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Activated Carbamate Reagent as Derivatizing Agent for Amino Compounds in High-Performance Liquid Chromatography

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Activated carbamate reagents were developed for the determination of amino compounds. The reagents, succinimido carbamates, were easily prepared by the reaction of disuccinimido carbonate (DSC) with the appropriate amine having a strong chromophore. Succinimido phenyl- and *p*-bromophenylcarbamates for spectrophotometric determination, and succinimido naphthylcarbamate for fluorometric determination, were synthesized as activated carbamate reagents. The carbamate reagents readily react with both primary and secondary amines such as alkyl amines or amino acids in mild conditions to give the corresponding urea derivatives. These derivatives are efficiently separated by reversed-phase liquid chromatography and were sensitively detected spectrometrically or fluorometrically.

Numerous derivatization reagents for amino compounds have been developed for high-performance liquid chromatographic (HPLC) study. Ninhydrin (*1*) was the most popular reagent for the colorimetric detection of amines only in postcolumn derivatization. Recently, fluorometric reagents such as *o*-phthalaldehyde (*2, 3*) or fluorescamine (*4, 5*), have applied to both precolumn and postcolumn derivatization procedures to replace ninhydrin. However, secondary amines do not fluoresce on reaction with these reagents. They should be converted into primary amines by an additional procedure such as oxidation prior to derivatization (*6, 7*). For the simultaneous determination of both primary and secondary amines, various types of reagents have been developed such as halogenosulfonate (*8–10*), formate (*11, 12*), nitroaryl halide (*13–15*), sulfonic acid (*16*), isocyanate (*17*), isothiocyanate (*18, 19*), etc.

Since isocyanate and isothiocyanate reagents readily react with amines under the mild conditions to give stable urea or thiourea derivatives without forming byproducts, various labeling reagents (*17–23*) of this type have been developed and applied to amino acid analyses, Edmann degradation (*18–21*), or chiral derivatization (*22–24*). However, these compounds were moisture-sensitive lachrymators, and their synthesis has required phosgen or thiophosgen.

On the other hand, a convenient synthetic method (*25*) for urea derivatives has recently been developed by using disuccinimide carbonate (DSC), which was essentially a synthetic reagent for the preparation of active carbonate in peptide chemistry (*26*). DSC reacts with amino compounds

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to give symmetric urea derivatives. This reaction proceeded through a carbamate compound as an intermediate, and the resulting compounds further reacted with the residual amine giving symmetric urea derivatives. This fact facilitated the synthesis of asymmetric urea derivatives. One amino compound was first coupled with an equimolar amount of DSC as follows: the product was then allowed to react with another, structurally different, amino compound.

The present paper deals with the application of the above-mentioned reactions to the analysis of amino compounds. Activated carbamate reagents prepared by the reaction of DSC with aromatic amines were investigated in their practicabilities as precolumn derivatization reagents for amino compounds in HPLC.

EXPERIMENTAL SECTION

Chemicals. Aromatic and alkyl amines, amino acids, HPLC-grade methanol, and other reagents were obtained from Wako Pure Chemicals Industry (Osaka, Japan). HPLC-grade acetonitrile was obtained from Kanto Chemical Co., Inc. (Toyo, Japan). *N,N'*-Disuccinimidocarbonate (DSC) was obtained from Chemiscience Co., Inc. (Tokyo, Japan). Water was purified by passage through a Milli-R/Q system, followed by a final cleanup through a Milli-QII system (Nihon Millipore, Ltd., Tokyo, Japan).

Synthesis of Activated Carbamate Reagent. Activated carbamate reagents were synthesized according to the previous paper (*25*).

