Identification and Interaction of Amino Acids with Leucine-Anthracene Reagent by TLC and Spectrophotometry: Experimental and Theoretical Studies

Animesh Sahana, Sudipta Das, Raja Saha, Moumita Gupta, Subrata Laskar, and **Debasis Das***
Department of Chemistry, The University of Burdwan, Burdwan 713104, India

Abstract

A new reagent has been synthesized by coupling anthracene moiety to L-leucine. The reagent is characterized by different analytical techniques. It is capable for easy identification of various amino acids on thin-layer chromatography plates by developing distinguishable colors (detection limit between 0.1–0.5 µg at cold condition and 0.1–0.4 µg after heating). This reagent also binds with different amino acids very strongly in solution (methanol). Estimation of equilibrium binding constants of this new reagent with different amino acids has also been carried out. The values of the binding constants are lowest for L-Tyrosine (6.86×10^3 dm^3 mol^{-1}) and highest for L-Arginine monohydrochloride (8.86×10^5 dm^3 mol^{-1}) at 25°C. A theoretical study (Hartree–Fock) has been performed to investigate the interaction of the reagent with a representative amino acid, glycine.

Introduction

In recent times, amino acid derivatives have attracted immense interest as they can be used in the development of redox driven chirality switch (1–3) to modeling the structure and function of zinc enzymes like carboxypeptidase (4), alkaline phosphatase (5), and L-fuculose1-phosphate aldolase (6). Because of its rich chemistry and the wide area that remains uncovered, we are presently working on the synthesis of different derivatized chiral amino acids, their structural characterization, metal ion binding properties, and analytical applications of the these new series of ligands. Identification of amino acid is a key necessity in the evaluation of protein structure, as these compounds are the structural units of proteins and also for determination of the C-terminal units of degraded proteins. Thin-layer chromatography (TLC) is an important tool used for such a purpose, using various specific and non-specific reagents (7). Among the reagents used, ninhydrin as a non-specific reagent is the most well-known and widely used for its remarkable high sensitivity (8). But, it produces the same purple/violet color with all amino acids, except proline and hydroxyproline. An attempt has been made to overcome this problem by combining the newly synthesised N-anthracenylmethyl L-leucine (AML) and ninhydrin to form a new reagent, which affords distinguishable colors with many amino acids and enables convenient and easy detection of such compounds on silica "G" for TLC with very high sensitivity (detection limit between 0.1–0.5 µg at cold condition and 0.1–0.4 µg after heating). The equilibrium binding constants of the newly synthesized reagent with different amino acids has been determined, which indicates a molecular level interaction and formation of a molecular assembly between the reagent and the free amino acids.

Herein, we report the preparation of N-anthracenylmethyl L-leucine (AML), its characterization by different spectroscopic techniques, elemental analysis and analytical application for the trace level detection of amino acids on TLC plates. Estimation of equilibrium binding constants with different free amino acids has also been carried out. The mode of interaction of the newly synthesized reagent with a representative amino acid (glycine) has also been studied by Hartree–Fock method with Gaussian 03 program (9).

Experimental

Apparatus

IR spectra were recorded on a Jasco FT-IR (model: FTIR-H20) spectrometer. pH measurements were performed with Systronics digital pH meter (model 335). UV-vis spectra were obtained from a Shimadzu Spectrophotometer (model: UV2101PC). A Kratos MALDI-TOF 1 mass spectrometer using the matrix α-ACHC and an extraction voltage of 4 kV was used for mass spectrometry. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 200 MHz and 50 MHz Varian Gemini 200 and optical rotations were determined on a Perkin-Elmer model 341 polarimeter at 589 nm. TLC plates (20 × 20 cm, thickness 0.1 mm) were prepared using silica gel "G" (Merck, India) and a Unoplan coating apparatus (Shandon Scientific, London, UK). Sample solutions were spotted on to the plates by means of a graduated micropipette (5 µL). A theoretical calculation (Hartree–Fock) has been performed using Gaussian software (9).

Reagent

L-leucine (SRL, India), L-leucine methyl ester hydrochloride (Acros, USA), 9-anthracenecarboxyaldehyde (Aldrich), and $NaBH_4$ (Aldrich) were used as received. Standard amino acids and ninhydrin were procured from Sigma (USA) and n-propanol

*Author to whom correspondence should be addressed: email ddas100in@yahoo.com.

from Merck (India). Spectroscopic grade KBr (Aldrich) was used to record FTIR. $CDCl_3$ blended with TMS (internal standard) (Aldrich) was used for ^{1}HNMR and ^{13}CNMR spectroscopic studies. All other chemicals and solvents were used as received. N-anthracenylmethyl L-leucine (AML) was synthesized in our laboratory as described below.

