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Furan-based acetylating agent for the chemical modification of proteins

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ABSTRACT

We have synthesized a furan-based acetylating agent, 2,5-bisacetoxymethylfuran (BAMF) from carbohydrate derived 5-hydroxymethylfurfural (HMF) and studied its acetylation activity with amines and cytochrome *c*. The results show that BAMF can modify proteins in biological conditions without affecting their structure and function. The modification of cytochrome *c* with BAMF occurred through the reduction of heme center, but there was no change in the coordination property of iron and the tertiary structure of cytochrome *c*. Further analysis using MALDI-TOF-MS spectrometer suggests that BAMF selectively targeted lysine amino acid of cytochrome *c* under our experimental conditions. Kinetics study revealed that the modification of cytochrome *c* with BAMF took place at faster rates than aspirin.

1. Introduction

Protein aggregation and misfolding is a serious concern in the recent time. Many neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and prion diseases are associated with this.[1,2] Hydrophobic interaction between amino acids in different globin chains causes hemoglobin polymerization and results sickle cell disease (SCD).[3] Sometimes abnormal change in protein structure can lower the solubility of protein in the body fluid which causes supersaturation. Example includes sickle cell disease where sickle hemoglobin aggregates due to structural change in β-globin unit.[4] To overcome this problem, the only remedy is the modification of protein with proper modifying agent.

Protein modification is of major interest in chemical biology. In this regard, post-translational modification (PTM) is essential for regulating the function, stability, localization and targeting of many eukaryotic proteins.[5,6] Phosphorylation, farnesylation, ubiquitination, glycosylation, acetylation, formylation, amidation, sumoylation, biotinylation are the examples of PTM. Many chemical methods have been developed for such purpose by carefully balancing the reactivity and selectivity.[7–9] Site-selective chemical modification of a protein requires an efficient reaction and an interesting molecule to attach. Experimental facts have proved that Nt-acetylated proteins are more stable in vivo than non-acetylated proteins.[10] Also the N-terminus of a protein has unique pH-dependent reactivity and is thus an attractive target for single-site modification.[11] Blocking of the N-terminus by Nt-acetylation potentially prevents N-terminal ubiquitination, and thus stabilizes the protein.[12,13] Lysine contains a primary amine group which is protonated under biological pH, but it can still react as a nucleophile. Acetylation of lysine groups in proteins has been extensively used for modifying enzymatic properties, immunological reactivity, and proteolytic digestion patterns. Lysine acetylation has emerged as a major post-translational modification for histones in modulating chromatin-based transcriptional control. It is a reversible posttranslational modification (PTM), which neutralizes the positive charge of this amino acid, changing protein function in diverse ways.[14,15] Lysine acetylation is also important for p53 functions and interactions and for microtubule stabilization.[16]

Enormous number of enzymatic acetylating agent is known these days for chemical acetylation of biomolecules. However, only a few numbers of chemical acetylating agents is reported. Acetic anhydride is the simplest example of acetylating agent which can modify protein molecules by N-terminal acetylation of lysine residues.[17,18] But it is very hard to control its action due to its rapid reaction in aqueous environment. High reactivity also decreases the selectivity towards the target molecules. Aspirin is currently one of the most frequently used drugs which has been shown to acetylate proteins and biomolecules such as hemoglobin, DNA, RNA and histones, as well as several plasma constituents, including

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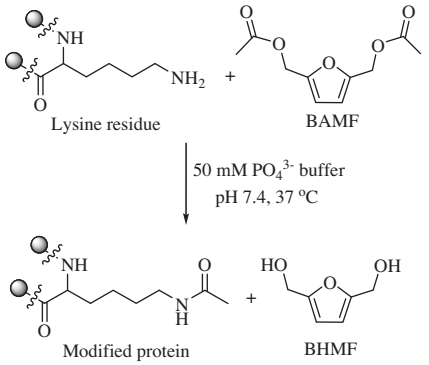

Scheme 1. Modification of lysine residues in protein leading to adduct formation via amidation reaction.

hormones and enzymes.[19] Aspirin is reported as an antisickling agent which modifies sickle hemoglobin through βLys-82 acetylation.[20] Walder et al. reported a bromo aspirin derivative, acetyl-3,5-dibromosalicylic acid (dibromoaspirin), as an antisickling agent which demonstrated potential acetylating ability for intracellular hemoglobin in vitro.[21] The bromoaspirin effectively targets the amine groups of Hb S unit to get acetylated, which increases the oxygen affinity to exert the antisickling effects. But sometimes the use of aspirins can cause fatal gastrointestinal bleeding, hemorrhagic strokes, nephrotoxicity, and adverse effects on the central nervous system. Thus investigations for new acetylating agents will remain continue in the context of suitable application with minimum side-effects.