A solution of aromatic amine (aniline, *p*-bromoaniline, or 1-naphthylamine; 40 mmol/40 mL of acetonitrile) was added dropwise to DSC solution with stirring (50 mmol/60 mL of acetonitrile) over a period of 3–4 h at room temperature. After the addition, the mixture was further stirred for 1 h at room temperature, and then acetonitrile was evaporated. The resulting residue was redissolved in about 100 mL of ethyl acetate, and the ethyl acetate solution was washed consecutively with 1 N hydrochloric acid, 4% sodium bicarbonate, and distilled water, and the organic layer was dried over anhydrous sodium sulfate. After evaporation of the ethyl acetate, crystals of the activated-carbamate reagent were obtained. succinimido phenylcarbamate (SIPC), succinimido *p*-bromophenylcarbamate (SIBr-PC), and succinimido 1-naphthylcarbamate (SINC) were formed from aniline, *p*-bromoaniline, and 1-naphthylamine, respectively. They were recrystalized from the benzene–acetone system, and their structures were identified by using infrared and mass spectroscopy and elemental analysis.

Preparation of Urea Derivatives. To an acetonitrile solution of SIPC (1 mmol/5 mL) was added each solution of various alkylamines (1.1 mmol/3 mL of acetonitrile), and the resulting mixture was allowed to stand for 10 min at room temperature. *N*-Hydroxysuccinimide precipitated and was filtered off, and the clear solution containing an urea derivative was evaporated. Each urea derivative was purified by recrystallization for the methanol–water system, and the structure was identified by mass spectrometry.

Derivatization Procedure. Reagent solution was prepared by dissolving SIPC, SIBr-PC, or SINC in acetonitrile in the concentration of 5 mM.

For alkyl amines. Sample solutions of alkylamine were prepared by dissolving various alkylamines in acetonitrile (1 mg/mL) and diluting with acetonitrile before use. To 20 μL of the sample solution was added 10 μL of the reagent solution. The reaction mixture was allowed to stand for 2 min at room temperature and then was mixed with 10 μL of 1% monoethanolamine acetonitrile solution. After being allowed to stand for 2 min, an aliquot (5–10 μL) of the mixture was injected directly into the HPLC.

For amino acids. Amino acids were dissolved in 0.1 N aqueous hydrochloride solution at a concentration of 1 mg/mL and diluted with the same hydrochloride solution before use. To 20 μL of the sample solution was added 30 μL of 0.3 M borate buffer (pH 9.5) and 50 μL of the reagent solution. The reaction mixture was allowed to stand for 5 min at room temperature. An aliquot (5 μL) of the resulting mixutre was injected directly into the HPLC.

Apparatus. An HPLC system consisted of an LC-3A high-pressure pump (Shimadzu Seisakusho, Kyoto, Japan), a valve universal injector (Sanuki industry, Tokyo, Japan), and an Develosil ODS-5 packed column (Nomura Chemical Co., Ltd., Seto-Shi, Japan, 150 × 4.6 i.d. mm). An SPD-2A spectrophotometric detector (Shimadzu) and an RF-530 spectrofluoromonitor (Shimadzu) were utilized properly. All chromatographic studies were performed at room temperature.

Ultraviolet absorption spectra and fluorescence spectra were measured by using a Model 200-20 spectrophotometer (Hitachi Seisakusho, Japan) and an RF-500 spectrofluorophotometer (Shimadzu), respectively.

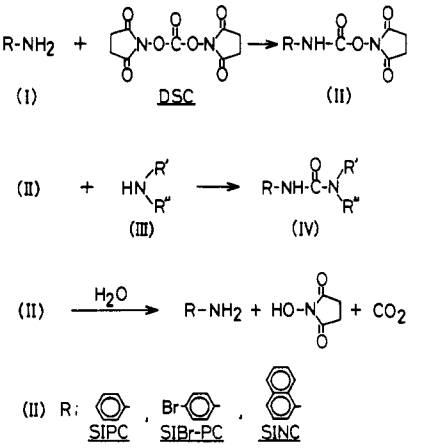

Figure 1. Mode of reactions for preparation of activated carbamate reagent, formation of urea derivative from activated carbamate reagent with amine, and alkaline hydrolysis of the reagent.

