ISSN 2706-6819 Vol.6, Issue 12: 45-52, DOI: 10.25236/IJFM.2024.061207

Design and Synthesis of Molecular Probes for the Determination of the Target of the Anti-HBV Lead PA-XY1

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Abstract: *Despite the availability of effective vaccines, hepatitis B virus (HBV) infection remains a significant global public health issue, causing nearly 1 million deaths annually. Nucleoside analogs, which act as competitive inhibitors of viral DNA polymerase and have been approved for the treatment of chronic HBV infection, show no inhibitory effect on the expression of HBeAg or HBsAg. Furthermore, the rapid development of drug-resistant viruses has become a major challenge. Therefore, there is an urgent need to discover novel chemical entities with new mechanisms of action or new therapeutic targets against HBV. Based on structure-activity relationship studies and computer-aided design, structural modifications of PA led to the development of a highly effective and low-toxicity anti-HBV molecule—PA-XY1. Further mechanistic studies indicated that PA-XY1 significantly downregulates the expression of hepatocyte nuclear factor-4α (HNF-4α), a mechanism distinct from traditional interferon-based and nucleoside analog anti-HBV drugs. This novel discovery suggests that PA-XY1 may act on a new therapeutic target; however, the specific molecular target remains unclear. Therefore, it is essential to further investigate the molecular target of the anti-HBV lead compound PA-XY1 to support the development of novel anti-HBV drugs.*

Keywords: *Hepatitis B virus; Active molecule; PA-XY1; Molecular probe; Design and synthesis*

1. Introduction

The hepatitis B vaccine is an effective measure for preventing Hepatitis B Virus (HBV) infection; however, it has no effect on individuals already infected with HBV. Clinically, the most commonly used anti-HBV drugs include interferon-based therapies and nucleoside analogs. However, both types of drugs suffer from issues such as significant toxicity or drug resistance. Interferons can inhibit viral replication, but they are ineffective in the majority of infected patients and often cause flu-like symptoms [1], cytopenia, neuropsychiatric symptoms, gastrointestinal disturbances, and endocrine disorders, among other side effects. Nucleoside analogs target HBV DNA polymerase but cannot destroy the viral DNA; as a result, they only suppress HBV replication. Once treatment is discontinued, viral rebound frequently occurs. Therefore, current mainstream anti-HBV drugs are limited by structural diversity, significant side effects, and suboptimal therapeutic outcomes [2].

Phyllanthusol A, a natural anti-hepatitis B virus active molecule isolated and identified from Phyllanthus emblica (referred to hereafter as PA), has shown the ability to inhibit the secretion of hepatitis B e antigen (HBeAg). However, PA is limited by high toxicity and insufficient activity. Therefore, our research group performed structural modifications of PA, resulting in the discovery of the lead anti-HBV compound PA-XY1. Further studies revealed that PA-XY1 significantly downregulates the expression of hepatocyte nuclear factor-4α (HNF-4α), which differs from the mechanisms of action of traditional interferon-based and nucleoside analog anti-HBV drugs [3]. This novel mechanism of action suggests that PA-XY1 may target a new molecular site, necessitating the identification of its target to lay the foundation for the development of innovative anti-HBV drugs.

2. ABPP molecular probe technology

Affinity-based protein profiling (ABPP) is a technology used to study proteins that interact with drugs, primarily employing affinity-based probes (ABPs) as research tools to investigate the effects of drugs on protein structure and function. The experimental design of ABPP molecular probes involves the

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following steps: First, an active small-molecule probe containing both a reactive group and a reporter group is constructed; Secondly, the small-molecule probe binds to the target protein through covalent interaction facilitated by the reactive group. Finally, the reporter group in the active small-molecule probe is utilized to efficiently isolate the target protein from the proteome by exploiting its physical, chemical, or biological properties. The isolated target protein is subsequently analyzed and identified using techniques such as LC-MS/MS [4].

ABPP is currently a widely used and rapidly evolving technique. A crucial component of this method is the design and synthesis of small-molecule probes (ABPs). These probes typically consist of a reporter group (e.g., biotin, fluorescein), a linker group (e.g., hydrophilic chains, hydrophobic chains, peptide chains), and a reactive group (active small molecule). Generally, ABP probes are designed by directly introducing a reporter group into the active small molecule to capture the target protein. However, this approach has certain limitations. The incorporation of large reporter groups, such as biotin or fluorescein, often interferes with the activity of the small molecule, leading to reduced or lost activity. Additionally, large reporter groups may hinder the probe's ability to penetrate the cell membrane, making it difficult to identify intracellular target proteins. Consequently, these probes are typically used in cell lysates and can only capture target proteins that interact covalently with the small molecule. With the advancement of technology, the synthesis of probes in ABPP methods is no longer limited to traditional approaches [5]. The introduction of photoaffinity labeling (PAL) and click chemistry (CC) has expanded and supplemented conventional ABPP methodologies.

