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# Synthesis and Properties of 4-Diazomethyl-7-methoxycoumarin as a New Fluorescent Labeling Reagent for Alcohols and Carboxylic Acids[1)]

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4-Diazomethyl-7-methoxycoumarin (DMC) was synthesized as a new fluorescent labeling reagent for hydroxyl and carboxyl compounds for use in high-performance liquid chromatography. Treatment of 4-formyl-7-methoxycoumarin with *p*-toluenesulfonohydrazide gave the tosylhydrazone in 87% yield, and this was derived to DMC in 78% yield by treatment with 0.2 N NaOH. DMC was practically nonfluorescent in solution and possessed excellent storage properties. DMC reacted with alcohols in dichloromethane at room temperature in the presence of $HBF_4$ as a catalyst to give the corresponding fluorescent coumarin ethers and also reacted with carboxylic acids in acetonitrile on heating to give the esters. DMC derivatives of cholestanol and cholesterol were satisfactorily separated by high-performance liquid chromatography. The present studies indicate that DMC is a useful fluorescent reagent for the labeling of alcohols and carboxylic acids.

**Keywords**——coumarin diazomethane; coumarin ether fluorescence; fluorescent labeling of alcohols; fluorescent labeling of carboxylic acids; HPLC of alcohols

The development of new highly sensitive and selective fluorescent labeling reagents is required in order to utilize the full potential of high-performance liquid chromatography (HPLC) with fluorescence detection. A number of reagents have been developed for the labeling of amino,[2)] carbonyl,[3)] carboxyl,[4)] and thiol[5)] groups. However, only a few reagents for alcoholic hydroxyl groups have been reported.[6)]

Thus, in order to obtain a new fluorescent labeling reagent for alcoholic hydroxyl groups, 4-diazomethyl-7-methoxycoumarin (DMC) (III) was synthesized and its properties as a labeling reagent were examined. Furthermore, as esterification of carboxyl groups with diazoalkanes is well known,[7)] the labeling of carboxylic acids with DMC was also examined.

## Results and Discussion

### Synthesis and Properties of DMC

The synthesis of DMC(III) was carried out as shown in Chart 1. Treatment of 4-formyl-7-methoxycoumarin(I)[8)] with *p*-toluenesulfonohydrazide (tosylhydrazine) gave the tosylhydrazone(II) in 87% yield, and this was derived to the desired DMC in 78% yield by treatment with 0.2 N sodium hydroxide according to Cava's method.[9)] The structure of DMC was confirmed as follows. DMC showed the infrared (IR) absorption band due to the azo group at 2070

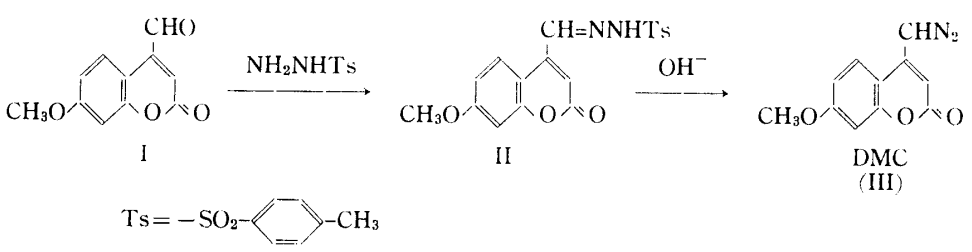
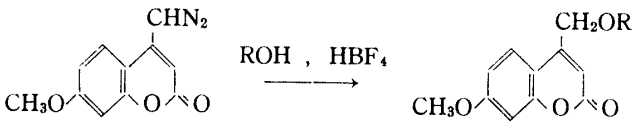



Chart 1

$cm^{-1}$ and nuclear magnetic resonance (NMR) signals due to methoxy, azomethin, and aromatic ($C_3$–H and $C_5$–H) protons at 3.85, 5.70, 6.43, and 7.65 ppm, respectively. These spectral data and elemental analysis data were consistent with expected structure of DMC. DMC was practically nonfluorescent in solvents such as acetonitrile, benzene, ethanol, and tetrahydrofuran (THF). It was stable in dichloromethane for a few days and in a desiccator for a few months.

