Journal of Chromatography, 606 (1992) 133–135
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 312

Short Communication

Sensitive and stable Cookson-type reagent for derivatization of conjugated dienes for high-performance liquid chromatography with fluorescence detection

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(First received March 24th, 1992; revised manuscript received April 24th, 1992)

ABSTRACT

A sensitive and stable Cookson-type reagent, 4-substituted 1,2,4-triazoline-3,5-dione, having 6-methoxy-2-phenylbenzoxazole as a fluorophore, was prepared for high-performance liquid chromatographic measurements of conjugated dienes. The reagent was purified by sublimation to give stable purple crystals. The reagent quantitatively produced the adduct with provitamin D_3 in 5 min under ice cooling, which was highly responsive to fluorescence detection [detection limit 2 fmol per injection (signal-to-noise ratio = 5)].

INTRODUCTION

In a previous paper we reported the preparation of Cookson-type reagents, 4-substituted 1,2,4-triazoline-3,5-diones, having a chromophore, fluorophore or electrophore at the 4-position, for high-performance liquid chromatographic (HPLC) measurements of vitamin D-related compounds having a conjugated diene [1,2]. The reactivity of these reagents and the properties of their adducts were examined by using provitamin D_3 (7-dehydrocholesterol; 7-DHC) as a model compound. The adduct with 4-[2-(1-pyrenyl)ethyl]-1,2,4-triazoline-3,5-dione was most responsive to detection {fluorescence (FL): detection limit 25 fmol per injection [signal-to-noise ratio (S/N) = 5]}. However, the reagent could not be purified by sublimation or recrystallization, so the reaction mixture of a precursor and an oxidizing agent was used as the derivatization reagent [1,2]. The reagent sometimes gave the problem in the derivatization of sulphated vitamin D metabolites that a deconjugation reaction occurred with the remaining oxidant or its degradation products [3].

This paper deals with the preparation and properties of a new Cookson-type reagent (**IV**) having 6-methoxy-2-phenylbenzoxazole as a fluorophore for the determination of conjugated dienes (Fig. 1).

EXPERIMENTAL

Materials

Ethyl hydrazinecarboxylate and *tert.*-butyl hypochlorite were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of analytical-reagent grade. For thin-layer chromatography

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0021-9673/92/$05.00

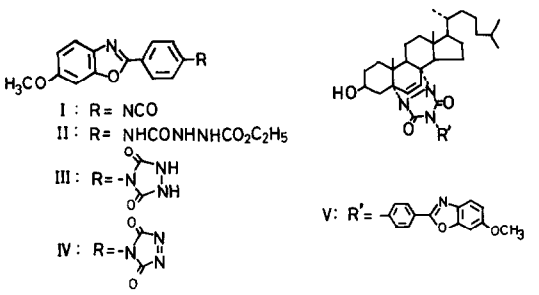

Fig. 1. Preparation of the Cookson-type reagent **IV** and its adduct with 7-DHC (**V**).

(TLC) silica gel HF_{254} precoated TLC plates (0.25 mm) (E. Merck, Darmstadt, Germany) were used and for silica gel column chromatography silica gel 60 (70–230 mesh) (E. Merck) was used.

Apparatus

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a Jeol (Tokyo, Japan) JNM-EX 270 spectrometer at 270 MHz using tetramethylsilane as an internal standard. The abbreviations used are s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet and br = broad. Electron impact ionization mass spectrometry (EI-MS) was carried out on a Hitachi (Tokyo, Japan) M-80 spectrometer.

HPLC was carried out on a Shimadzu (Kyoto, Japan) LC-6A chromatograph equipped with a Hitachi F-1050 FL detector ($\lambda_{ex.}$ 320 nm, $\lambda_{em.}$ 380 nm). A YMC-Gel C_8-120-S5 (5 μm) column (15 x 0.46 cm I.D.) (YMC, Kyoto, Japan) was used at ambient temperature at a flow-rate of 1.0 ml/min.

Preparation of the Cookson-type reagent

Compound II. 4-(6-Methoxy-2-benzoxazolyl) phenyl isocyanate (**I**), prepared according to the procedure described by Kondo *et al.* [4], was treated with ethyl hydrazinecarboxylate as described previously [2] to give the desired ethoxycarbonylsemicarbazide derivative (**II**) as a colourless amorphous substance (xylene): m.p., 218–220°C; ^{1}H NMR [C^2HCl_3–$C^2H_3O^2H$ (1:1)], δ 1.31 (3H, t, J = 7.3 Hz, $-CH_2CH_3$), 3.90 (3H, s, $-OCH_3$), 4.22 (2H, q, J = 7.3 Hz, $-CH_2CH_3$), 6.97 (1H, dd, J = 2.3, 8.7 Hz, benzoxazole-5H), 7.16 (1H, d, J = 2.3 Hz, benzoxazole-7H), 7.57 (1H, d, J = 8.7 Hz, benzoxazole-4H), 7.63 and 8.09 (each 2H, each d, J = 8.9 Hz, phenyl-H); EI-MS, m/z 370 (M^+).

Compound III. Compound **II** (10 mg) was dissolved in ethanol–water (1:1, v/v) (2 ml) containing 2 *M* KOH and stirred at room temperature for 20 min. The reaction mixture was acidified with 5% HCl, the resulting pale yellow precipitate was filtered and the precipitate was washed with water and dried *in vacuo*. Its homogeneity was confirmed by TLC [solvent system chloroform–methanol–water (80:20:2.5, v/v/v); R_F 0.56]; m.p. >300°C; EI-MS, m/z 324 (M^+). The compound was subjected to the following oxidation reaction without further purification.

