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A New Bromine-Containing Reagent for Cysteine Modification

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Abstract—5-Bromo-2[(2-iodoacetyl)amino]benzenesulfonic acid (AIBSA), a reagent for modification of free of cysteine thiol groups in proteins and peptides, was synthesized. Rate constants of its interaction with thiol groups were determined. The presence of a bromine atom allows an easy identification of the AIBSA-labeled peptides in mass spectra due to the characteristic isotope distribution. The compound is stable in solution and under exposure to light.

Key words: cysteine, isotope distribution, mass spectrometry, protein modification

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INTRODUCTION

Introduction of covalent label that gives special properties to the studied molecules of proteins and peptides is widely used in the investigations of polypeptides [1, 2].[2] The presence of a label attached to functional groups of a protein allows elucidation of its structure and functions, its quantitative determination and localization, etc.

Amino- or thiol functions of polypeptides are mainly modified by fluorescent, UV-absorbing, radioactive, biotin and other groups [3–5]. Type of the used label is often determined by the applied method of investigation. For example, the ^{15}N, ^{13}C, or ^{2}H nuclei are used in the NMR spectroscopy, whereas introduction of Se is convenient for the X-ray analysis [6]. Fluorescent groups provide a high sensitivity of analysis, and spectrophotometric detection is mostly used in chromatographic systems.

The majority of the recent achievements in the polypeptide chemistry is associated with the application and development of mass spectrometry. In this connection, the use of substances containing isotope labels for protein derivatization becomes widespread [7]. The application of $H_2^{18}O$ in the polypeptide cleavage proved to be especially effective [8, 9].

The use of bromine-containing substances for introduction of the label into a peptide is convenient, because two equally occurred isotopes with molecular masses of 79 and 81 are present in the bromine atom and form a characteristic multiplet isotope signal of the analyzed substance. Such form of the isotope distribution allows a facile identification of signals of the labeled peptides in complex mass spectra.

Application of the bromine-containing reagents for labeling oligonucleotides and DNA is described in literature [10–12]. The bromine label is seldom introduced into amino groups of proteins [13–15]. The reagent (4-bromo-2-iodoacetamide) exists; it is used for the introduction of the bromine-containing label into SH groups of proteins [16]. However, it is poorly soluble in water and can penetrate through cellular membranes and modify cytoplasmic and membrane cysteine-containing proteins of living cells. We synthesized AIBSA for the selective modification of membrane proteins by the bromine-containing label. This reagent can alkylate the thiol group of cysteine, gives hydrophobic properties to a protein molecule, and prevents its penetration into a living cell.

RESULTS AND DISCUSSION

2-Amino-5-bromobenzenesulfonic acid was prepared by sulfonation of *p*-bromoaniline. AIBSA was synthesized by the carbodiimide method (scheme). The structure of this compound was proved by NMR, UV, and IR spectroscopy and mass spectrometry.

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[2] Abbreviations: AIBSA, 5-bromo-2[(2-iodoacetyl)amino]benzenesulfonic acid; ESI TOF MS, a time-off-flight mass spectrometer equipped with an electrospray ionization source.)

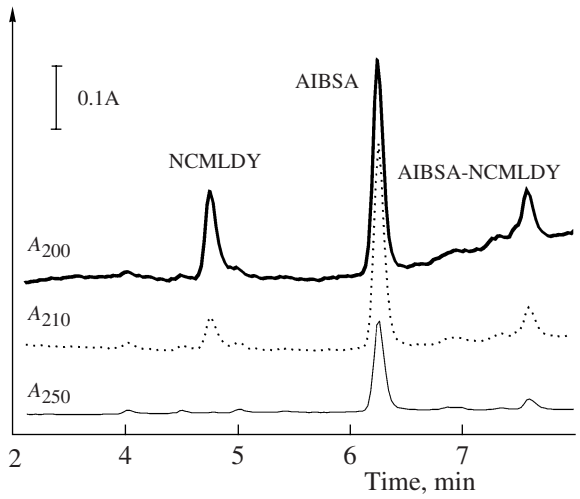
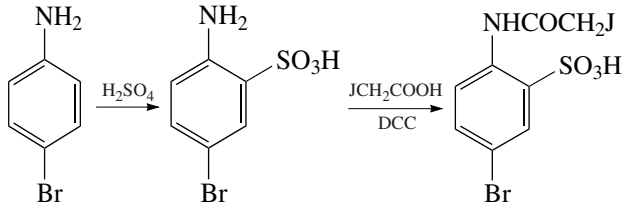


Fig. 1. Chromatogram of the reaction mixture of alkylation of 0.05 mM NCMLDY peptide with 0.5 mM AIBSA 16 min after beginning of the reaction.