**Labeling of Alcohols with DMC**

Methylation of alcohols with diazomethane catalysed by fluoboric acid has been carried out[10] and may be applicable to analytical procedures. DMC was found to react with alcohols at room temperature in the presence of fluoboric acid as a catalyst, giving highly fluorescent ethers, in our preliminary experiments. Chart 2 shows the labeling of different kinds of alcohol,




DMC(III)

IVa: R = $CH_3$
IVb: R = $CH_2CH_3$
IVc: R = $(CH_2)_3CH_3$
IVd: R = $CH(CH_3)_2$
IVe: R = $C(CH_3)_3$
IVf: R = $C_6H_{11}$
IVg: R = $C_{27}H_{47}$
IVh: R = $C_{27}H_{45}$
IVi: R = $CH_2C_6H_5$

Chart 2

*i.e.* primary, secondary, and tertiary alcohols. In order to find the most suitable reaction conditions, the labeling of cholesterol with DMC was examined in various solvents, dichloromethane, THF, and acetonitrile. The progress of the reactions was followed by measurement of the intensity at the fluorescence maximum (386 nm) of cholesteryl ether at regular intervals. The fluorescence intensities shown in Fig. 1 were measured in ethanol after isolation of cholesteryl ether from the reaction mixture in each solvent by thin-layer chromatography (TLC: benzene–ethyl acetate=4: 1). The fluorescence intensities reached the highest values in dichloromethane after 20 min, as shown in Fig. 1. The labeling of some other alcohols with DMC gave almost the same results as in the case of cholesterol.

Thus, the labeling of all alcohols with DMC was carried out under stirring in dichloromethane at room temperature for 30 min in the presence of fluoboric acid as a catalyst. The reaction yields and the fluorescent spectral data for the ethers (IVa—IVi) obtained are summarized in Table I. The structures of these ethers were confirmed by comparing their IR, NMR, and elemental analyses. As shown in Table I, the labeling with DMC gave satisfactory yields in the reactions with secondary and tertiary alcohols as well as primary alnohols. The linearity between the fluorescence intensities and the amounts of cholesterol in the reaction mixtures was maintained in the range of 10 to 100 nmol/ml, and the detection limit of cholestery ether was 500 pmol/ml (S/N=2). Figure 2 shows an example of an HPLC chromatogram of standard samples (IVg and IVh). The DMC derivatives were clearly separated.

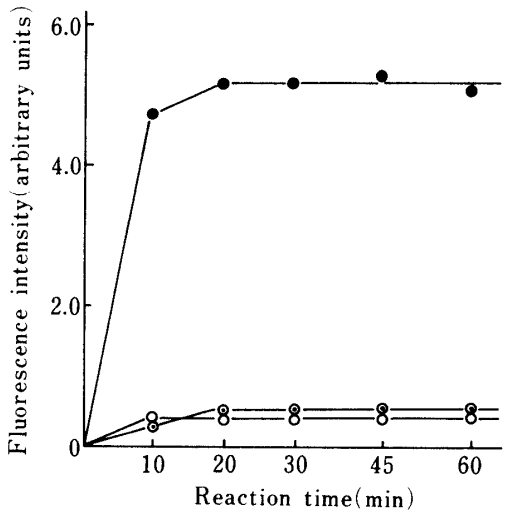
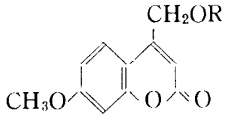



Fig. 1. Relationship between the Fluorescence Intensity of Cholesteryl Ether (IVh) and Reaction Time in Various Solvents

●, dichloromethane; ⊙, THF; ○, acetonitrile.

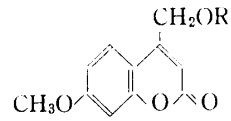
Table I. The Reaction Yields and Fluorescence Spectral Data of DMC Derivatives of Alcohols



| Compd. No. | R | Yield (%) | $F\lambda_{max}^{EtOH}$ nm[a] | Quantum yield |
|---|---|---|---|---|
| IVa | $CH_3$ | 61 | 386 | 0.074 |
| IVb | $CH_2CH_3$ | 63 | 384 | 0.088 |
| IVc | $(CH_2)_3CH_3$ | 74 | 386 | 0.098 |
| IVd | $CH(CH_3)_2$ | 46 | 386 | 0.082 |
| IVe | $C(CH_3)_3$ | 41 | 384 | 0.074 |
| IVf | $C_6H_{11}$ | 57 | 386 | 0.084 |
| IVg | $C_{27}H_{47}$ | 41 | 386 | 0.135 |
| IVh | $C_{27}H_{45}$ | 44 | 386 | 0.083 |
| IVi | $CH_2C_6H_5$ | 47 | 386 | 0.077 |

a) Excitation wavelength: 325 nm.