Compound IV. Compound **III** (4 mg) was suspended in ethyl acetate (1 ml) and treated with *tert.*-butyl hypochlorite (1.7 μl; 1.1 molar ratio) under ice cooling for 15 min. The reaction mixture was filtered to remove the unreacted precursor and the filtrate was evaporated *in vacuo*. The residue obtained was subjected to sublimation at 140°C (bath temperature) (0.2 mmHg) to give purple crystalls (1 mg): m.p., 148–150°C; EI-MS, m/z 322 (M^+); visible spectrum, λ_{max} ethyl acetate 528 nm [2]. The compound was stable for at least 1 month in a refrigerator.

Preparation of the authentic adduct (V) with 7-DHC

A solution of **IV** (5 mg) in ethyl acetate (0.5 ml) was added to a solution of 7-DHC (6 mg) in ethyl acetate (0.5 ml) and the reaction mixture was kept for 15 min under ice cooling. The mixture was applied to a silica gel column (10 x 0.6 cm I.D.) to decompose the excess of the reagent and the eluate was evaporated *in vacuo*. The residue obtained was subjected to preparative TLC using chloroform–ethyl acetate (1:1, v/v) as a developing solvent. The corresponding spot (R_F 0.36) was eluted with ethyl acetate to give the desired adduct (**V**, 4.5 mg) as a colourless amorphous substance (ethanol). ^{1}H NMR (C^2HCl_3), δ 3.20 (1H, dd, J = 4.3, 13.9 Hz, 9α-H), 3.89 (3H, s, $-OCH_3$), 4.46 (1H, br s, 3α-H), 6.26 and 6.43 (each 1H, each d, J = 8.3 Hz, 6,7-H), 6.96 (1H, dd, J = 1.9, 8.8 Hz, benzoxazole-5H), 7.12 (1H, d, J = 1.9 Hz, benzoxazole-7H), 7.64 (1H, d, J = 8.8 Hz, benzoxazole-4H), 7.69 and 8.25 (each 2H, each d, J = 8.3 Hz, phenyl-H).

Reactivity of IV with 7-DHC

A solution of an excess of the reagent (**IV**, about 20 equivalents) in ethyl acetate (0.1 ml) was added to a solution of 7-DHC (1 μg) in ethyl acetate (0.1 ml) under ice cooling. A portion of the reaction mixture was subjected to HPLC at various times. The rate of reaction was measured by comparing the peak area obtained with that of an authentic sample [2].

RESULTS AND DISCUSSION

The design of a useful derivatization reagent for conjugated dienes in HPLC–FL detection requires two structural features, *viz.*, a functional group reactive toward the conjugated dienes and a fluorophore responsive to FL detection with high sensitivity. In previous work we used 1,2,4-triazoline-3,5-dione as the functional group and anthracene or pyrene as a fluorophore, but the reagents obtained were not sensitive and stable as described above [1,2]. Subsequently, Shimizu *et al.* [5] and Jordan *et al.* [6] also reported the same type of reagent, but their reagents were not purified by recrystallization or sublimation.

Recently, Kondo *et al.* [4] and Naganuma *et al.* [7] reported 6-methoxy-2-phenylbenzoxazole as a sensitive fluorophore. These results prompted us to prepare a new Cookson-type reagent having this fluorophore at the 4-position of a 1,2,4-triazoline-3,5-dione, 4-[4-(6-methoxy-2-benzoxazolyl) phenyl]-1,2,4-triazoline-3,5-dione (**IV**), according to the procedure described previously [2]. The reagent was easily obtained from the known compound **I** and purified by sublimation to give stable crystals. The reactivity of the reagent was examined by using 7-DHC as a model compound and the reagent quantitatively produced the stable adduct **V** in 5 min under ice cooling as did other reagents of the same type [2]. The adduct **V** gave a single peak with the theoretical shape on HPLC and was highly responsive to FL detection [detection limit 2 fmol per injection (S/N = 5)] (Fig. 2).

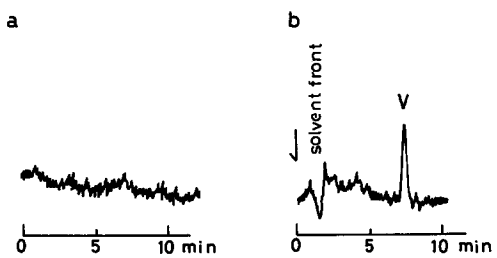

Fig. 2. HPLC of compound **V**. Solvent system: acetonitrile–water (9:1, v/v). For other conditions, see Experimental. (a) Blank chromatogram. (b) Compound **V** (1.3 fmol/in 10 μl of ethanol).

Although the Cookson reagent 4-phenyl-1,2,4-triazoline-3,5-dione was purified by sublimation [8], this is the first reported instance of a Cookson-type derivatization reagent having a sensitive fluorophore and being purified by sublimation. Studies of the application of this reagent to the determination of vitamin D_3 metabolites including conjugates are in progress.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan. We are indebted to Dr. Y. Kawahara (Sankyo, Tokyo) for valuable suggestions.

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