Synthetic Analytical Studies on Organic Reagents for Biomaterials

Shuzo Akiyama

School of Pharmaceutical Sciences, Nagasaki University, Nagasaki 852, Japan

“Synthetic analytical chemistry: Analytical chemistry with the aid of synthetic organic chemistry” is proposed by the present author as a new field of analytical chemistry. Such chemistry is developing on the basis of investigations of π-electron systems such as non-benzenoid aromatics (e.g. annulenes) or conjugated polyene and polyyne systems. Various kinds of organic reagents for analyses (ultraviolet/visible and near-infrared absorption, fluorescence, chemiluminescence and new silica gel column packings for high-performance liquid chromatography) were developed and applied to the analyses of many biomaterials. In this paper, synthetic analytical chemistry is illustrated mainly by the author’s own studies.

Keywords Organic reagent, light absorption, fluorescence, chemiluminescence, near-infrared dye, high-performance liquid chromatography, flow injection analysis, biomaterials, π–π interaction

1 Introduction

In a number of organic syntheses, the identification and structural determination of reaction products are routinely carried out by various instrumental analyses (nuclear magnetic resonance (NMR), mass spectra (MS), high-performance liquid chromatography (HPLC), etc.). Such development of synthetic organic chemistry is much indebted to that of analytical chemistry. Traditionally, various organic reagents for analysis have been used in analytical chemistry and the number and types of reagents have increased with the progress of synthetic chemistry. Reagents for analysis were clearly classified into either chemical or physical reagents by Prof. S. Ikeda in an article on modern analytical chemistry.1 The former means so-called conventional reagents and the latter refers to analytical techniques, the so-called instrumental analysis. Recently, I have proposed the possibility of a new genre, i.e., “Synthetic analytical chemistry: Analytical chemistry with the aid of organic synthesis”.2

Last year, the genre advocated by the author was illustrated by Prof. T. Nambara, one of the panelists, in a symposium entitled “Aiming at forthcoming analytical chemistry” held during the 41st Annual Meeting of the Japan Society for Analytical Chemistry in Kyoto.3 Many instrumental analyses can attain high performance by the assistances of chemical reagents, so improvements of the reagents bring about better efficiency. For accomplishment of this propose, analytical chemistry should be positively benefited by synthetic chemistry. Thus, analytical chemists should actively challenge the syntheses of novel and/or characteristic organic reagents for analysis.

In this review, I report the outline of some studies on “Synthetic analytical chemistry” developed empirically on the basis of the investigations of π-electron systems such as dehydroannulenes4, dehydroannulenodehydroannulenes5 and long conjugated polyene4 and polyyne5

1 Introduction

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systems having aromatic rings as the terminal groups. Many kinds of organic reagents for analysis [ultraviolet (UV)/visible and near-infrared (NIR) absorption, fluorescence (FL), chemiluminescence (CL) and new silica gel column packings for HPLC] were newly developed and applied to the analysis of biomaterials.

2 Development of Organic Reagents for Analysis (Ultraviolet/Visible and Near-Infrared Absorption, Fluorescence and Chemiluminescence)

2.1 Crowned compounds
Some crowned dinitrophenylazophenols (e.g., CDPA, Fig. 1) were examined in regard to the complexation with alkali and alkaline earth metals. CDPA showed a characteristic coloration with Li⁺ under the restricted conditions.8 To establish the determination of Li⁺, the complexation of CDPA was investigated in CHCl₃-dimethyl sulfoxide-triethylamine (TEA) and a sensitive colorimetric assay method for Li⁺ was first established in the range of 25 – 250 ppb of Li⁺.9 This method was applied to the analysis of a commercial pharmaceutical preparation, Li₂CO₃ (tablet).