Accordingly, ABPP techniques can now be further subdivided based on different probe synthesis strategies into ABPP, CC-ABPP, PAL-ABPP, A representative application of ABPP small-molecule probes in the identification of natural product targets is adenanthin, a diterpenoid compound isolated from Rabdosia adenantha of the Lamiaceae family, which can induce differentiation of leukemia cells. Researchers performed molecular modifications on adenanthin [6]and synthesized a biotin-labeled adenanthin probe after determining its active group. Studies showed that adenanthin covalently binds to the CR site of peroxiredoxin Prx I and Prx II. Adenanthin significantly inhibits the enzymatic activity of Prx II and, more prominently, Prx I, thereby increasing intracellular hydrogen peroxide (H_2O_2) levels. In the presence of the H_2O_2 scavenger N-acetyl-l-cysteine (NAC), adenanthin's differentiation-inducing effect is almost completely abolished, indicating that H_2O_2 plays a critical role in adenanthin-induced differentiation. The ABPP molecular probe schematic diagram of adenanthin is shown in Figure 1.

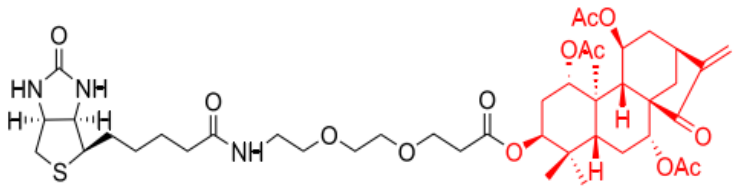

Figure 1: Schematic diagram of the ABPP molecular probe for adenanthin.

3. Design of PA-XY1 Molecular Probes

The interaction between PA-XY1 and target proteins is likely mediated by either irreversible covalent bonding or reversible non-covalent bonding. Additionally, it is important to consider that the same active group applied in different probe technologies may "capture" different proteins. Therefore, we synthesized four generations of probes using the ABPP (Activity-Based Protein Profiling) approach. First-generation probes were designed to label covalently bound target proteins. Second-generation probes were developed to label non-covalently bound target proteins. Third-generation probes were modified to address the potential issue of bulky labeling groups reducing labeling efficiency. In this case, an alkyne group was introduced as a potential reporter group to enhance labeling efficiency for non-covalently bound target proteins. Fourth-generation probes used rhodamine as a tag for intracellular localization of target proteins. The design process of the four generations of PA-XY1 molecular probes is as follows:

3.1. Design of the First-Generation PA-XY1 Molecular Probe

The interaction between PA-XY1 and its target proteins is hypothesized to occur through irreversible covalent bonding. Therefore, we referred to the ABPP strategy and designed the first-generation molecular probe based on the structural framework of ABPP molecular probes. The specific design structure is as follows: biotin was used as the reporter group. Biotin, also known as vitamin H or

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coenzyme A, is a water-soluble vitamin with a high affinity for avidin, enabling specific binding with streptavidin protein for labeling [7]. This interaction is characterized by high sensitivity, specificity, and minimal nonspecific interference, making it ideal for enriching, purifying, or identifying labeled proteins. Consequently, biotin has been widely used as a reporter group for chemical small-molecule probes. Additionally, the biotin-tagged complexes are easy to dissociate using solutions such as guanidine-HCl, biotin solution, or SDS solution. 1,4-diiodobutane was chosen as the linker group. A linker of appropriate length can prevent direct interference of the labeling group with the interaction between the probe molecule and the target protein, thereby maintaining the labeling efficiency of the probe molecule. Based on the above considerations and the structure-activity relationship analysis of PA-XY1's anti-HBV activity, we selected PA-XY1 as the active fragment. The probe molecule's additional fragments were constructed at the C-10 phenolic hydroxyl group of PA-XY1. Thus, the first-generation PA-XY1 molecular probe was designed, as illustrated in Figure 2.

