Precolumn Derivatization of Nucleotides Based on Fluorescent Carbamate Formation on the Sugar Moieties in High-Performance Liquid Chromatography

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The fluorescence derivatization of nucleotides with 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran in the presence of sodium azide and the separation of the derivatives by high-performance liquid chromatography are described. The reagent reacts with 5′-terminal hydroxyl groups of nucleotides to produce the corresponding fluorescent carbamates. The derivatives of mono- and oligonucleotides are separated by chromatography on a reversed phase column (TSKgel ODS-80T$_M$) and the derivatives of octa- and deca-nucleotides on a size exclusion column (TSKgel G3000SWXL). The detection limits (signal-to-noise ratio = 3) are 0.8—6.0 pmol on column. 5′-Phosphorylated nucleotide also gives a fluorescent derivative after alkaline phosphatase-mediated dephosphorylation.

Keywords precolumn fluorescence derivatization; 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran; sodium azide; nucleotide; high-performance liquid chromatography; alkaline phosphatase; 5′-terminal hydroxyl group

Ultraviolet (UV) detection has been extensively utilized for the quantification of nucleotides of biological importance by high-performance liquid chromatography (HPLC).[1,2] The nucleotides can be more sensitively quantified by precolumn fluorescence derivatization HPLC using chloroacetaldehyde[3] and bromoacetaldehyde[4] as fluorogenic reagents selective for adenine. A postcolumn derivatization method has also been reported for the determination of ribonucleotides based on periodate oxidation of the sugar moieties followed by a fluorescence reaction with 1,2-bis(4-methoxyphenyl)ethylenediamine as a fluorogenic reagent.[5]

We previously reported that 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OMB-COCl, Fig. 1) reacted with mono- and oligonucleotides to give the corresponding fluorescent products (OMB-esters), which could be separated by reversed phase HPLC.[6] We have recently found that the rates of the above reaction for octanucleotide and deca-nucleotide greatly decrease (20% or lower than those for mono- to tetra-nucleotides), but OMB-COCl reacts more readily, even with octa- and deca-nucleotides in the presence of sodium azide, to form different fluorescent products (OMB-carbamates). This study aims to establish a precolumn fluorescence derivatization HPLC of nucleotides by utilizing the above mentioned findings. The fluorescent products from mono- and oligonulceotides were separated by reversed phase HPLC and those from octa- and deca-nucleotides by size exclusion HPLC. 5′-Phosphorylated nucleotide also provides fluorescent derivatives after dephosphorylation by alkaline phosphatase (ALP)-catalyzed reaction.

Experimental

OMB-COCl was obtained from Dojindo Laboratories (Kumamoto, Japan). Adenosine-3′-monophosphate (Ap), cytidine-3′-monophosphate (Cp), guanosine-3′-monophosphate (Gp), uridine-3′-monophosphate (Up), 2′-deoxyadenosine-3′-monophosphate (dAp), 2′-deoxycytidine-3′-monophosphate (dCp), 2′-deoxyguanosine-3′-monophosphate (dGp), thymidine-3′-monophosphate (Tp), adenosine-5′-monophosphate (pA), cytidine-5′-monophosphate (pC), guanosine-5′-monophosphate (pG), uridine-5′-monophosphate (pU), 2′-deoxyadenosine-5′-monophosphate (pdA), 2′-deoxycytidine-5′-monophosphate (pdC), 2′-deoxyguanosine-5′-monophosphate (pdG), thymidine-5′-monophosphate (pT), 2′-deoxyadenylyl(3′-5′)-2′-deoxyadenosine [d(AA)], 2′-deoxyadenylyl(3′-5′)thymidine [d(AT)] and ALP (EC 3.1.3.1, from bovine intestine, 1.9 units/mg) were purchased from Sigma (St. Louis, MO, U.S.A.), a tetra-nucleotide [d(AGCT)], an octa-nucleotide [d(TTGGCCAA)] and a 5′-phosphorylated deca-nucleotide [d(pCCAAGCTTGG)] from Takara Shuzo (Kyoto, Japan), and a deca-nucleotide [d(CCAAGCTTGG)] from Yuki Gosei Kogyo (Tokyo, Japan). All other chemicals were of reagent grade. Pyridine and other organic solvents were distilled and dried as described previously.[6]

Apparatus An HPLC system was essentially the same as described previously. The fluorescence spectrometer was operated at an excitation wavelength of 340 nm and an emission wavelength of 420 nm. In reversed phase HPLC, the column was a TSKgel ODS-80T$_M$ (150 × 4.6 mm i.d.; particle size, 5 μm; Tosoh, Tokyo, Japan), and the mobile phase was a mixture of acetonitrile and 50 mM McIlvaine buffer (50 mM citric acid and 50 mM disodium hydrogen phosphate, pH 3.0) (2 : 3, v/v), which was pumped at a flow rate of 1.0 ml/min. In size exclusion HPLC, the column was a TSKgel G3000SWXL (300 × 7.6 mm i.d.; particle size, 5 μm; Tosoh) and the mobile phase was a mixture of acetonitrile and 50 mM phosphate buffer (pH 6.5) (1 : 9, v/v) which was pumped at a flow rate of 1.0 ml/min. The column temperature was ambient (25 ± 2 °C). Peak heights were used for the quantification of nucleotides.