RESULTS AND DISCUSSION

Figure 1 shows the reaction course of DSC with amines (I and III), by way of succinimido carbamate (II). Carbamate compounds (II) prepared by reaction of DSC with chromogenic amines (I) are generally called "activated carbamate reagents". The activated carbamate reagent (II) afforded N,N'-substituted urea derivative (IV) on reaction with both primary and secondary amines (III) under mild conditions. The urea derivatives (IV) formed were the same as those obtained from the corresponding isocyanate type reagent. All the activated carbamate reagents examined were crystalline solid and had no lachrymatory activity. Both SIPC and SIBr-PC were stable for at least a several months in the solid state and for several weeks in acetonitrile solution at room temperature. SINC was somewhat unstable on exposure to light; nevertheless, the reagent could be used for at least 1 week in acetonitrile solution when stored in refrigerator.

Urea derivatives of SIPC and SIBr-PC showed absorption maxima at 240 and 250 nm, and their molar extinction coefficients were about 26 000 and 28 000, respectively. Accordingly, urea derivatives can be sensitively detected spectrophotometrically. The transition of conjugated π electrons of the aromatic ring and urea derivative bonding are responsible for these absorbances, which cannot be given by the amide formed on reaction of amines with carboxylic succinimido ester type reagent.

Activated carbamate reagents themselves showed irregular peaks on chromatograms when water-containing mobile phase was used (Figure 2A) . Isocyanate type reagents such as phenyl isocyanate also behaved similarly. The excess reagents interfered with the detection of urea derivatives (Figure 2B). This interference could be avoided by addition of a large amount of monoethanolamine to the reaction mixture, since the reaction product of monoethanolamine with the excess reagent eluted faster than any of the alkylamine derivatives (Figure 2C). A similar approach has previously been done (*24*) for chiral derivatization of amino acid enantiomers with peracetylated glyco isothiocyanate.

N-Hydroxysuccinimide was formed as the product of a derivatization reaction, but it did not interfered with the

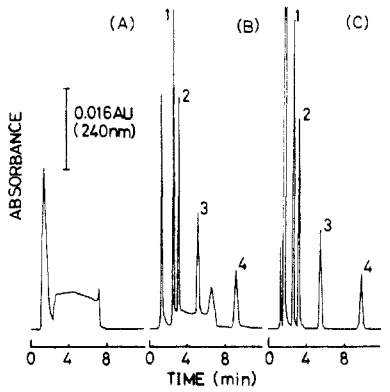

Figure 2. interference with detection of alkylamine derivatives by activated carbamate reagent removal using monoethanolamine: mobile phase, acetonitrile/water = 50/50; flow rate, 1.0 mL/min; (A) 5 μL of 5 mM SIPC/acetonitrile solution; (B) 5 μL of derivatization mixture without ethanolamine; (C) 5 μL of derivatization mixture to which was added ethanolamine as described in the Experimental Section; peak assignment, (a) *N*-hydroxysuccinimide, (b) ethanolamine, (1) *n*-propylamine, (2) isobutylamine, (3) di-*n*-propylamine, (4) diisobutylamine.

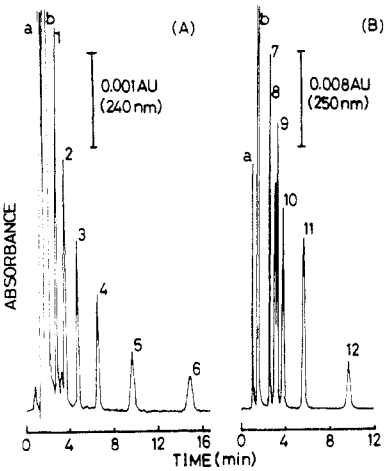

Figure 3. Chromatogram of urea derivatives formed from various alkylamines with SIPC (A) or SIBr-PC (B): mobile phase, acetonitrile/water, 50/50 (A) and 55/45 (B); flow rate, 1.0 mL/min (A) and 1.5 mL/min (B); peak assignment, (1) *n*-propylamine, (2) *n*-butylamine, (3) *n*-amylamine, (4) *n*-hexylamine, (5) *n*-heptylamine, (6) *n*-octylamine, (7) isobutylamine, (8) diethylamine, (9) isobutylamine, (10) *tert*-butylamine, (11) diisopropylamine, (12) diisobutylamine. Each numbered peak corresponds to 1 ng (A) and 10 ng (B) of alkylamine.

determination, since its peak appeared immediately after the solvent front.