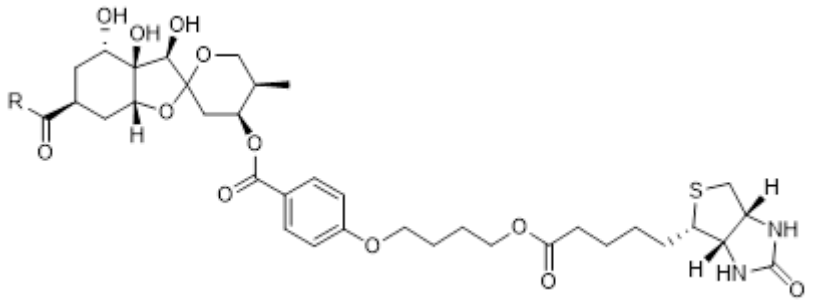

Figure 2: Schematic Diagram of the First-Generation PA-XY1 Molecular Probe.

3.2. Design of the Second-Generation PA-XY1 Molecular Probe

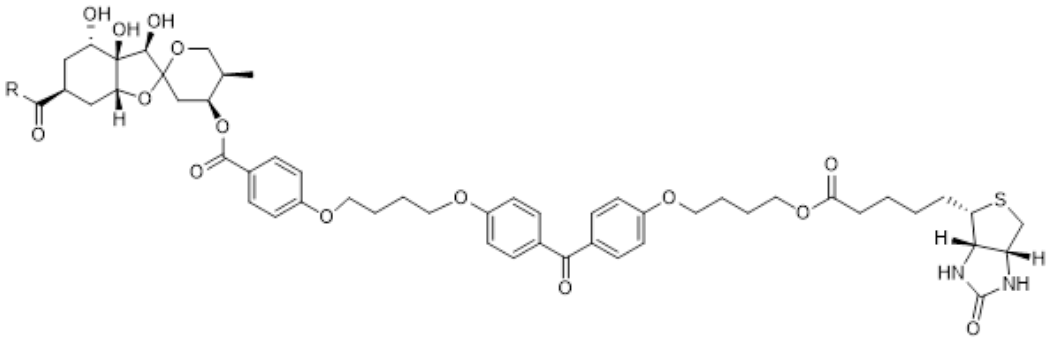

Figure 3: Schematic Diagram of the Second-Generation PA-XY1 Molecular Probe.

The interaction between PA-XY1 and target proteins is more likely to occur via reversible non-covalent bonding, which allows easy dissociation of the probe from the target protein during the separation process. Therefore, we referred to the ABPP (Activity-Based Protein Profiling) strategy and designed the second-generation molecular probe based on the structural framework of PAL-ABPP molecular probes. The specific design structure is as follows: Benzophenone-based photoaffinity labeling group. Benzophenone-based photoaffinity groups are widely used, highly efficient photoaffinity groups. Upon photolysis, they form a triplet biradical intermediate that undergoes minimal rearrangement reactions. These groups primarily react with C-H bonds in protein molecules [8] and are particularly stable in protic solvents, exhibiting negligible reactivity with water. This property ensures that the excited state of the probe selectively interacts with the target protein. Moreover, benzophenone-based probes exhibit high labeling efficiency and are easy to store. Biotin as the reporter group. Biotin, also known as vitamin H or coenzyme A, is a water-soluble vitamin with a high affinity for avidin, enabling specific binding with streptavidin protein for labeling. This reaction is characterized by high sensitivity, specificity, and minimal nonspecific interference, making it ideal for enriching, purifying, or identifying labeled proteins. Consequently, biotin is widely used as a reporter group for chemical small-molecule probes. Additionally, the biotin-tagged complexes can be conveniently dissociated using guanidine-HCl solution, biotin solution, or SDS solution. 1,4-diiodobutane was chosen as the linker group. A linker of appropriate length prevents the labeling group from directly interfering with the interaction between the probe molecule and the target protein, thereby ensuring the labeling efficiency of the probe molecule. Based on the above considerations and the structure-activity relationship analysis of PA-XY1's anti-HBV

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activity, we selected PA-XY1 as the active fragment. The other components of the probe molecule were constructed at the C-10 phenolic hydroxyl group of PA-XY1, resulting in the design of the second-generation PA-XY1 molecular probe. The detailed structure is shown in Figure 3.

