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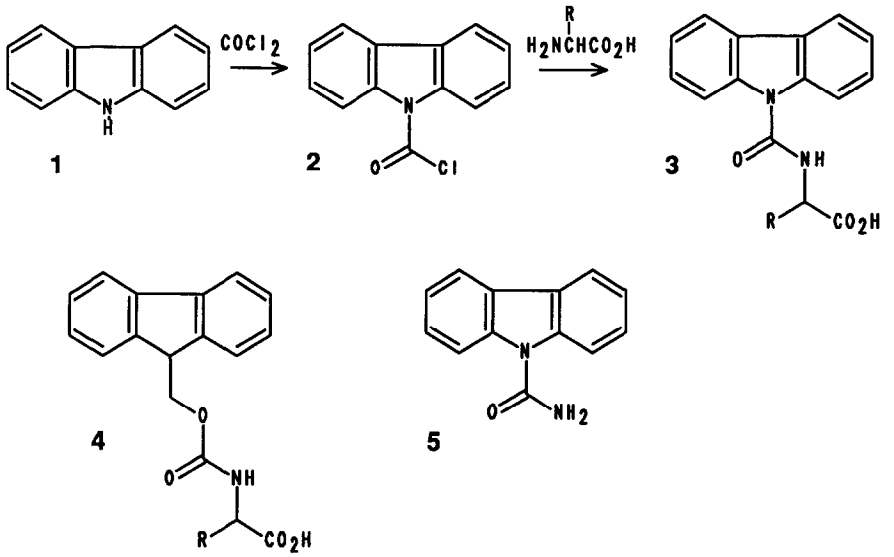
A USEFUL REAGENT FOR DERIVATIZATION OF AMINO ACIDS

Stig Allenmark

Laboratory of Microbiological Chemistry, University of Gothenburg, Guldhedsgatan 10A,
S-41346 Gothenburg, Sweden

SUMMARY: N-(Chloroformyl)-carbazole, which is readily synthesized by phosgenation of carbazole, has been shown to be an excellent reagent for fluorogenic labelling of amino acids. The derivatization reaction is carried out at room temperature in a borate buffer of pH 9 and yields highly fluorescent derivatives with good chromatographic properties. Further, the derivatives of amino acid enantiomers are well separated on BSA-silica (Resolvosil®) analytical columns.

Due to its well-known photophysical properties (strong fluorescence and long phosphorescence),[1] carbazole (**1**) is an interesting starting material for the synthesis of derivatization reagents. Since it is a secondary amine, reaction with phosgene gives a N-chloroformyl derivative (**2**) which will not undergo any subsequent elimination reactions. Compound **2** should, however, possess a high reactivity for nucleophilic halogen substitution and accordingly be useful for derivatization of amino acids. The amino protective chloroformate reagents, eg. 9-fluorenylmethyl chloroformate (FMOC-Cl), are primarily based on the same principle.[2] The carbamoyl chloride **2** presented in this communication, although described briefly in recent patent literature,[3] has not been used earlier for analytical derivatization purposes, however. The urea-linkage makes the amino acid derivatives (**3**) much more stable than those (**4**) containing the base-sensitive FMOC group.[4] The reactions used are outlined in Scheme 1.



Scheme 1.

Synthesis of the reagent. Carbazole (1.7 g), recrystallized from methanol and dried, was dissolved in a mixture of toluene (7.5 ml) and pyridine (1.5 ml) and heated to 90°C under stirring. A 20% (1.93 M) solution of phosgene in toluene (8 ml) was added during 20 min. After further 10 min the possible excess of phosgene was removed by a stream of nitrogen and the mixture allowed to cool. The precipitated pyridine hydrochloride was removed by filtration and the filtrate evaporated. Recrystallization of the crude product from toluene gave some unreacted carbazole. Evaporation of the mother liquor followed by repeated recrystallization of the remaining product from toluene + hexane, gave **2** as beautiful, long colourless needles of m.p. 102.5-3.0 °C (lit.[3]: m.p. 90-1°C). Final purification of the compound may also be achieved by flash chromatography in dichloromethane on a short silica gel column. ^{1}H NMR (δ(TMS) ppm; $CDCl_3$): 8.43 (d, 2H, J=7.5 Hz), 7.99 (d(split), 2H, J=7.5 Hz), 7.52 (t(split), 2H, J=7.5 Hz), 7.45 (t(split), 2H, J=7.5 Hz). TLC ($CHCl_3$/EtOAc 2/1): Rf=0.95, tailing to 0.10 . Treatment of an ether solution of **2** with an excess of conc. ammonia by shaking at room temperature prior to TLC, leads to a complete disappearence of the previous spots and instead a single new spot is found at Rf=0.51, corresponding to the urea derivative (**5**). Some carbazole (**1**) may be formed from **2** under hydrolytic conditions via the intermediate N-carboxy-carbazole. It (**1**) is easily identified at Rf=0.89 on the TLC plate.

Derivatization of amino acids. The procedure is a slight modification of the method used for derivatization with FMOC-Cl.[5] The reagent **2** is used as a 15 mM acetone solution. To 200 µl of the amino acid (1-100 µM) in 200 mM borate buffer (pH 9), is added 200 µl of the reagent solution and the mixture is shaken for 1 min. Removal of reagent excess is effected by extraction with 3x200 µl of hexane. An aliquot of the remaining aqueous phase is diluted (50x) with mobile phase prior to injection.