The coloration reactions of CDPA-Ba²⁺ complex with 28 different amines were examined in MeOH and a new sensitive determination method of various aliphatic amines was developed with a range of 0.2 to 4 µg/ml.10 For the determination of trimethylamine (TMA) (0.6 – 3.0 µg/50 µl for TMA), a simple and sensitive flow injection spectrophotometric method was designed and applied to the determination of total volatile amines, such as the content of TMA in fish.11

2.2 Compounds related to pyrimidine
A series of N,N'-disubstituted 2-thiobarbituric acids was systematically prepared and examined in regard to the condensation with malondialdehyde (MDA) to yield the corresponding pigments (Fig. 2). The molar absorptivity (ε) of the 1,3-diphenyl-2-thiobarbituric acid (DPTBA) condensate (R₁=R₂=C₆H₅) was fairly large (ε=1.96×10⁵ (EtOH)). DPTBA improved the sensitivity as a derivatizing reagent for MDA. The details will be given later (3.1).

2.3 5-(p-Dimethylaminophenyl)-2,4-pentadienal (DAPDA)
DAPDA was conveniently prepared from p-dimethylaminobenzaldehyde by using readily available 4-(t-butylthio)-3-buten-2-one as the chain lengthening agent which was developed for a synthon for α,β,γ,δ-unsaturated aldehydes.17 DAPDA condenses easily with primary amines to yield their Schiff bases; upon protonation, these bases undergo dramatic bathochromic shifts and their ε values are intensified (Fig. 4).18,19 DAPDA is a useful reagent for detection and colorimetric determination of many pharmaceuticals, e.g. sulfur drugs having primary amino group(s).18
2.4 2-(4-Substituted phenyl)benzofurans

In order to develop sensitive and useful fluorogenic reagents, the synthesis of derivatives of 2-phenylbenzofuran (PB, Fig. 5) was carried out. These compounds were expected to exhibit strong FL due to the presence of a latent trans-stilbene skeleton. Fifteen kinds of PB were prepared and their FL spectral properties were investigated. Among them N-[4-(dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM, Fig. 5) has been found to give fluorescent products when reacted selectively with certain biological thiols, e.g. reduced glutathione (GSH) and cysteine (Cys). The application of DBPM to HPLC is discussed in 3.5.

2.5 Reagents related to chemiluminescence

Our group is interested in peroxyoxalate CL (PO-CL) and we are trying to develop new characteristic aryl oxalates and FL reagents for enhancement of CL.

2.5.1 Aryl oxalates

Eight aryl oxalates having an alkoxy moiety were synthesized to improve the solubility in acetonitrile (MeCN) for the PO-CL reaction system. Among them, bis[2-(3,6-dioxahexyl)oxy]carbonyl-4-fluorophenyl]oxalate showed the highest solubility (1686 mM); bis[2-(3,6-dioxahexyl)oxy]carbonyl-4-bromophenyl]oxalate gave the highest intensity, which was about 100 times lower than that of bis(2,4-dinitrophenyl)oxalate under the condition of PO-CL reaction in the presence of hydrogen peroxide, TEA and 9,10-diphenylanthracene in MeCN. However, the CL intensities of the oxalates synthesized continued for a far longer period of time than those of commercially available oxalates.

2.5.2 Fluorescence reagents for enhancement of chemiluminescence

a) Pyrimido[5,4-d]pyrimidine (PP)

Imai et al. found that dipyridamole (DP, Fig. 6) shows the lowest detection limit in a study on the estimation of the detection limits of fluorescent compounds by the PO-CL reaction system, in which the presence of a coexisting fluorescent substance having an intensive FL is very important. Our group synthesized other fluorescent compounds having a PP skeleton and evaluated their UV and FL spectra with respect to their possible use as CL reagents. Of these compounds, morpholino compound (MP) and thiomorpholino compound (TMP) were found to be suitable for the use of the CL method (Fig. 6).

b) Styrylstilbenes, tolyltolans and their related compounds

For the development of new fluorescent and chemiluminescent probes for analytical and/or biological studies, we have investigated a series of styrylstilbenes (SS), distylylanthracenes (DSA), bis(phenylethynyl)benzenes (BPB) and bis(phenylethynyl)anthracenes (BPA) (Fig. 7). These compounds were synthesized and the absorption, FL and CL spectroscopic properties were examined.