General Methods

Synthesis of N-anthracenylmethyl L-leucine (AML).

Method 1 (10): To a solution of 2.0 g (15.25 mmol) L-leucine dissolved in 50 mL 4 M NaOH was added 3.15 g (1 eqv) 9-anthracenecarboxyaldehyde under stirring condition over a period of about 1 h. The stirring was continued for additional 2 h followed by addition of 0.5 g $NaBH_4$ in small portions. Stirring was continued till foaming ceases. Then the pH of the solution was adjusted to 3.0 with 6 M HCl. Some suspended particulates were separated by filtration. The volume of the filtrate reduced to 20 mL by vacuum distillation and kept overnight. The orange crystalline product was isolated and dried: the yield was 55%. M.P. 85 ± 1°C:

Method 2 (11): To a 100 mL dichloromethane solution containing L-leucine methyl ester hydrochloride (1 g, 6.89 mmol), 1 equivalent of 9-anthracenecarboxyaldehyde (1.42 g, 1 eqv) was added. To this mixture, 20 mL triethylamine and 6 g anhydrous magnesium sulphate were added under stirring condition for 1 h at room temperature. Filtration, removal of solvent, water–ether partitioning, and finally removal of ether yielded 70% of the Schiff base ester. This ester (1.0 g) was dissolved in THF and subjected to hydrolysis with 0.5 mol/L (20 mL) aqueous LiOH at room temperature for 4 h. The solvent was removed under reduced pressure and subjected to water–dichloromethane partitioning to remove any un-reacted ester or other impurities. To the aqueous part, 0.3 g $NaBH_4$ in small portion was added under stirring condition till foaming ceases. The pH of the aqueous part was maintained at 7.0–7.5 with dilute HCl and extracted with ethyl acetate. Traces of water were removed by anhydrous Na_2CO_3. Solvent was removed under reduced pressure and the desired compound was crystallized from methanol. Yield: 65%.

^{1}H-NMR (200 MHz, $CDCl_3$, peaks of the spectrum are relatively broad): δ 1.15 (6H, m, CH_3); 1.85 (1H, m, CH); 1.60 (2H, m, CH_2); 4.35(1H, t, J = 7 Hz, CH); 3.62 (2H, m, CH_2); 8.45(1H, s); 7.95(2H, d); 7.45(4H, t); 7.92(2H, d); ^{13}C-NMR (50 MHz, $CDCl_3$); δ 21.9, 23.8, 40.1, 56.2, 64.2, 122.6, 125.5, 128.3, 130,130.2, 175. MS (m/e): 322.63 $(M+H)^+$. Anal. Calcd. for $C_{21}H_{23}NO_2$: C(78.47%) H(7.21%) N(4.36%). Found: C(78.87%) H(6.74%) N(4.23%). FT-IR data (cm^{-1}): ν 1622 (CO); 3393 (OH); 1642 and 1552 (anthracene part). UV-vis data (in acetonitrile): λ (nm), 390 (ε = 90000 $dm^3mol^{-1}cm^{-1}$); 360 (ε = 75000 $dm^3mol^{-1}cm^{-1}$); $[\alpha]^{25}D = +54°$ (c 0.033 g /100 mL, acetonitrile).

Detection of amino acids on TLC plates

Standard solutions (1 mg/mL) of amino acids were prepared in 0.01 mol/L phosphate buffer (pH 8.0) and spotted on the TLC plates. Spotting volume was always 1 µL; the solutions were diluted approximately when necessary. Plates were air-dried and subjected to TLC using n-propanol-water, 70:30 (v/v) as mobile phase. After development plates were dried and sprayed with 0.01% N-anthracenylmethyl L-leucine (AML) in acetone (Reagent 1) and again dried in air for complete evaporation of solvent. The plates were then sprayed with 0.25 % ninhydrin in acetone (Reagent 2), dried in air and colors were noted (Table I). The plates were then heated at 110°C for 10 min in an oven and the colors were recorded again. Colors were always observed visually. Detection limits for the amino acids after use of ninhydrin alone are also given in Table I.