Table II.



| Compd. No. | R | mp (°C) | Formula | Analysis (%) Calcd (Found) C | H | NMR ($CDCl_3$) $\delta$ $C_3$-H | $C_4$-$CH_2$ | $C_5$-H | $C_7$-$OCH_3$ | R |
|---|---|---|---|---|---|---|---|---|---|---|
| IVa | $CH_3$ | 142—143 | $C_{12}H_{12}O_4$ | 65.44 (66.51 | 5.49 5.50) | 6.33 | 4.50 | 7.43 | 3.86 | 3.50($CH_3$) |
| IVb | $CH_2CH_3$ | 102—103 | $C_{13}H_{14}O_4$ | 66.65 (66.65 | 6.02 6.01) | 6.36 | 4.63 | 7.40 | 3.86 | 1.30($CH_2CH_3$) 3.65 |
| IVc | $(CH_2)_3CH_3$ | 74—75 | $C_{15}H_{18}O_4$ | 68.68 (68.34 | 6.92 6.79) | 6.40 | 4.62 | 7.46 | 3.87 | 0.70—1.85($C_4H_9$) 3.55 |
| IVd | $CH(CH_3)_2$ | 78—79 | $C_{14}H_{16}O_4$ | 67.73 (67.44 | 6.50 6.25) | 6.42 | 4.62 | 7.46 | 3.86 | 1.25 ($C_3H_7$) 3.50—4.00 |
| IVe | $C(CH_3)_3$ | 79—80 | $C_{15}H_{18}O_4$ | 68.68 (68.50 | 6.92 6.92) | 6.46 | 4.60 | 7.43 | 3.86 | 1.30($C_4H_9$) |
| IVf | $C_6H_{11}$ | 68—69 | $C_{17}H_{20}O_4$ | 71.98 (72.02 | 6.92 6.71) | 6.46 | 4.65 | 7.46 | 3.86 | 1.00—2.24($C_6H_{11}$) 3.10—4.00 |
| IVg | $C_{27}H_{47}$ | 186—187 | $C_{38}H_{56}O_4$ | 79.12 (79.02 | 9.79 10.03) | 6.43 | 4.66 | 7.46 | 3.86 | 0.50—3.60($C_{27}H_{47}$) |
| IVh | $C_{27}H_{45}$ | 188—189 | $C_{38}H_{54}O_4$ | 79.40 (79.90 | 9.47 9.31) | 6.50 | 4.75 | 7.53 | 3.90 | 0.50—3.50($C_{27}H_{45}$) 5.40 |
| IVi | $CH_2C_6H_5$ | 95—97 | $C_{18}H_{16}O_4$ | 72.96 (72.49 | 5.44 5.48) | 6.41 | 4.65 | 7.41 | 3.83 | 4.67($CH_2C_6H_5$) 7.35 |

## The Fluorescence Properties of DMC Derivatives of Alcohols

Figure 3 shows a typical excitation and fluorescence spectrum of a labeled compound (IVh) in ethanol. DMC derivatives of alcohols showed fluorescence maxima in the 384—386 nm region with fluorescence quantum yields of 0.074—0.135, as shown in Table I.

## *Labeling of Carboxylic Acids with DMC*

The labeling of carboxylic acids with DMC was carried out by heating at 80°C in acetoni-

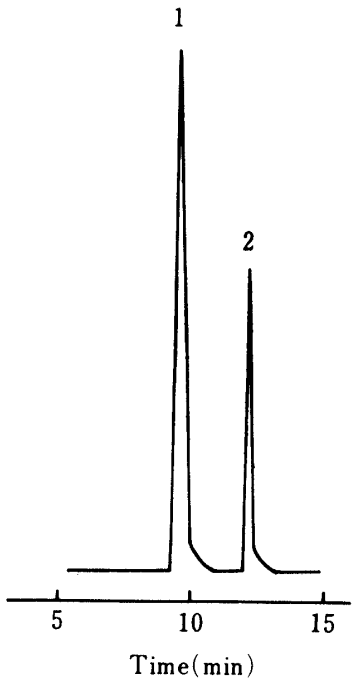



Fig. 2. HPLC Chromatogram of DMC Derivatives

1, cholesteryl ether (IVh); 2, cholestanyl ether (IVg).