Scheme. Synthesis of AIBSA.

The alkylating ability of AIBSA was proved on the synthetic peptide NCMLDY. The alkylation was monitored by HPLC (Fig. 1). The rate constant of alkylation of thiol groups was 102 l mol^{-1} min^{-1} and corresponded to the halftime of conversion of 13.5 min at the concentrations of reagent and substrate of 0.5 and 0.05 M, respectively. The structure of the preparatively purified derivative was confirmed by the ESI TOF MS (Fig. 2).

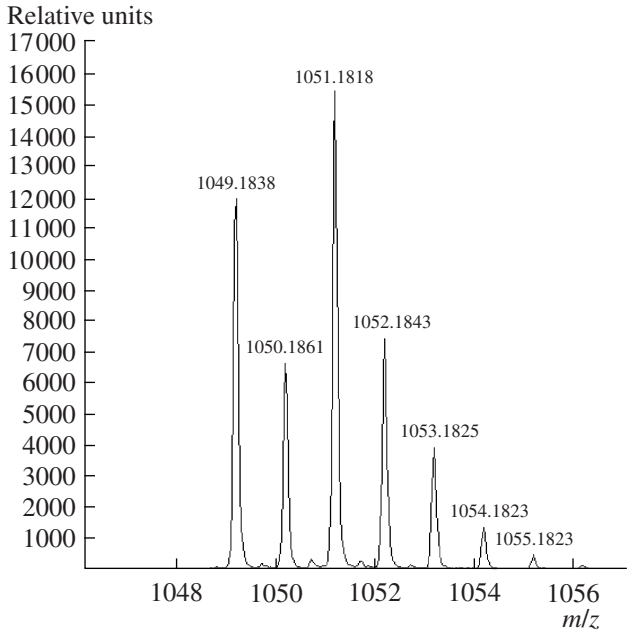


Fig. 2. A fragment of mass spectrum of the product of interaction of the NCMLDY peptide with AIBSA.

The efficiency of AIBSA for labeling the protein fragments containing SH groups was demonstrated by the example of lysozyme. The trypsinolysis of lysozyme and subsequent analysis of peptides were achieved by the standard procedure [17] with the replacement of iodoacetic acid on the stage of methylation of carboxyl groups of the reduced cysteine residues of lysozyme by AIBSA. The peaks of the cysteine-free peptides preserved their positions on the HPLC chromatogram (Fig. 3), whereas those subjected to the modification had increased retention times. For example, the absence on the chromatogram of NLCNTPCSALLSSDITASVNCAK peptide, usually detected among the trypsinolysis products, can be explained by the attachment of three AIBSA residues, which results in its long retention time (Fig. 2).

The peptides formed after the trypinolysis were separated by HPLS (Fig. 3), and their modified structures were confirmed by ESI TOF MS (table, Fig.4). Isotopic multiplet signals of the modified bromine-containing peptides have a special pattern and can be easily distinguished from the signals of similar peptides without the label. Additional chromophore increases the sensitivity of determination of the AIBSA-labeled peptides in the long-wave UV area.

EXPERIMENTAL

The NCMLDY hexapeptide was synthesized by N.N. Karpyshev in the NPO Vector. All the solutions

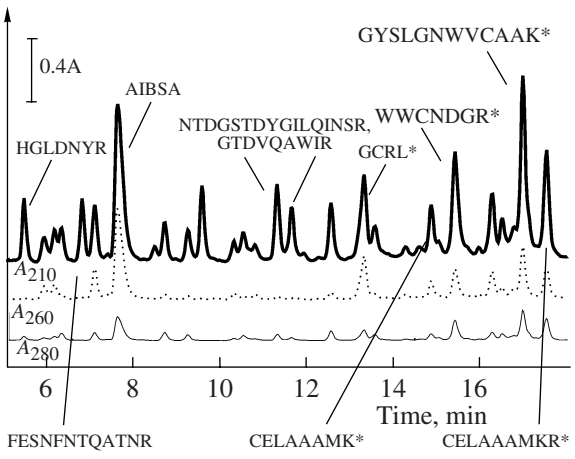


Fig. 3. Chromatogram of the peptides obtained after the trypsinolysis of lysozyme alkylated by AIBSA. Peptides containing the modified cysteine are indicated by asterisks.

were prepared in laboratory glassware filled with argon using water saturated with argon.