Determination of equilibrium binding constants (12)

Standard solutions (1×10^{-5} M) of amino acids were prepared at pH 8.0 using phosphate buffer. The concentration of the reagent solution in methanol was fifty times higher than that of amino acids. Keeping the reagent concentration fixed, the concentration of an amino acid was varied in different sets. The total volume of the mixture was kept constant (10 mL). It is interesting to note that the absorbance of the mixture increases with time and hence the mixtures were kept overnight for the attainment of equilibrium as verified by the stable value of absorbance.

Table I. Colors formed by amino acids on TLC plates with AML–ninhydrin as spray reagent with detection limits for ninhydrin reagent and their R_f-values in *n*-propanol-water (70:30) system

Amino acids	Color observed before heating*	Color observed after heating*	Detection limits for Ninhydrin reagent†	R_f‡ values
Arginine	Red (0.10)	Blood red (0.10)	0.01	0.02
Cysteine	Brownish violet (0.50)	Lilac (0.40)	0.02	0.38
Cystine	Pale violet (0.40)	Pale pink (0.20)	0.01	0.32
Histidine	Yellowish orange (0.20)	Dark orange (0.10)	0.05	0.20
Isoleucine	Yellowish orange (0.10)	Dark orange (0.10)	0.20	0.53
Glutamine	Pale violet (0.30)	Pink (0.10)	0.10	0.15
Lysine	Pink (0.20)	Brownish violet (0.20)	0.005	0.03
Asparagine	Pale pink (0.30)	Reddish pink (0.20)	0.10	0.14
Tryptophan	Lilac (0.30)	Deep violet (0.20)	0.05	0.62
Phenylalanine	Light brownish violet (0.40)	Violet (0.20)	0.05	0.58
Serine	Reddish pink (0.20)	Brick red (0.10)	0.008	0.35
Threonine	Lilac (0.40)	Deep violet (0.20)	0.05	0.37
Alanine	Reddish violet (0.10)	Brick red (0.10)	0.009	0.37
Glutamic acid	Orange violet (0.10)	Reddish violet (0.10)	0.04	0.35
Valine	Violet (0.30)	Reddish violet (0.10)	0.01	0.45
Methionine	Brick red (0.40)	Deep Reddish violet (0.30)	0.01	0.51
Aspartic acid	Blue (0.40)	Violet (0.20)	0.10	0.33
Tyrosine	Violet (0.40)	Brownish violet (0.30)	0.03	0.57
Leucine	Brick red (0.10)	Blood red (0.10)	0.01	0.55
Glycine	Orange (0.10)	Reddish orange (0.10)	0.001	0.32
Proline	Yellowish green (0.20)	Yellow (0.20)	0.10	0.26
Hydroxyproline	Yellowish brown (0.20)	Brownish violet (0.10)	0.05	0.34

* Values in the parentheses are the detection limits (in µg) of individual amino acids.
† Reference (2).
‡ in *n*-propanol–water (70:30).

The highest wavelength band (382) of the reagent undergoes a red shift (390 nm), after mixing with the amino acid. The extent of red shift is insignificant for different amino acids. The absorbance of each set was measured at 390 nm (the wavelength where the absorbance was changing with time, immediate after mixing, indicating some sort of interaction between the reagent and amino acid) after keeping the mixtures overnight. Then the binding constants were calculated using the Benesi- Hildebrand equation [13] for a cell having 1 cm optical path length:

$[A]_0\ [B]_0/d = [B]_0 / \varepsilon + 1/k\varepsilon$; where $[A]_0$ and $[B]_0$

are the initial concentrations of the ligand and amino acid respectively; d is the absorbance of the complex measured against the free ligand having concentration $[A]_0$, k is the binding constant and ε is the molar extinction coefficient of the molecular complex. A representative plot is shown in Figure 1.