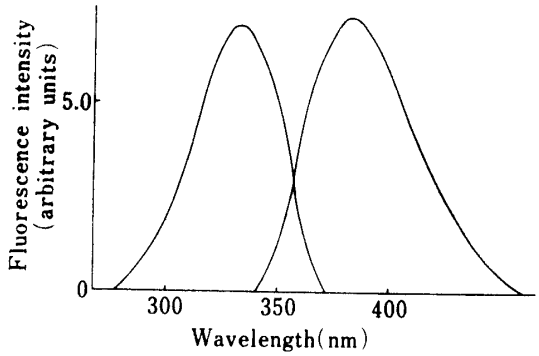


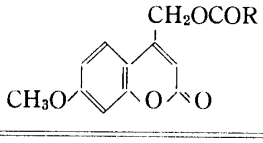

Fig. 3. Excitation and Fluorescence Spectra of Cholesteryl Ether (IVh) in Ethanol


Va: R = $CH_3$
Vb: R = $CH_2OH$
Vc: R = $(NHCOCH_3)CH_3$
Vd: R = $CH_2Cl$
Ve: R = $(CH_2)_{10}CH_3$
Vf: R = $(CH_2)_{12}CH_3$
Vg: R = $(CH_2)_{14}CH_3$
Vh: R = $(CH_2)_{16}CH_3$

Vi: R = $CH=CH_2$
Vj: R = $C_6H_5$
Vk: R = $p$-$CH_3C_6H_4$
Vl: R = $p$-$ClC_6H_4$
Vm: R = $p$-$OHC_6H_4$
Vn: R = $p$-$CH_3OC_6H_4$
Vo: R = $C_5H_4N$
Vp: R = iso-$C_5H_4N$

Chart 3

TABLE III. The Reaction Yields of DMC Derivatives of Carboxylic Acids




| Compd. No. | R | Yield (%) | Compd. No. | R | Yield (%) |
|---|---|---|---|---|---|
| Va | $CH_3$ | 24.9 | Vi | $CH=CH_2$ | 44.0 |
| Vb | $CH_2OH$ | 66.9 | Vj | $C_6H_5$ | 89.3 |
| Vc | $CH(NHCOCH_3)CH_3$ | 48.1 | Vk | $p$-$CH_3C_6H_4$ | 70.3 |
| Vd | $CH_2Cl$ | 67.6 | Vl | $p$-$ClC_6H_4$ | 99.7 |
| Ve | $(CH_2)_{10}CH_3$ | 56.9 | Vm | $p$-$OHC_6H_4$ | 74.0 |
| Vf | $(CH_2)_{12}CH_3$ | 52.5 | Vn | $p$-$CH_3OC_6H_4$ | 60.5 |
| Vg | $(CH_2)_{14}CH_3$ | 51.4 | Vo | $C_5H_4N$ | 31.3 |
| Vh | $(CH_2)_{16}CH_3$ | 53.7 | Vp | iso-$C_5H_4N$ | 72.0 |

trile, giving highly fluorescent esters as shown in Chart 3. The yields of esters (Va—Vp) are summarized in Table III. The esters other than Vo and Vp were identified by comparison of their melting points and spectral data with those of previously obtained samples.[11] Aromatic acids reacted more readily with DMC than did aliphatic acids, as shown in Table III. Kinoshita *et al.*[4c] reported a fluorescence labeling method with anthryldiazomethane, which reacts with carboxylic acids at room temperature. The reactivity of DMC was less under the same conditions.

The labeling of phenols with DMC was examined under various reaction conditions, but no reaction products were obtained.

In the present study, DMC could be easily synthesized from 4-formyl-7-methoxycoumarin (I)[8] and was adequately stable. DMC reacted with alcohols and carboxylic acids under mild conditions, and thus is a useful new fluorescent labeling reagent for alcohols and carboxylic acids.

## Experimental

**Synthesis**——7-Methoxy-4-(*p*-toluenesulfonyl)hydrazonomethylcoumarin (II): A mixture of I (3.0 g)[8] and tosylhydrazine (3.0 g) in MeOH (150 ml) was stirred at room temperature for 1 h, then allowed to stand overnight in refrigerator. The resulting yellow precipitates were collected by filtration and washed with cold MeOH. Recrystallization from MeOH gave 4.8 g (87%) of II as pale yellow needles, mp 155—156°C. *Anal.* Calcd for $C_{18}H_{16}N_2O_5S$: C, 58.06; H, 4.33; N, 7.52. Found: C, 57.72; H, 4.31; N, 7.51. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3420. NMR (DMSO-$d_6$) $\delta$: 2.40 (3H, s, $CH_3$), 3.88 (3H, s, $OCH_3$), 6.45 (1H, s, $C_3$–H), 7.47 (2H, d, benzene ring), 7.83 (2H, d, benzene ring), 8.03 (1H, s, $C_4$–CH), 8.23 (1H, d, $C_5$–H), and 12.30 (1H, br, NNH).