Chromatography. The analytical chromatographic system[6] was equipped with a variable wavelength fluorescence detector coupled to an integrator. The optimum wavelengths found for **3** in the aqueous mobile phases used, were $\lambda_{exc.}$=287 nm and $\lambda_{emiss.}$=340 nm.

Derivatization of an equimolar mixture of six amino acids showed the reaction to be uniform and quantitative. The derivatives show a good chromatographic behaviour on an octadecylsilica column, Fig. 1. The solution injected was 200 nM. A direct comparison with the FMOC-derivatives (**4**), run under identical chromatographic conditions, but with the use of $\lambda_{exc.}$=260 nm and $\lambda_{emiss.}$=313 nm, gave the following results: 1) For each amino acid derivative the fluorescence intensity ratio **3**/**4** was ca. 3; 2) the chromatographic capacity factors ($k' = V_R/V_0 - 1$) were constantly lower for compounds **3** by a factor of ca. 0.62±0.06; 3) both types of derivatives showed good peak symmetry under the conditions used.

Since it could be expected that the derivatives **3** of racemic amino acids should be possible to separate into enantiomers on columns utilizing bovine serum albumin (BSA) as the chiral stationary phase,[8] some preliminary experiments were performed. All of the ten amino acids investigated (Ser, Thr, Ala, Val, Leu, Ileu, Phe, Glu, Asp, Met), were baseline resolved into the enantiomers, except for Asp, which separated only partially under the conditions used. A typical chromatogram is shown in Fig. 2. The elution order of the enantiomers was determined by subsequent injection of a derivatized L-serine sample.

The chromatographic behaviour on the BSA phase was extremely dependent on the nature of the α-substituent R in **3**. The elution order (in terms of k_1'-values) of the derivatized amino acids investigated resembled the one obtained on octadecylsilica, thus demonstrating the large contribution of hydrophobic interaction to the retention process.

Some of the derivatized amino acids (threonine, alanine, phenylalanine) could be optically resolved with enantiomeric separation factors ($\alpha = k_1'/k_2'$) exceeding 3.

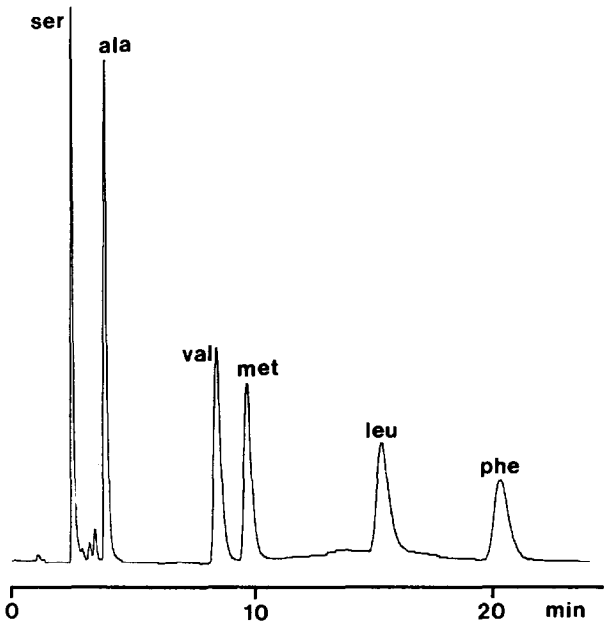


Fig. 1. Analytical reversed-phase chromatography of derivatized amino acids. Column[7]: 4.6x150 mm Nucleosil C18; mobile phase: Phosphate buffer (5 mM, pH 6.0, 25% acetonitrile), 1.5 ml/min. Each peak corresponds to 4 pmol.

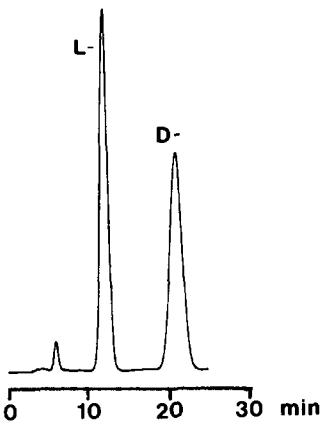


Fig. 2. Separation of the enantiomers of derivatized serine. Column[7]: 4.6x150 mm Resolvosil®BSA-7; mobile phase: Phosphate buffer (50 mM, pH 8.5, 8% 1-propanol), 1.5 ml/min.

The derivatives of the individual amino acids were readily isolated by collection of eluted fractions of a more concentrated sample (ca. 10 μM in each analyte) from a C18 column. The enantiomer composition of each amino acid could then be determined by reinjection of the individual fractions, without concentration, on a BSA column.

Due to the facts that: 1) The derivatization reaction proceeds without any detectable amino acid racemization and 2) the limit of detection is very low (<100 fmol), the method should be valuable for a precise determination of the optical purity of small amounts of amino acids.

Further work in this field is in progress.

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6. This was composed of a constant flow, high-pressure pump (ERC mod. 64), a 20 µl volume loop injector (Rheodyne mod. 7125), the analytical column, a fluorimetric detector (Shimadzu mod. 510 alt. mod. 535) and an integrator (Waters mod. 740 alt. Shimadzu mod. C-R5A Chromatopac).

7. From Macherey-Nagel GmbH, Düren, FRG.

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