Application

Detection of amino acids present in A. excelsa seed protein on TLC plate

Preparation of protein hydrolysate: Seed protein (4 mg) was hydrolysed with 6 mol/L HCl (2 mL) in an evacuated sealed glass tube by heating at 110°C for 24 h in a temperature controlled oven. The hydrolysate was filtered and excess HCl was removed under reduced pressure at 40–50°C. To remove traces of HCl (if any) from the thin film of hydrochlorides of amino acids, it was placed in a vacuum desicator over solid anhydrous KOH for 36 h. The hydrolysate was then dissolved in warm water, filtered and evaporated to dryness with great care. Finally, it was dissolved in 1 mL of 10% *n*-propanol. This solution of protein hydrolysate and standard amino acids were spotted on the TLC plate with graduated micro-pipette (25 µL) and subjected to run with *n*-propanol–water (70:30; v/v) as mobile phase. The plates were then dried and sprayed with the 0.01% solution of *N*-anthracenylmethyl L-leucine (AML) in acetone. The plates were then air died and heated to 110°C for 10 min and sprayed with 0.25% Ninhydrin solution in acetone. The plates were again air dried and the colors were noted. Finally, the plates were again heated to 110°C for 10 min and colors were noted.

From the observed colors of amino acids (both seed protein hydrolysate and standard amino acids) and also comparing the Rf values with the standard amino acids, it was possible to identify fourteen amino acids present in the seed protein A. excelsa.

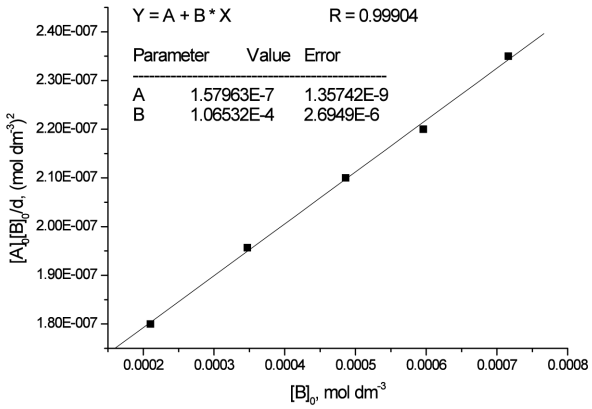

Figure 1. Benesi Hildebrand plot for the estimation of binding constant of the reagent AML with L-tyrosine.

Theoretical studies

Hartree–Fock method (11) using STO 3-21G* basis set have been applied to study the interaction and obtain an energy minimized structure of the molecular complex between the ligand AML and the simplest amino acid, glycine.

Results and Discussion

Synthesis

Synthesis of the *N*-anthracenylmethyl L-leucine (AML) [IUPAC: 2-[(Anthracen-9-ylmethyl)-amino]-4-methyl-pentanoic acid] derivative by the two methods are shown in Figure 2.

Structural properties of the ligand

Both ^{1}H-NMR and ^{13}C-NMR spectra show the agreement with the proposed structure of the ligand whereas FT-IR and UV-vis spectra support the functionalities present in the molecule. Elemental analysis and mass spectral data also confirms the molecular formula as assigned. Combining all these, the structure of the ligand is designated as 1.

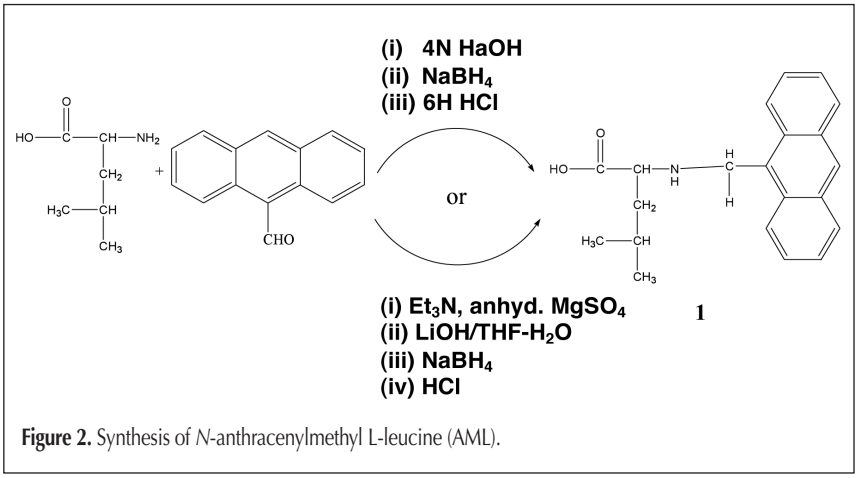

Figure 2. Synthesis of *N*-anthracenylmethyl L-leucine (AML).