4-Diazomethyl-7-methoxycoumarin (III):[12] II (0.5 g) was dissolved in dichloromethane (12 ml), and 0.2 N NaOH (24 ml) was added dropwise at 0°C. The mixture was stirred at room temperature for 40 min, then the organic layer was separated, washed with cold water, dried over $Na_2SO_4$ and evaporated to dryness. Recrystallization of the residue from THF gave 0.23 g of III as yellow needles, mp 160°C (dec.). *Anal.* Calcd for $C_{11}H_8N_2O_3$: C, 61.11; H, 3.73; N, 12.96. Found: C, 61.36; H, 3.83; N, 12.74. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 2070. NMR (DMSO-$d_6$) $\delta$: 3.86 (3H, s, $OCH_3$), 5.70 (1H, s, $C_4$–CH), 6.46 (1H, s, $C_3$–H), and 7.63 (1H, d, $C_5$–H).

**Labeling Procedure of Alcohols with DMC**——A mixture of an alcohol (0.1 mmol), DMC (0.3 mmol), and a drop of concentrated $HBF_4$[10] in dichloromethane (15 ml) was stirred at room temperature for 30 min, then concentrated under reduced pressure. Standard samples were prepared by recrystallization from EtOH after separation by TLC (solvent system: benzene (or cyclohexane)–ethylacetate=4: 1) on a preparative scale.

**Labeling Procedure of Carboxylic Acids with DMC**——A mixture of a carboxylic acid (0.1 mmol) and DMC (0.3 mmol) in acetonitrile (3 ml) was heated at 80°C for 1 h. The ester was obtained by the same procedure as in the case of alcohols (TLC solvent system: benzene–ethylacetate=4: 1). The structures of Vo and Vp were confirmed by the following data. Vo: mp 200—202°C. *Anal.* Calcd for $C_{17}H_{13}NO_5$: C, 65.59; H, 4.21; N, 4.50. Found: C, 65.73; H, 4.08; N, 4.56. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1709. NMR ($CF_3COOD$) $\delta$: 4.06 (3H, s, $OCH_3$), 5.95 (2H, d, $C_4$–$CH_2$); 7.80 (1H, d, $C_5$–H), and 8.80—9.31 (4H, q, pyridine ring). Vp: mp 174—176°C. *Anal.* Calcd for $C_{17}H_{13}NO_5$: C, 65.59; H, 4.21; N, 4.50. Found: C, 65.20; H, 4.17; N, 4.56. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1709. NMR ($CF_3COOD$) $\delta$: 4.03 (3H, s, $OCH_3$), 5.93 (2H, d, $C_4$–$CH_2$), 7.75 (1H, d, $C_5$–H), and 8.10—9.70 (4H, m, pyridine ring).

**Apparatus and Measurements**——All melting points were measured with a Yanagimoto micro-melting point apparatus, and are uncorrected. IR spectra were taken with a JASCO DS-301 (JASCO, Ltd., Tokyo, Japan). NMR spectra were taken with a JEOL C-60H spectrometer (JEOL, Ltd., Tokyo, Japan), employing tetramethylsilane as an internal standard. The abbreviations used are as follows: s, singlet; d, doublet; q, quartet; m, multiplet. Absorption and fluorescence spectra were measured with a Hitachi 556s dual-wavelength spectrophotometer (Hitachi, Ltd., Tokyo, Japan) and a Hitachi 650-60 fluorescence spectrophotometer (Hitachi, Ltd.), respectively. Fluorescence quantum yields were determined according to the method of Parker and Rees[13] using quinine sulfate in 1 N $H_2SO_4$ as the standard. A Model 635 HPLC machine (Hitachi, Ltd.) equipped with a Model 204-S fluorescence detector (Hitachi, Ltd.) was used in the reversed phase mode at room temperature. A stainless steel column (4.0×150 mm i.d.) was packed with Lichrosorb RP-18 (particle size 5 μm, E. Merck, Darmstadt). Acetonitrile–THF–water (50: 40: 10) was used as the mobile phase at a flow rate of 1.0 ml/min. The fluorescence was measured at 386 nm, with excitation at 325 nm.

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