Fluorescence spectra and fast atom bombardment mass (FAB-MS) spectra were measured as described previously.[6]

Preparation of the Fluorescent Products from Ap and d(AA) Ap (50 mg, 0.15 mmol) or d(AA) (50 mg, 0.09 mmol) and sodium azide (310 mg, 4.8 mmol) were dissolved in a mixture of pyridine (12.5 ml) and 6 mM OMB-COCl in benzene (37.5 ml). The solution was refluxed for 24 h in the dark. The solvents were removed *in vacuo*, and the residue was dissolved in water (10 ml). A portion (500 μl) of the resulting solution was subjected to preparative HPLC on a TSKgel ODS-120T (a preparative column; 30 × 6 cm i.d., Tosoh) using a mixture of acetonitrile and 1 M acetic acid (2 : 3, v/v) as a mobile phase, which was pumped at a flow rate of 6.0 ml/min. The HPLC procedure was repeated approximately 20 times, and the fractions containing the fluorescent product were combined and concentrated *in vacuo* to give a fluorescent compound [compound I from Ap: formula $C_{23}H_{20}N_7O_{12}P$; FAB-MS (*m/z*), $(M+H)^+ = 618$ (base peak), $M^+ - HPO_3 = 537$; mp > 300 °C; yield 15 mg (16%): compound II from d(AA): formula $C_{33}N_{12}O_{13}P$; FAB-MS (*m/z*), $(M+H)^+ = 835$ (base peak); mp > 300 °C; yield 10 mg (14%)].

Derivatization Procedure A mixture of nucleotide solution (0.05—10 nmol per 0.1—1 ml water) and 0.15 M sodium azide (0.3 ml) placed in a screw—capped vial (3.5 ml) was lyophilized. To the lyophilizate was added a mixture of pyridine and 3 mM OMB-COCl in benzene (1 : 3, v/v) (0.5 ml). The vial was tightly closed and heated at 100 °C for 90 min in the dark. The reaction mixture was dried in a stream of nitrogen at 60 °C, and the residue was dissolved in the mobile phase (1 ml) for HPLC.

ALP-Mediated Hydrolysis of 5′-Phosphorylated Nucleotide To an aqueous solution (0.2 ml) of 5′-phosphorylated nucleotide placed in a screw-capped vial were added 40 mM Britton–Robinson buffer (pH 9.0) (0.1 ml) and 7.5 units/ml ALP (0.4 ml). The mixture was incubated at 37 °C for 1 h. To the reaction mixture were added 10% (w/v) sodium dodecylsulfonate (50 μl) and 0.1 M ethylenediaminetetraacetic acid disodium salt (EDTA · 2Na) (10 μl), and the mixture was heated at 70 °C