As shown in Fig. 8, we found a novel one-stage conversion of the CH=CH bond to the C=C bond; the
compounds BPA \((R=R'=\text{NEt}_2\) and \(\text{CN}\); \(R=\text{MeO}, R'=\text{NO}_2\)) were obtained from the corresponding olefinic compounds BPB by treatment with \(\text{t-BuOK}\) in dimethylformamide (DMF) at pressure. For examination of the above direct conversion reaction, we chose 4-substituted 4'-nitrostyryl system (NS) to give the corresponding tolans (NT) and their phenols (NP), and are now exploring the scope and the limitation of this process (Fig. 8).\(^{27a,b}\) We have found the ESR signals showing the presence of the corresponding anionic radical species derived from the stilbene\(^{27b,c}\) and are now continuing the ESR study on the mechanism. The use of NS and the tolans (NT and NP) will be helpful for nonlinear optics\(^{28}\) and reagents for analysis.

The applications of these fluorescent compounds, referred to in a) and b) of this section, are described in 3.2.

2.6 Lophine derivatives

2,4,5-Triphenylimidazole ("lophine") is a well-known compound which reacts with an oxidizing agent in the presence of a strong base to exhibit CL.\(^{29}\) A series of four aliphatic carboxylic esters (acetate, caproate, laurate and palmitate) of 2-(4-hydroxyphenyl)-4,5-diphenyl-imidazole was prepared. Usefulness of these esters as fluorescent substrates for the measurement of lipase activity was evaluated using an HPLC method.\(^{30}\) For the assay of saturated free fatty acids, a new fluorescent

Fig. 7 Synthesis of acetylenic compounds from the corresponding ethylenic compounds.

Fig. 8 Reaction of 4-substituted 4'-nitrostyrenes with \(\text{t-BuOK}\) in DMF under pressure.

Fig. 9 Triphenylmethane dye ethynyllogs (TPMDE) and an allene-quinoid structure.
derivation reagent with a "lophone" skeleton, 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI) has been synthesized and successfully applied.\(^{31}\)

**2.7 Triphenylmethane dye ethynyllogs\(^{32,33}\)**

Dye systems which contain acetylenic bond(s) in triphenylmethane dyes (triphenylmethane dye ethynyllogs, TPMDE, Fig. 9) were systematically prepared and their spectroscopic properties were examined to apply as analytical reagents and/or non-linear optic materials.

The introduction of an acetylenic bond into the resonating system of triphenylmethane dyes makes the cation part coplanar by releasing steric congestion between ortho-hydrogen atoms of phenyl groups in the dyes and induces a strong bathochromic effect on the peak in the visible region of the spectra. The unique properties seem to be explained by the contribution of the allene-quinoid structure in the system (Fig. 9).

**2.7.1 Reagents for ion pair extraction**

By the use of 1,1-bis(p-dimethylaminophenyl)-3-phenyl-2-propynylium chloride, which was derived easily from the reaction of the bis-ethynyllogs of Malachite Green (MG) with HCl, micro amounts of perchlorate ions could be extracted into chlorobenzene and determined spectrophotometrically at 690 nm; results showed a larger bathochromic shift (ca. 70 nm) than in the case of MG.\(^{33}\)

An extraction–fluorometric determination method for perchlorate ions based on their extraction into chlorobenzene with 2,6-di-p-tolyl-4-phenylpyrylium chloride (DTPP+Cl\(^-\), Fig. 10) has been developed.\(^{34}\) DTPP+Cl\(^-\), a new reagent derived from the study of the dye ethynyllogs, reacts with perchlorate ions to form a 1:1 ion pair (DTPP+ClO\(_4\)-), which can be extracted into chlorobenzene with an excitation maximum at 376 nm and an emission maximum at 495 nm. The determination range of percholrate was 0.01 – 1.0 ppm.

