 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl azide; the determination was made by high performance liquid chromatography (HPLC). This method is sufficiently sensitive for this purpose, though it takes 60 min at 120°C to convert the alcohol into the desired urethane derivative.

The present paper deals with the preparation of a sensitive, highly reactive reagent having a pyrene moiety as a chromophore or fluorophore and a triazolinedione as a reacting group toward the diene moiety, and its applicability to the derivatization of 7-DHC for HPLC.

Experimental*Chemicals*

Pyrene-1-carboxaldehyde and carbazic acid ethyl ester were obtained from Wako Pure Chemical Co. (Osaka, Japan) and Tokyo Kasei Co. (Tokyo, Japan), respectively. Other chemicals used were also commercially available and of reagent grade.

Apparatus

Proton nuclear magnetic resonance (¹H-NMR) spectra were obtained with a JEOL JNM-FX 100S spectrometer (JEOL Co., Tokyo) at 100 MHz using tetramethylsilane as an internal standard. The abbreviations used are s=singlet, t=triplet, q=quartet, dd=doublet of doublets, m=multiplet and br=broad. Mass spectra

(MS) were measured on a Hitachi M-80 spectrometer (Hitachi Ltd., Tokyo). Infrared (IR) spectra were recorded with a JASCO A-202 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo).

HPLC was carried out on a Shimadzu LC-6A chromatograph equipped with a Shimadzu SPD-6AV UV (240 nm)(Shimadzu Co., Ltd., Kyoto, Japan) or a Hitachi F-1050 fluorescence detector (Ex 270 nm, Em 370 nm) was used. A YMC-GEL C₈-120-S5 (5 μm) column (15 cm×0.46 cm i.d.)(YMC Co., Kyoto) was used with CH₃CN as a mobile phase under an ambient temperature at a flow rate of 1 ml/min.

Synthesis of 4-(1-pyrenyl)-1,2,4-triazoline-3,5-dione (V)

The compound was synthesized according to a procedure described by Cookson *et al.*⁷ Pyrene-1-carbonyl azide (I)⁸(765 mg) in toluene (15 ml), prepared from pyrene-1-carboxaldehyde via pyrene-1-carboxylic acid⁹, was heated at 100°C to give pyrene-1-isocyanate (II). The reaction mixture was added to a solution of carbazic acid ethyl ester (308 mg) in benzene (15 ml) under ice-cooling. The reaction mixture was kept at room temperature for 1 h and then at 100°C for 1 h. After filtration, the obtained precipitate was washed with benzene and dried *in vacuo* to give 4-(1-pyrenyl)-1-carbethoxysemicarbazide (III) as a colorless amorphous substance (803 mg). mp>300°C. IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3275, 1720, 1650, 1570.¹⁰ ¹H-NMR (DMSO-d₆) δ: 1.24 (3H, t, J=7.1 Hz, -CH₃), 4.11 (2H, q, J=7.1 Hz, -CH₂-), 7.70–8.40 (9H, m, pyrene-H). MS *m/z*: 347 (M⁺). A suspension of III (475 mg) was treated with 4 M KOH (7 ml) at 80°C for 1.5 h. The reaction mixture was acidified with conc. HCl, and the obtained precipitate was collected, washed with H₂O and dried *in vacuo*. The product was reprecipitated from dimethylformamide-H₂O to give 4-(1-pyrenyl)urazole (IV) as a colorless amorphous substance (377 mg). mp>300°C. IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3050, 1665, 1460.¹⁰ MS *m/z*: 301 (M⁺). *t*-Butyl hypochlorite (8.2 μl: 69 μmol) was added to the suspension of IV (19.5 mg: 65 μmol) in AcOEt (5 ml) under an N₂ gas stream, and the reaction mixture was

Fig. 1 Structures of the derivatization reagent, its synthetic intermediates and a derivatization reaction with 7-dehydrocholesterol.

stirred for 6 h at room temperature. The mixture was centrifuged to remove any unreacted IV; a dark-red supernatant containing 4-(1-pyrenyl)-1,2,4-triazoline-3,5-dione (V) was used for the derivatization. The solution was stable for at least 7 d in a refrigerator, which was confirmed by measuring the intensity of the UV absorption ($\lambda_{\text{max}}^{\text{AcOEt}}$ 537 nm).¹⁰ After evaporation of the solvent, V was obtained as a dark-red amorphous substance (15 mg). mp > 300°C. MS m/z : 299 (M^+).¹¹

Preparation of the authentic 7-dehydrocholesterol derivative

A solution of V in AcOEt was added to a solution of 7-DHC (12.5 mg) in AcOEt (2 ml) at room temperature until the red color of the excess reagent remained in the reaction mixture. The mixture was washed with 1 M NaOH, then H₂O, and finally dried over anhydrous Na₂SO₄ and evaporated *in vacuo*. The residue obtained was twice subjected to preparative thin-layer chromatography using benzene-AcOEt (3:2; R_f 0.40) and then CHCl₃-MeOH (100:2; R_f 0.30) as a developing solvent. The corresponding spot was eluted with AcOEt to give the desired adduct as a pale-yellow solid (14.3 mg). ¹H-NMR (CDCl₃) δ : 0.80 and 0.87 (each 3H, each s, 18- or 19-CH₃), 3.14 (1H, dd, J =2.9, 14.3 Hz, 9 α -H), 4.36 (1H, br s, 3 α -H), 6.14 and 6.33 (each 1H, each dd, J =8.6, 2.3 Hz, 6- or 7-H), 7.60–8.30 (9H, m, pyrene-H).

Results and Discussion

The design of a promising derivatization reagent requires structural features having both a group sensitive to a detector and functional group reactive toward the diene moiety. 4-Phenyl-1,2,4-triazoline-3,5-dione, a Cookson reagent⁷, has been used as a protecting group of the diene moiety for the synthesis of VD₃-related compounds.¹² The reagent gave a stable Diels-Alder adduct quantitatively under mild condi-

tions within a short time. These data prompted us to develop a derivatization reagent possessing pyrene and triazolinedione as a chromophore or fluorophore and a reacting group, respectively.

The desired derivatization reagent, 4-(1-pyrenyl)-1,2,4-triazoline-3,5-dione (V), was synthesized from pyrene-1-carbonyl azide (I) according to a known method.⁷ The reactivity of V (260 nmol/ml) was then investigated with 7-DHC (13 nmol/ml) in ethyl acetate under ice-cooling (Fig. 1). An aliquot of the resulting solution was applied to HPLC. The reaction rate was estimated by a comparison with the peak area of the standard derivative. Under the above-mentioned condition derivatization was completed within 5 min. The resulting adduct was responsive to both UV and fluorescence detectors with a detection limit of 30 fmol and 2.4 pmol (t_R =5.0 min: at signal to noise ratio=5), respectively. The adduct was stable at room temperature for at least 1 d.

The newly developed derivatization reagent having a dienophile for HPLC has proved to be satisfactory with respect to reactivity. The sensitivity of the adduct using the UV detector is much higher than that obtained with the fluorescence detector, the details of which are now under investigation in our laboratories. The use of this type of reagent is promising for the selective labeling of the cisoid diene moiety in 7-DHC, VD₃ and related compounds. The synthesis of this type of reagent having another chromophore or fluorophore and the application of the present method to the determination of bio-active substances in biological fluids will be presented in a future communication.

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References and Notes

1. This paper is dedicated to Professor Toshio Nambara (Tohoku University, Sendai, Japan) on the occasion of his retirement.
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