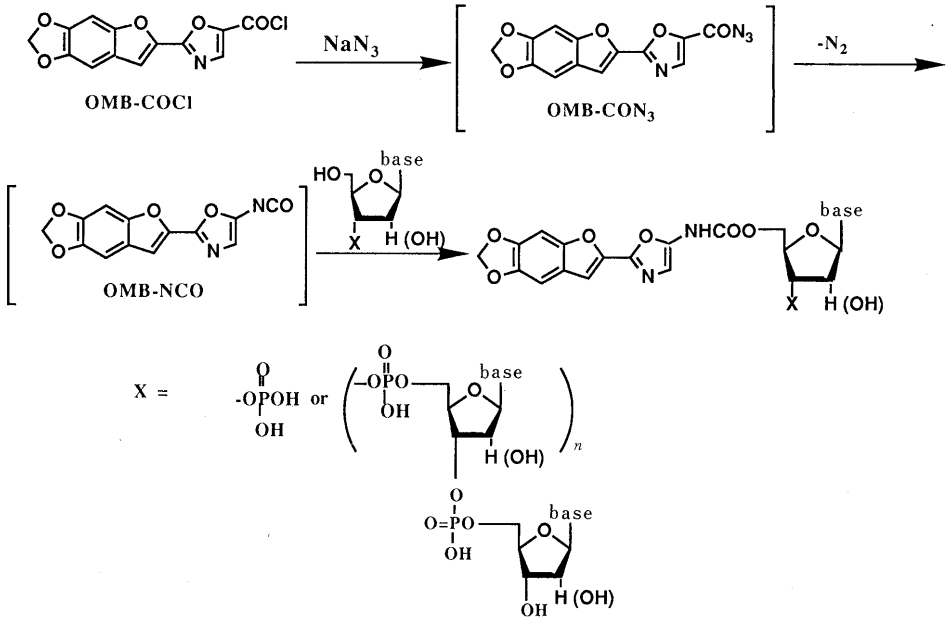

Fig. 1. Possible Route of Fluorescence Derivatization of Nucleotides with OMB-COCl and Sodium Azide

for 15 min to inactivate the enzyme. The resulting solution was treated by the derivatization procedure.

Results and Discussion

Characterization of Compounds I and II The retention times (2.8 and 11.8 min) of compounds I and II, and their excitation (both 340 nm) and emission (both 420 nm) maxima were identical with those for the fluorescent compounds produced from Ap and d(AA) under the conditions of the derivatization procedure, respectively: the maxima were in shorter wavelengths than those of OMB esters (360 and 475 nm, respectively). The FAB-MS spectral data supported that compounds I and II were the OMB-carbamates of Ap and d(AA), respectively. OMB-COCl did not work on the 5′-phosphorylated nucleotide. These observations indicate that the fluorescent compounds yielded under the derivatization conditions are the carbamates of 5′-terminal hydroxyl groups. It seems that OMB-COCl reacts with sodium azide to form OMB-CON_3, which is converted to OMB-NCO, and then reacts with nucleotides (Fig. 1).

Derivatization Conditions The derivatization conditions were investigated using Ap, Up, dAp, Tp d(AT), d(AGCT) and d(TTGGCCAA). The highest peaks were obtained at OMB-COCl and sodium azide concentrations greater than 2 and 60 mM, respectively; 3 and 90 mM were used as optima. Of the solvents examined for the derivatization (benzene, acetonitrile, ethyl acetate, dimethylsulfoxide, toluene, acetone, dimethylformamide, dioxane and tetrahydrofuran), benzene provided the most intense peaks for all the nucleotides. Acetonitrile also afforded an intense peak for d(TTGGCCAA); benzene could be replaced with acetonitrile. Pyridine, which accelerated the reaction, afforded maximum peak heights at concentrations of 2.5—4.1 M in the reaction mixture; 3.1 M was used in the procedure. Higher temperatures permitted the reaction to proceed more rapidly in the range 60—120 °C. At a selected temperature of 100 °C, the peak heights reached maxima after heating for 1 h.

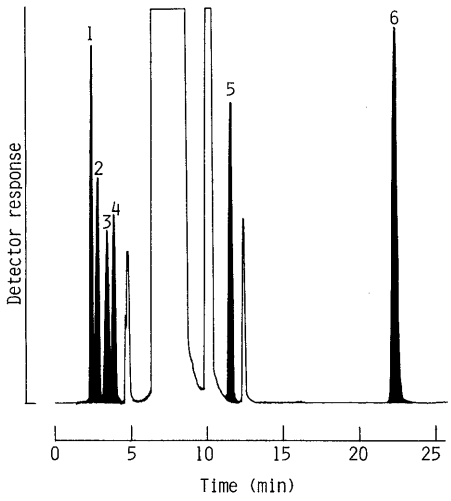

Fig. 2. Chromatogram of the OMB-carbamates of Six Nucleotides

The peaks for the nucleotides are shaded. Peaks: 1 = Up, 2 = Ap, 3 = Tp, 4 = dAp, 5 = d(AT), 6 = d(AGCT), others = the reagent blank. Concentrations (on column): 35 pmol each.

Under the present conditions, OMB-COCl readily reacted with the octa- and deca-nucleotides (Table I). This is probably due to the high reactivity of the isocyanate moiety of the above mentioned OMB-NCO.

HPLC Separation Reversed Phase HPLC: The OMB-carbamates of Ap, Up, dAp, Tp, d(AT) and d(AGCT) used for the investigation of HPLC conditions could be separated on a TSKgel ODS-80T_M with isocratic elution using a mixture of acetonitrile and 50 mM McIlvaine buffer (pH 3.0) (2:3, v/v) as a mobile phase. At pH of greater than 4.0 in McIlvaine buffer (50 mM) caused an early elution of the reagent blank, of which peaks were overlapped with the peaks for Ap, Up, dAp and Tp. Concentrations (25—100 mM) of McIlvaine buffer (pH 3.0) did not affect their separation and peak heights. Figure 2 shows a typical chromatogram obtained with the OMB-carbamates of the six nucleotides under the HPLC con-