**2.7.2 Near-infrared dyes\(^{35,36}\)**

The first examples of xanthenilium dye ethynyllogs (XDE) [4-(dimethylamino)phenylethynylxanthenyl, -thioxanthenylium and -selenoxanthenylium perchlorates\(^{37a,b}\) and triphenylmethane dye ethynylvinylogs (TPMEV)\(^{37c}\) absorbing in the NIR (up to 814 nm in CH\(_2\)Cl\(_2\)) have been synthesized (Fig. 11). Their absorption spectral properties and chemical structures have been investigated and compared with the results of MO calculations.\(^{37b}\) The contribution of positive charge-delocalized structures (resonance hybrids) is effective. Their usefulness for the NIR optical information recording materials was examined.\(^{38}\)

We have been interested in bridging effects as shown in the above dye system (XDE) and have subsequently developed fluorenylium dye ethynyllogs (FDE). A series of FDE, which absorb NIR light (up to 1096 nm in CH\(_2\)Cl\(_2\)), has been systematically prepared and their characteristic absorption spectroscopic and the other properties have been studied.\(^{39}\)

### 3 Application to the Determination of Biomaterials

Some selected examples of the applications to the determination of the compounds related to biomaterials are described below.

#### 3.1 MDA

After the study\(^{13}\) mentioned in 2.2, a sensitive and selective HPLC-visible detection method for the DPTBA-MDA condensate (pigment) was developed.\(^{40}\)
The CL detection method of fluorescent TBA-MDA condensates by a flow injection analysis method has been developed in order to find a suitable reagent for MDA among TBAs. Then, 1,3-diethyl-2-thiobarbituric acid (DETBA) and DPTBA were selected to condense with MDA and the HPLC-CL detection of the resulting condensates was developed by using bis(2,4,6-trichlorophenyl)oxalate (TCPO) and hydrogen peroxide as postcolumn chemiluminogenic reagents in McCN. The detection limits for DETBA-MDA and DPTBA-MDA were 20 and 200 fmol, respectively, per 20 µl injection, S/N>2. As shown in Table 1, this detection system was applied to the determination of MDA in rat brains by using DETBA as a fluorescent derivatizing reagent.

### 3.2 Hydrogen peroxide, glucose and uric acid

An HPLC method with PO-CL detection was developed for the determination of hydrogen peroxide. Hydrogen peroxide separated by a reversed phase column was detected with the CL produced by the PO-CL reaction using a mixture of TMP and bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl]oxalate or TCPO in acetonitrile as a postcolumn chemiluminogenic reagents in MeCN. The detection limits for DETBA-MDA and DPTBA-MDA were 20 and 200 fmol, respectively, per 20 µl injection, S/N>2. As shown in Table 1, this detection system was applied to the determination of MDA in rat brains by using DETBA as a fluorescent derivatizing reagent.

### 3.3 Thiols

In biochemistry, medicine and pharmacology, it is important to determine thiols and the related compounds selectively, sensitively and easily. For such purposes, DBPM was developed; both its reactivity with thiols and the FL stability of the reaction products seemed to be superior to those of the known maleimide reagents. By using HPLC, six kinds of thiols could be separated within 12 min and detected at fmol levels. An alternative method to fluorometry, CL is more remarkable at the point of the sensitivity. Until recently, no CL method has been utilized for the determination of thiols by HPLC. Then, five kinds of thiols were investigated with DBPM by the PO-CL method consisting of TCPO and hydrogen peroxide using HPLC. The labeling reaction of DBPM with thiols was carried out at 60°C for 30 min at pH 8.5; then a sample of the resulting reaction mixture was subjected to HPLC. Five kinds of labeled thiols were separated and detected in the ranges from 500 fmol to 2 pmol/100 µl (Cys and N-acetylcysteine), to 3 pmol/100 µl (Cys) to 5 pmol/100 µl (GSH and D-penicillamine). The lower detection limits were from 7 fmol (Cys) to 113 fmol (GSH) per 100 µl (S/N>2). This method was applied to the determination of thiols in a rat liver. The amounts of GSH and Cys were 1.23±0.15 µmol/g (n=5) and 0.15±0.04 µmol/g (n=5), respectively.