Trypsin TPCK was from Sigma (United States). IR spectra were recorded on an Infralum FT-801 IR Fourier spectrometer. ^{1}H NMR spectra were registered on a VXR-500S spectrometer (Varian). Chemical shifts (δ, ppm) were recorded relative to 2,2-dimethyl-2-silapentane-5-sulfonic acid. Chromatograms were obtained on a Milikhrom A-02 liquid chromatograph (ZAO EkoNova, Novosibirsk) equipped with a Silasorb SPH C-18 column (75 × 2 mm). TOF mass spectra were recorded on a MX-5303 mass spectrometer with electrospray ionization (Institute of Analytical Instrument Making, Russian Academy of Sciences, St. Petersburg).

5-Bromo-2[(2-iodoacetyl)amino]benzensulfonic acid. a. Sodium salt of 2-amino-5-bromobenzenesulfonic acid was synthesized according to the modified procedure [18]. 4-Bromoaniline (5 g, 29 mmol) (Aldrich, United States) was mixed with dry quartz sand (10 ml) with a particle size less than 1 mm in a porcelain mortar. Concentrate sulfuric acid (2.85 g, 29 mmol) was added, and the mixture was carefully ground. The reaction mixture was placed in a round-bottom flask and heated to 170–190°C at 10 mm Hg pressure for 5 h. Then, the flask was cooled, and the reaction mixture with the sand was transferred into a KOH solution (2 g per 250 ml of water), stirred for 30 min, and filtered. Hydrochloric acid (10%) was added dropwise to the filtrate to pH 3. The precipitate was filtered and dried in a vacuum. 2-Amino-5-bromobenzenesulfonic acid was obtained in yield 4.54 g (62%); mp (with decomposition) 275–280°C. This value corresponded to the literature data [19]. The resulting compound (5 g, 19.8 mmol) was mixed with water (5 ml) and $NaHCO_3$ (1.67 g, 19.8 mmol). Water was removed by azeotropic distillation with toluene (50 ml), and the residue was separated and dried in a vacuum. Sodium salt of 2-amino-5-bromobenzenesulfonic acid (5.43 g) was prepared in a quantitative yield; IR (KBr, ν, cm^{-1}): 3072, 3051, 3020, 1482, 1467, 817, 720 (benzene ring), 1230, 1185, 1147, 1033 (SO_3^-) group), 3423, 3300, 1624, 1289, 1119, 627 (amino group), 528 (C–Br).

b. AIBSA. A solution of sodium 2-amino-5-bromobenzenesulfonate (5.43 g) in DMF (75 ml) was cooled to 0°C, and iodoacetic acid (4.81 g, 25.9 mmol) (ACROS) was added to the solution. The mixture was stirred to solution, and *N*,*N*'-dicyclohexylcarbodiimide (7.69 g, 37.3 mmol) (Aldrich) was added. The reaction mixture was kept for 24 h at 0°C. The precipitate was removed. The filtrate was evaporated at a residual pressure of 1 mm of Hg, and the residue was recrystallized from ethanol. The purity of the product was determined by HPLC. AIBSA was prepared as its sodium salt in yield of 2.5 g (29%); mp 238–241°C; IR (KBr, ν, cm^{-1}): 3290, 1674, 1524 (amide), 1239, 1185, 1150, 1025 (sulfonic group), 3038, 3025, 3060, 821 (benzene), 2971, 2932, 2855 (methylene group), 528 (C–Br), 680 (C–I); ^{1}H NMR (δ, ppm): 3.00 (2 H, s, CH_2); 7.00–8.30 (3 H, m, Ar), and 10.50 (1 H, s, NH). Found, %: C 22.0, H 1.2, N 3.1, S 7.4. Calcd. for $C_8H_6BrINNaO_4S$, %: C 21.7, H 1.4, N 3.2, S 7.3.

Identified tryptic peptides of lysozyme

Amino acid sequence	MH^+	
	calculated	determined
HGLDNYR	874.42	874.42
FESNFNTQATNR	1428.65	1428.64
NTDGSTDYGILQINSR	1753.84	1753.91
GTDVQAWIR	1045.54	1045.55
GCRL*	739.23	739.15
CELAAAMK*	1127.4	1127.54
WWCNDGR*	1227.38	1227.53
GYSLGNWVCAAK*	1559.61	1560.12
CELAAAMKR*	1284.23	1284.35

Note: * Peptides containing the modified cysteine.