TABLE I. Retention Times and Detection Limits of the OMB-carbamates of Nucleotides

Nucleotide	Separation mode[a]	Mobile phase[b]	Retention time (min)	Detection limit[c] (pmol on column)
Ap	RP	A	2.8	4.0
Cp	RP	A	2.6	4.5
Gp	RP	A	2.6	5.0
Up	RP	A	2.4	3.0
dAp	RP	A	3.8	3.8
dCp	RP	A	3.8	4.7
dGp	RP	A	3.5	6.0
Tp	RP	A	3.3	1.2
d(AT)	RP	A	11.6	1.2
d(AA)	RP	A	11.8	1.3
d(AGCT)	RP	A	22.3	0.8
d(TTGGCCAA)	SE	B	9.5	1.0
d(CCAAGCTTGG)	SE	B	9.0	1.5

a) RP, reversed phase (on TSKgel ODS-80T$_M$); SE, size exclusion (on TSKgel G3000SWXL). *b*) A, acetonitrile–50 mM McIlvaine buffer (pH 3.0) (2:3, v/v); B, acetonitrile–50 mM phosphate buffer (pH 6.5) (1:9, v/v). *c*) At signal-to-noise ratio (S/N)=3.

ditions; the retention times are listed in Table I and those obtained with the other nucleotides examined are also shown in Table I.

Size Exclusion HPLC: The OMB-carbamates of the deca- and octa-nucleotides were separated from the reagent blank peaks (the retention times, 13—35 min) on a TSKgel G3000SWXL, using a mixture of acetonitrile and 50 mM phosphate buffer (pH 6.5) (1:9, v/v) as a mobile phase (for the retention times, see in Table I). Varing pHs (2.5—7.5) and concentrations (0.01—0.15 M) of the phosphate buffer had no effect on separation and peak heights.

Hydrolysis of the 5′-Phosphorylated Nucleotide The hydrolysis was carried out in a Britton–Robinson buffer (pH 9.0, 5.7 mM). The hydrolysis (%) of 3 nmol/ml d(pCCAAGCTTGG) was 80.2±1.6% (n=5), which was calculated in comparison with d(CCAAGGTTGG).

Calibration Curves, Precision and Detection Limits The relationships between the peak heights and the amounts of nucleotides were linear from 10 pmol up to at least 1 nmol on column. The precision was established by repeated determinations (n=10) using the nucleotides (concentrations, 8.75 nmol/ml each). The relative standard deviations (%) were 1.4 (Ap), 0.6 (Cp), 1.5 (Gp), 2.0 (Up), 1.3 (dAp), 1.5 (dCp), 1.8 (dGp), 3.0 (Tp), 5.0 [d(AA)], 4.8 [d(AT)], 5.2 [d(AGCT)], 4.6 [d(TTGGCCAA)] and 5.5 [d(pCCAAGCTTGG)]. The detection limits were 0.8—6.0 pmol on column (Table I): the resulting fluorescence intensities did not shown any significant difference among the nucleotides, but the limits differed depending on background noise.

In conclusion, this paper provided the reaction of nucleotides (mono- to deca-nucleotides) with OMB-COCl in the presence of sodium azide to give the corresponding OMB-carbamates of the 5′-terminal hydroxyl groups, and the reaction is probably applicable to longer nucleotides. The derivatives of mono- and oligonucleotides could be separated by reversed phase HPLC. The method should be applicable to the determination of mono- and oligonucleotides released from polynucleotides by nuclease-mediated hydrolysis. The derivatives of octa- and deca-nucleotides were separable from the reagent blank by size exclusion HPLC. This technique should be applicable to the preparation of fluorescent deoxyribonucleic acid (DNA) probes for hybridization assays based on direct fluorescence labeling to the 5′-terminal hydroxyl groups.

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References

1) P. R. Brown, A. M. Krstulovic and R. A. Hartwick, *Ad. Chromatogr.*, **18**, 101 (1980).
2) R. C. Sympson and P. R. Brown, *J. Chromatogr.*, **379**, 269 (1985).
3) M. Yoshioka and Z. Tamura, *J. Chromatogr.*, **123**, 220 (1976).
4) M. Yoshioka, K. Nishidate, H. Iizuka, A. Nakamura, M. M. El-Merzabani, Z. Tamura and T. Miyazaki, *J. Chromatogr.*, **309**, 63 (1984).
5) Y. Umegae, H. Nohta and Y. Ohkura, *Anal. Sci.*, **6**, 519 (1990).
6) H. Nagaoka, H. Nohta, M. Saito and Y. Ohkura, *Anal. Sci.*, **8**, 565 (1992).