Detection

It is observed from Table I that detection limits obtained after uses of the reagent are very low both before (0.1–0.5 µg) and after (0.1–0.4 µg) heating and various distinguishable colors were produced. Figure 3 (see page 4A) shows a representative digital picture of a TLC plate on which some of the colors of the AML-amino acid molecular complex has been developed. Sometimes the detection limit is same before and after heating and in other cases it is somewhat different. It should be noted that identification of amino acids by ninhydrin is in practice difficult, in spite of the high sensitivity of ninhydrin. The advantage of the new reagent is that it can differentiate visually different amino acids as listed in Table I.

Determination of equilibrium binding constant

Table II shows the values of equilibrium binding constants of different amino acids with the newly synthesized ligand. The values are fairly high. The results indicate molecular association between the reagent and amino acids. Most possibly, there may be a hydrogen bonding interaction between the two components. The widely different binding constant values may provide a useful analytical tool for the separation of different amino acids by column chromatography using our reagent as a immobilized stationary phase on silica or alumina.

Application of the developed method for detection of amino acids present in A. excelsa seed protein on TLC plate

It is found that use of combo reagent (Spraying of 0.01% solution of *N*-anthracenylmethyl L-leucine (AML) in acetone followed by spraying with 0.25% ninhydrin solution in acetone) detected fourteen amino acids viz. arginine, isoleucine, glutamine, lysine, asparagine, phenylalanine, serine, alanine, glutamic acid, valine, aspartic acid, leucine, glycine, and proline. The results are also in conformity with their respective Rf values.

Though the combo reagent is very useful for the detection of amino acids by their respective colors, the color of the amino acids present in lesser concentration may be masked by that of higher concentration amino acids having closer R_f values.

Molecular level interaction

Stereoscopic view of the energy minimized structure of the molecular complex between the ligand AMM and glycine using Hartree–Fock method (STO 3-21G* basis set) have been shown in Figure 4A (See Page 4A). Contour diagram and Highest Occupied Molecular Orbital interactions are presented in the Figure 4B (see page 4A) and S-1 (supplementary materials) respectively. Total dipole moment of the associated molecular complex is 1.6927D, which plausibly indicates a charge separated species formed in the energy minimized form of the molecular complex. Values for various bond moments are presented as supplementary materials (S1).

Table II. Equilibrium Binding Constants (k) and Molar Extinction Coefficients (ε) of the Molecular Complexes Between the Reagent (AML) and Different Amino Acids at pH 8.0 (phosphate buffer) at 25°C.

Sl. No.	Amino acids	k ($dm^3\ mol^{-1}$)	ε ($dm^3 mol^{-1} cm^{-1}$)
1	L-Glycine	9.72×10^4	2.39×10^3
2	L-Alanine	3.49×10^4	8.38×10^3
3	L-Phenylalanine	8.35×10^4	9.09×10^3
4	L-Methionine	6.93×10^4	8.90×10^3
5	L-Leucine	7.23×10^4	1.69×10^3
6	L-Isoleucine	8.39×10^4	8.98×10^3
7	L-Serine	9.93×10^4	7.89×10^3
8	L-Tyrosine	6.86×10^3	9.26×10^3
9	L-Histidine monohydrochloride	7.90×10^4	6.17×10^4
10	L-Tryptophan	9.11×10^4	9.17×10^3
11	L-Valine	9.77×10^4	2.18×10^3
12	L-Threonine	7.46×10^4	8.65×10^3
13	L-Proline	6.14×10^5	8.92×10^4
14	L-Hydroxyproline	8.36×10^4	8.30×10^3
15	L-Cystine	7.86×10^4	8.83×10^3
16	L-Glutamic acid	5.59×10^4	8.89×10^3
17	L-Aspertic acid	3.26×10^4	9.49×10^3
18	L-Glutamine	1.44×10^4	8.78×10^3
19	L-Arginine monohydrochloride	8.86×10^5	7.81×10^4
20	L-Aspergine	4.16×10^4	9.79×10^3

Conclusion

The newly synthesised *N*-anthracenylmethyl L-leucine (AML) in combination with ninhydrin turns out to be a very effective for detection of different amino acids by affording distinguishable colors on silica "G" for TLC with very high sensitivity (detection limit between 0.1–0.5 µg at cold condition and 0.1–0.4 µg after heating). The reagent AML also binds with different amino acids very strongly in solution (aqueous methanol–phosphate buffer mixture, pH 8.0). The molecular level interaction of the reagent with different amino acids (simplest amino acid glycine have been chosen) have been established by theoretical (Hartree Fock method) studies.

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