The reaction yields of SIPC derivatives with various primary and secondary amines were examined by measuring their peak heights, which reached a plateau in 1–2 min. The peak heights were the same with those of the corresponding authentic samples. The reaction of both primary and secondary amines proved to be extremely rapid and quantitative.

Figure 3 shows typical chromatograms of urea derivatives of SIPC and SIBr-PC. Each derivative was resolved efficiently according to their hydrophobisity. Since SIBr-PC derivatives were retained more strongly than SIPC derivatives, an increased amount of acetonitrile was required for the elution of a SIBr-PC derivative at the same retention time as a SIPC derivative of the corresponding amine. Figure 3A indicates that a sub-nanogram level of the urea derivatives is detectable.

SINC prepared form napthylamine also reacted with amino compounds giving urea derivatives having blue fluorescence in the same manner as SIPC and SIBr-PC. Figure 4 shows the excitation and fluorescence spectra of SINC and its urea derivative of *n*-propylamine. The excitention and emission maxima of the urea derivative were at 305 and 378 nm, respectively. Figure 5 shows the chromatogram of SINC derivatives of various alkyl amines detected fluorometrically. The detection limits of these derivatives were 3–8 pg (S/N = 2).

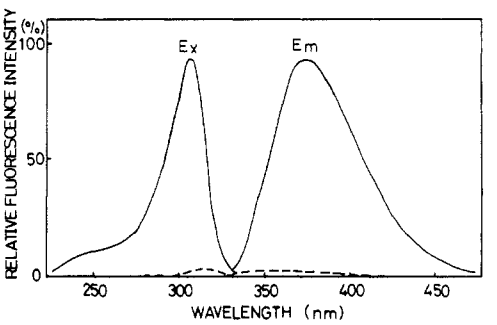

Figure 4. Excitation and fluorescence spectra of SINC (- - -) and its urea derivative of *n*-propylamine (——) in acetonitrile. The concentrations of two compounds are identical.

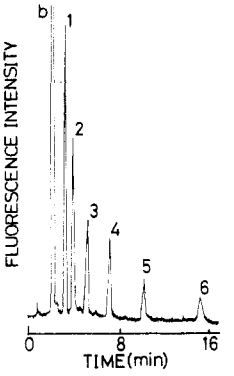

Figure 5. Chromatogram of urea derivatives formed from SINC with various alkylamines by using fluorometric detection: mobile phase, acetonitrile/water 55/45; flow rate, 1.5 mL/min; detector wavelength, λ_{Ex} = 305 nm and λ_{Em} = 378 nm. Peak assignment is same as in Figure 3. Each numbered peak corresponds to 25 pg of alkylamine.

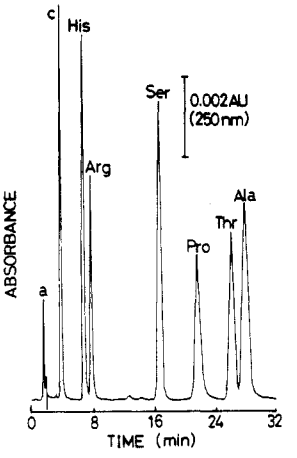

Figure 6. Chromatogram of urea derivatives formed from SIBr-PC with various amino acids: mobile phase, methanol/0.1 % aqueous phosphoric acid 45/55; flow rate, 1.0 mL/min; peak assignment (a) *N*-hydroxysuccinimide; (b) *p*-bromoaniline. Each peak corresponds to 50 ng of free amino acid.