Alkylation of the NCMLDY peptide (Asn-Cys-Met-Leu-Asp-Tyr). A solution of the NCMLDY peptide (1 mM, 10 μl) in 0.1 M hydrochloric acid was placed in a glass tube of a 250-μl volume; 0.1 M $NaHCO_3$ (50 μl), H_2O (120 μl), and 5 mM solution of AIBSA (20 μl) in 50% acetonitrile were added to the reaction mixture under vigorous stirring. The reaction was monitored by HPLC (Fig. 1) at the interval of 12 min. The chromatographic conditions were: 0.05 M KH_2PO_4 (pH 6.0) in 5% aqueous acetonitrile served as eluent A and methanol served as eluent B. The column was eluted with a gradient of B in A (from 15 to 75% for 9 min) at a flow rate of 200 μl/min; the column temperature was 45°C; detection was at 200, 210, 220, 250, 260, 270, 290, and 330 nm. Areas of peaks of the NCMLDY peptide (T_r 4.7 min) and the reaction product (T_r 7.6 min) were determined at a wavelength of 220 nm. The fraction of the reaction product was preparatively separated, and its structure was confirmed by ESI TOF MS.

The rate constant of the reaction was determined from the slope of the time dependence of $\log(C_0/C)$, where t is time from the reaction start, min; C_0 the starting area of the NCMLDY peptide peak; and C is the area of NCMLDY peptide peak at the time t.

Alkylation of lysozyme. Lysozyme was reduced by mercaptoethanol by a standard procedure [17]. The thiol groups of reduced lysozyme (5.6×10^{-2} mM) were modified by AIBSA (30 mM of AIBSA in 0.1 M Tris-

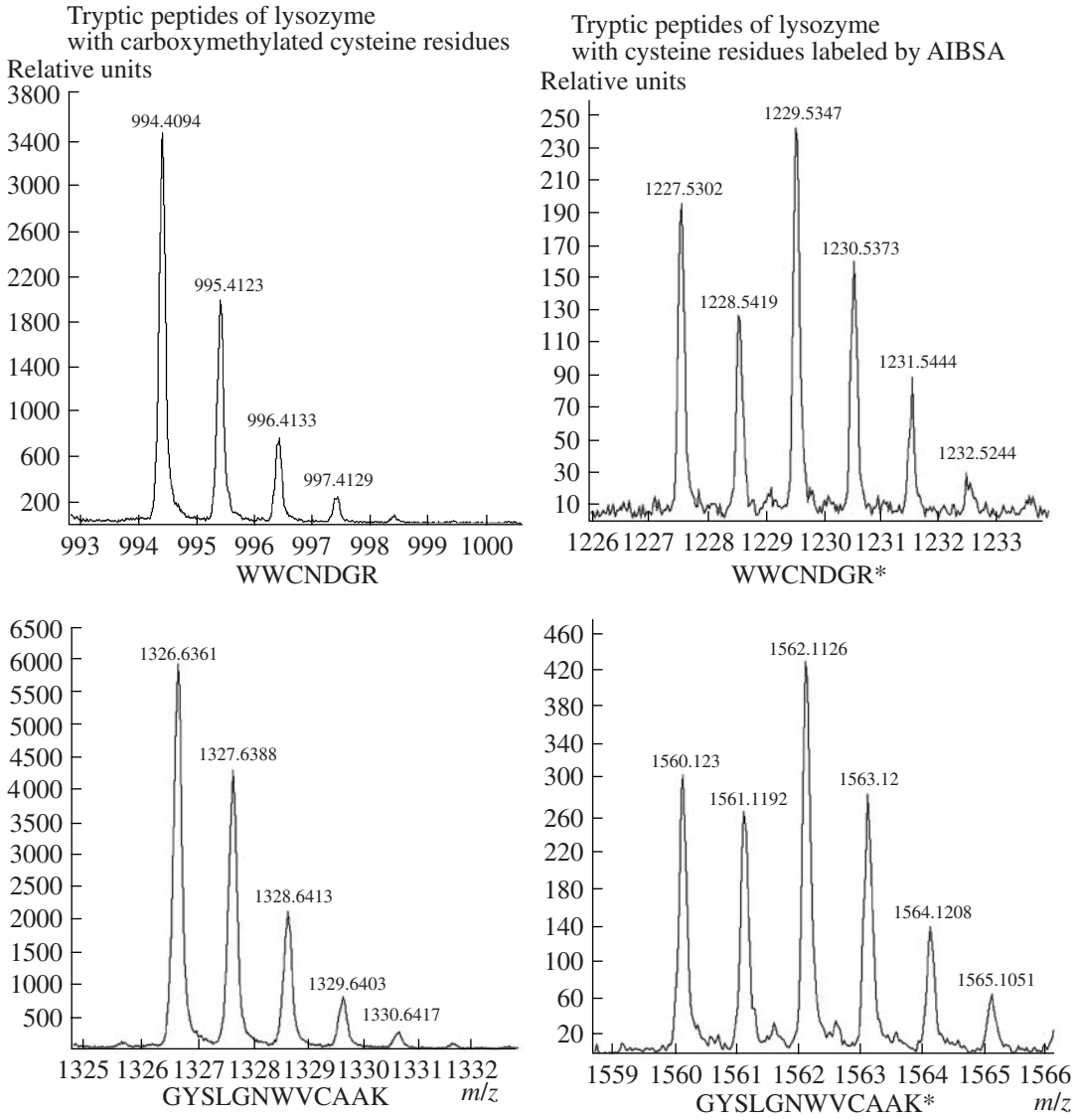


Fig. 4. Fragments of mass spectra of the lysozyme tryptic peptides.