### Table 1 Assay of MDA in rat brain

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Amount of MDA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>47.7±12.1</td>
</tr>
<tr>
<td>Midbrain</td>
<td>66.0±16.8</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>35.2±8.2</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>52.6±8.0</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>66.4±20.8</td>
</tr>
<tr>
<td>Striatum body</td>
<td>86.6±24.3</td>
</tr>
<tr>
<td>Pons/medulla oblongata</td>
<td>59.9±20.2</td>
</tr>
</tbody>
</table>

a. Amount of MDA is expressed as mean±S.D. (n=4) in terms of nmol MDA/g wet wt.
4 Silica Gel Column Packing Materials for Water-Soluble Bioactive Compounds

4.1 Development

An octadecylsilylated silica gel (ODS) column is now widely applied to various fields and continues to dominate all other HPLC modes. Recent trends have been directed toward the development of specialty phases for realization of selectivity. Our group has aimed at the development of new HPLC column packing materials for separation of water-soluble compounds. As shown in Fig. 12, the first silica gels developed for HPLC (MPS, PZS and PDS) were synthesized by the reaction of silica gel with the corresponding 3-(substituted propyl)trimethoxysilane in toluene under reflux; these gels were suitable for the simultaneous separation of several water-soluble vitamins in a single analysis by a single buffer elution with UV detection, in addition to shortening the analysis time. These columns have a reversed-phase type character; their ion-exchange behavior is like a zwitter-ionic stationary phase. In anticipation of the appearance of a π-π interaction, seven kinds of 3-(N-substituted)aminopropylsilyl silica gels [(NAPS and AAPS), (ACNQ and AAQI), (PAIP, NCIP and NAIP); cf. Fig. 12] were prepared; these were substituted with π-π electron systems at the amino group by the reaction of the gel with two species of aroyl halides (1-naphthoyl- or 1-anthroyl chloride), organic dyes (5-amino-2,3-dicyano-1,4-naphthoquinone or 1,4-diamino-9,10-anthraquinone-2,3-dicarboxylic anhydride) or aromatic dicarboxylic anhydrides (phthalic, 2,3-naphthalenic or 1,8-naphthalenic anhydride). Basic examinations of the characteristics of the HPLC columns prepared by using these gels were carried out in order to separate compounds related to nucleic acids. Gels immobilized with organic dyes will be available for separation by a π-π interaction mode in HPLC.

4.2 Applications

As indicated in Fig. 13, MPS could quite easily separated six water-soluble vitamins. The pH greatly influences the retention times of thiamine-HCl (TA), hydroxocobalamin acetate (HC) and flavin adenine dinucleotide (FAD), but not the times of ascorbic acid (AA), nicotinic acid (NA) and pyridoxine-HCl (PY).
detection, using a phosphate buffer), and these vitamins could be completely separated within fewer than 12 min. MPS can quite easily separate water-soluble vitamins contained in commercially available beverages. By pH change experiments, separation of the combined peak of nicotinamide with caffeine can be realized at pH 3.0, through at the sacrifice of the analysis of the other vitamins. This result gave the possibility of caffeine analysis. MPS could be applied to separation and determination of compounds (PY, FAD, cyanocobalamin, chlorophenylamine, naphazoline and neostigmine) in an eye lotion and 5'-ribonucleotide compounds (inosine (5'-IMP), guanylic (5'-GMP) and 5'-xanthyllic (5'-XMP) acids) in a seasoning related to "Umami." MPS was successfully used also for the separation of sugars (rhamnose, xylose, arabitol, glucose, mannose, sucrose, maltose, melibiose, raffinose and maltotriose) and the determination of glucose or and sucrose in an infusion, drinks for sports and soft drinks; these analyses were carried out with elution with acetonitrile/10 mM imidazole-borate buffer (pH 6.5) (90/10, v/v) by refractive index detection.

NAPS and AAPS were applied to analysis of the compounds (adenosine, guanosine and inosine), (adenosine, AMP, ADP and ATP) and (AMP, 5'-GMP, 5'-IMP and 5'-XMP) related to nucleic acids, in which adenosine nucleotides were separated in a single analysis by a single buffer solution with UV detection. NAPS could be successfully used for the measurement of ATPase activity in fish meat (Sillago japonica). The mobile phase used in the analysis was throughout an aqueous phosphate buffer.

The HPLC columns prepared by using the gels (ACNQ, AAQI, PAIP, NCIP and NAIP) were examined and applied to the separation of adenine derivatives, i.e., ATP, ADP, ATP, adenosine and adenine as shown in Fig. 14 by ACNQ (a dye column) as a representative example. In expectation of the separation in terms of a π-π interaction, these columns are now being examined for the analysis of pharmaceuticals.

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