Activated carbamate reagent readily reacted also with amino acids in an weak alkaline aqueous media giving urea derivatives quantitatively. Figure 6 shows a chromatogram of SIBr-PC derivatives of several amino acids, and standard curves for these derivatives are illustrated in Figure 7 . Histidine gave only a monosubstituted derivative. Under this deivatization condition, excess reagent was decomposed by hydrolysis in 3–4 min to give corresponding aromatic amines (Figure 1). Since the reagent was used in large excess and its

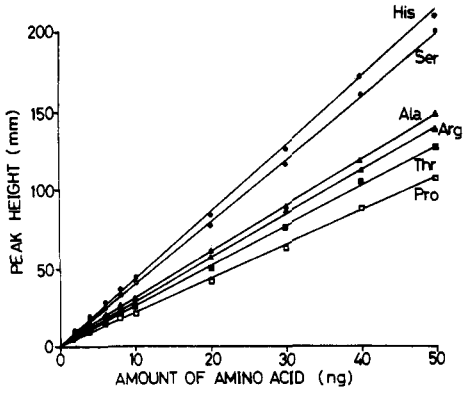

Figure 7. Standard curves for SIBr-PC derivatives of amino acids.

hydrolysis was much slower than the formation of urea derivatives, the determination of amino acids was performed without any influence of the hydrolysis (Figures 6 and 7). The resulting aromatic amine generated from the derivatization reagent gave a single peak that was widely apart from the peaks of amino acid derivatives (Figures 6 and 7). When SINC was used in the same aqueous media, excess reagent was also hydrolyzed giving fluorescent naphthylamine, which was also observed as a single peak. Coefficients of variation of the peak height given by 10 ng of each amino acids was in the range of 2.2–2.8 (n = 6). Detection limits of amino acids in this procedure were 0.15–0.3 ng (S/N = 2).

Many attempts (*27–30*) have recently been made to accomplish amino acid analysis by using precolumn derivatization with isothiocyanate type reagent. The present methods are also promising tools for the same purpose.

Activated carbamate reagent(s) was found to have enormous value in the liquid chromatographic analysis of amino compounds. Further, the reaction proceeding through an activated carbamate compound as an intermediate, as it is one of the bifunctional reactions should be applicable to the introduction of an appropriate probe to desired location of macromolecules such as biopolymers and fine supports.

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Liquid Chromatographic Determination of Amino Acids after Gas-Phase Hydrolysis and Derivatization with (Dimethylamino)azobenzenesulfonyl Chloride

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The level of amino acid background in the blank sample is one of the limiting factors for high sensitivity amino acid analysis. By use of the (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) precolumn labeling/HPLC method, the potential sources of contaminants during hydrolysis and derivatization were systematically investigated. The data indicate that the most likely source of amino acid contaminants is aqueous 6 N HCl. Gas-phase acid hydrolysis can reduce the background generated by normal liquid-phase hydrolysis to about 25%. A typical blank sample, after gas-phase hydrolysis, yields an average of 0.5–3 pmol of Asp, Glu, Ser, and Gly. This low level of background together with the improved DABS-Cl method permits routine and reliable amino acid analysis of low nanogram quantities of protein hydrolysates.

The sensitivity limit of amino acid analysis depends upon the background of contaminants. When nanogram quantities of proteins were isolated and hydrolyzed for amino acid composition analysis, the background could result from the contaminants introduced in protein purification, in 6 N HCl, or in the glasswares etc. Despite strenuous pruification and cleaning of the solvents and the glasswares, there are low picomole levels of Gly, Ser, and Asp that are very difficult to remove (*1–5*). With the emerging new technique of precolumn derivatization/RP-HPLC, which is now capable of detecting femtomole levels of amino acid derivatives (*6–26*), these low picomole amounts of amino acid contaminants have become the major obstacle for high sensitivity amino acid analysis.

In order to address this problem, we have investigated the

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