In the present work we report the synthesis of a furan-based acetylating compound 2,5-bisacetyloxymethylfuran (BAMF) from 5-hydroxymethylfurfural (HMF) which is a dehydration product of hexose sugars.[22,23] Although BAMF was first isolated as a natural product from an ethyl acetate extract of the terrestrial *Streptomyces* species,[24] the chemical synthesis of this compound has not been reported yet. We have shown that this compound acetylates primary amine groups in the biological pH range. The preliminary results have motivated us to further study its efficiency in the acetylation of cyclohexylamine and L-lysine (Scheme 1). Cytochrome *c* and lysozyme were selected as model proteins because of their different chemical characteristics, high content and easy accessibility of surface lysine residues, hydrophobicity indices and extensive structural information. Cytochrome *c* also plays a key role in the energy production in mitochondria and also has so many enzymatic activities in animals. BAMF has successfully modified both the proteins without changing their structure and activity.

2. Experimental

2.1. Materials

5-Hydroxymethylfurfural, cytochrome *c* (from bovine heart) and lysozyme (from chicken egg white) were purchased from Sigma–Aldrich. Sodium borohydride and sodium acetate were purchased from SRL, India. Acetic anhydride, ethyl acetate and dichloromethane were supplied by S D Fine-Chem, India. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate and potassium chloride were obtained from Merck. Guanidinium chloride (GdmCl) was the ultrapure sample from MP Biomedicals. Unless otherwise stated, all chemicals were used without further purification and distilled water was used as aqueous phase.

2.2. Synthesis of 2,5-bishydroxymethylfuran (BHMF)

In a 50 mL round bottom flask 0.63 g (5 mmol) HMF was dissolved in 10 mL distilled water. In another flask, 0.19 g (5 mmol) $NaBH_4$ was dissolved in 3 mL distilled water. Then $NaBH_4$ solution was added drop wise to the HMF solution with continuous stirring. The reduction process was very fast. After the complete reduction, the product (BHMF) was isolated by extracting with ethyl acetate (4 × 20 mL). Here the aqueous phase was saturated with NaCl for the quantitative extraction of BHMF. After evaporation of ethyl acetate, BHMF was obtained as white solid product (0.64 g, 100% yield) and characterized by NMR studies. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.16 (s, 2H, 2C*H*), 4.50 (s, 4H, 2CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.0, 108.5, 57.4.

2.3. Synthesis of 2,5-bisacetyloxymethylfuran (BAMF)

In a 10 mL round bottom flask 0.512 g (4 mmol) BHMF was dissolved in 1.89 mL (20 mmol) of acetic anhydride. Then sodium acetate (0.066 g, 0.8 mmol) was added into the mixture as a catalyst and it was stirred for 6 h at room temperature. After the reaction, 2 mL of distilled water was added into the reaction mixture very slowly to consume the excess acetic anhydride. Reaction product was isolated by extracting with dichloromethane (5 × 8 mL). After evaporation of dichloromethane BAMF was obtained as white solid product (0.611 g, 72% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.35 (s, 2H, 2C*H*), 5.01 (s, 4H, 2CH_2), 2.06 (s, 6H, 2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.5 (*C*=O), 150 (*C*–O), 111.4 (*C*H), 57.9 (CH_2), 20.7 (CH_3). Melting point = 62 °C.

2.4. Acetylation of cyclohexylamine

In a 10 mL glass vial 19.8 mg (0.2 mmol) cyclohexylamine and 21.2 mg (0.1 mmol) BAMF were taken. To it 1 mL of 50 mM potassium phosphate buffer (pH = 7.4) was added and the reaction mixture was stirred at 37 °C for 6 h. After the completion of reaction, the aqueous phase was saturated with NaCl and the product was isolated by extracting with ethyl acetate. Product was characterized by ^{1}H NMR (Fig. S3) and yield of acetylated cyclohexylamine was calculated by using mesitylene as an external standard. Yield = 82%.

2.5. Acetylation of L-lysine

In a 10 mL glass vial 29.2 mg (0.2 mmol) L-lysine and 21.2 mg (0.1 mmol) BAMF were taken. To it 1 mL of 50 mM potassium phosphate buffer (pH = 7.4) was added and the reaction mixture was stirred at 37 °C for 6 h. Due to high solubility of lysine and its amide in aqueous phase, it was not possible to characterize the product by ^{1}H NMR. In this case UV–vis spectroscopy was used since the lysine amide shows an absorbance at 215 nm, while free lysine does not show any absorbance in this range (Fig. S4). Absorbance of free BAMF was subtracted from the acetylated lysine.

2.6. Preparation of protein samples

Lysozyme and cytochrome *c* solutions were dialyzed extensively against 0.1 M KCl at pH 7.0 at ~4 °C. Protein stock solutions were filtered using 0.22-μm millipore filter paper. All the proteins gave a single band during polyacrylamide gel electrophoresis. Concentration of the protein solutions was determined experimentally using the molar absorption coefficient (ε) values (3.9×10^4 M^{-1} cm^{-1} at 280 nm for lysozyme, and 1.06×10^5 M^{-1} cm^{-1} at 410 nm for cytochrome *c*). The concentration of GdmCl stock solution was determined by refractive index measurements. All solutions for optical measurements were prepared in the desired degassed buffer. For desired pH range, 50 mM phosphate buffer (pH 7.4) was used. Since pH of the protein solution may change on the addition of co-solvents, pH of each solution was also measured after the denaturation experiments.