HCl, pH 8.0, and 7 M urea). The reaction mixture was kept for 1 h at room temperature, and mercaptoethanol (2 μl) was then added. The reaction mixture was kept overnight in a refrigerator and 3.5-fold diluted with distilled water. The precipitate of modified lysozyme was separated by centrifugation, washed with water, suspended in 2 M urea (0.5 ml), and treated with trypsin (30 μl, 1 mg/ml) at 38°C. The trypsinolysis was monitored by HPLC (Fig. 2). The peptides were preparatively separated, and their structures were confirmed by ESI TOF MS (Fig. 3).

Thus, a new reagent for alkylation of Cys residues in proteins and peptides is created. This reagent provides for a selective detection of the labeled compounds by mass spectrometry. The selectivity was achieved due to the presence of bromine in the reagent that gives characteristic isotopic multiplet signal.

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REFERENCES

1. Roffman, E., *Biochem. Biophys. Res. Commun.,* 1986, vol. 136, pp. 80–85.
2. Khalfan, H., Abuknesha, R., Rand-Weaver, M., Price, R.G., and Robinson, D., *Histochem.*, 1986, vol. 18, pp. 497–499.
3. Earl Hudson, N. and Weber, G., *Biochemistry,* 1973, vol. 12, pp. 4154–4161.

4. Lux, B. and Gerard, D., *Biol. Chem.*, 1981, vol. 256, pp. 1767–1771.
5. Bruch, R.C. and White, H.B., *Biochemistry*, 1982, vol. 21, pp. 334–341.
6. *J. Comput. Biol.*, 2001, vol. 8, pp. 19–36.
7. Shevchenko, A., Chernushevich, I., Shevchenko, A., Wilm, M., and Mann, M., *Mol. Biotechnol.*, 2002, vol. 20, pp. 107–118.
8. Shevchenko, A. and Shevchenko, A., *Anal. Biochem.*, 2001, vol. 296, pp. 279–283.
9. Lin, D., Tabb, D.L., and Yates, J.R., III, *Biochim. Biophys. Acta*, 2003, vol. 1646, pp. 1–10.
10. Vroegh, J., Erik, F.J., de Vries, Elsinga, P.H., and Vaalburg, W., *Appl. Radiation and Isotopes*, 2003, vol. 58, pp. 469–476.
11. Steinberg, J.J., Oliver, G.W., Jr., Farah, N., Simoni, P., Winiarsky, R., and Cajigas, A., *J. Chrom. B,* 1997, vol. 694, pp. 333–341.
12. Rosato, M.T., Jabbour, A.J., Ponce, R.A., Kavanagh, T.J., Takaro, T.K., Hill, J.P., Poot, M., Rabinovitch, P.S., and Faustman, E.M., *J. Immunol. Methods*, 2001, vol. 256, pp. 35–46.
13. Hollemeyer, K., Heinzle, E., and Tholey, A., *Proteomics*, 2002, vol. 11, pp. 1524–1531.
14. Weiss, S.J., Test, S.T., Eckmann, C.M., and Ross, D., *Science*, 1986, vol. 234, pp. 200–202.
15. Wu, W., Chen, Y., d'Avignon, A., and Hazen, S.L., *Biochemistry*, 1999, vol. 38, pp. 3538–3548.
16. Adams, J.S. and Deebe, G.F., *J. Chem. Eng. Data*, 1967, vol. 12, pp. 619–621.
17. Practical Protein Chemistry: A Handbook, Darbre, A., Ed., J. Wiley & Sons, 1987. Translated under the title: *Prakticheskaya khimiya belka*, Moscow: Mir, 1989.
18. *Preparativnaya organicheskaya khimiya*, Polaczkowa, W., Ed., Warszawa: Panstwowe Wydavnictwa Techniczne, 1954. Translated under the title: *Preparativnaya organicheskaya khimiya* Moscow: Goskhimizdat, 1959.
19. Scott, J.R. and Cohen, J.B., *J. Chem. Soc. Trans,* 1922, vol. 121, pp. 2034–2051.