3.3. Design of the Third-Generation PA-XY1 Molecular Probe

The interaction between PA-XY1 and target proteins is more likely to occur via reversible non-covalent bonding, which makes the probe prone to dissociation during the separation process. However, considering that the photoaffinity group and tag size of PAL-ABPP small-molecule probes may reduce labeling efficiency, we designed a third-generation molecular probe based on the PAL-CC-ABPP molecular probe structural framework, drawing on the ABPP strategy. The third-generation probe incorporates an alkyne group as a potential reporter group. After the probe molecule binds to the target protein, the alkyne group allows the introduction of a reporter group through click chemistry, thereby improving labeling efficiency. This design addresses the issue of steric hindrance caused by large photoaffinity groups or tags, ensuring more effective identification of target proteins. The specific design structure is as follows: Benzophenone-based photoaffinity labeling group. Benzophenone-based photoaffinity groups are a class of commonly used and highly efficient photoaffinity groups. Upon photolysis, these groups generate a triplet biradical intermediate that undergoes minimal rearrangement reactions. They primarily react with C-H bonds in protein molecules and are particularly stable in protic solvents, exhibiting negligible reactivity with water. This ensures that the excited state selectively interacts with target proteins, resulting in high labeling efficiency. Furthermore, benzophenone-based groups are convenient for storage.

Biotin, also known as vitamin H or coenzyme A, is a water-soluble vitamin with a high affinity for avidin, enabling specific binding with streptavidin proteins for labeling. This interaction is characterized by high sensitivity, specificity, and minimal nonspecific interference, making biotin ideal for enriching, purifying, or identifying labeled proteins. As a result, biotin has been widely used as a reporter group for chemical small-molecule probes. Additionally, the dissociation of tag reporter groups is convenient and can typically be achieved using solutions such as guanidine hydrochloride (guanidine-HCl), biotin solutions, and SDS solutions. Therefore, we selected azido-functionalized biotin, which allows for the incorporation of biotin through "Click Chemistry" after the probe molecule labels the target protein. This approach enhances the specificity and sensitivity of the labeling process [9]. Based on this information and the structure-activity relationship analysis of PA-XY1's anti-HBV activity, we designed the third-generation small-molecule probes of PA-XY1, using PA-XY1 as the active fragment and constructing other probe molecule segments at its C-10 phenolic hydroxyl group. The specific design is shown in Figure 4.

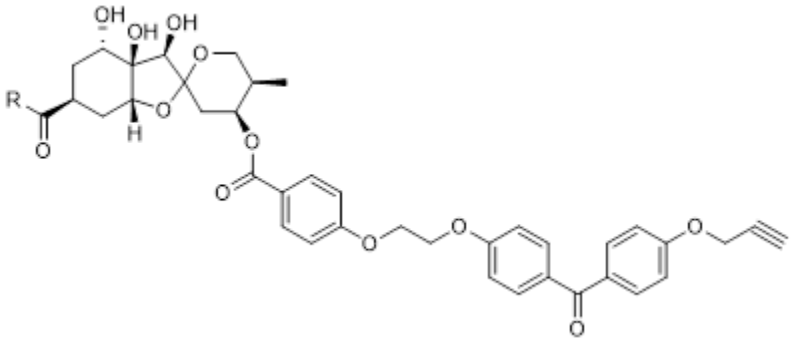

Figure 4: Schematic Diagram of the Third-Generation PA-XY1 Molecular Probe.

3.4. Design of the Fourth-Generation PA-XY1 Molecular Probe

Rhodamine B, also known as rose bengal B or basic rose bengal, is a synthetic dye with a vivid peach-red color. It is easily soluble in water and ethanol, slightly soluble in acetone and chloroform, and sparingly soluble in hydrochloric acid and sodium hydroxide solutions. It appears as a red to violet powder, producing a blue-red solution in water, which emits strong fluorescence when diluted. Its alcoholic solution exhibits red fluorescence. Due to its strong fluorescence in solution, Rhodamine B is commonly used as a fluorescent dye for cell staining in laboratory experiments. Within cells, the localization of Rhodamine B can be visually observed through cellular imaging, which helps confirm the exact position of target proteins in the cell [10]. To investigate the cellular localization of the target

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proteins responsible for PA-XY1's anti-HBV activity, this study references the activity-based protein profiling (ABPP) method to design the fourth-generation molecular probes. These probes follow the ABPP molecular probe structural model and incorporate a fluorescent group as the reporter group. The specific design utilizes 2-bromoethanol as the linker group. A linker group of sufficient length prevents the direct introduction of the labeling group from interfering with the binding efficiency between the probe molecule and the target protein, thereby ensuring the labeling efficiency of the probe molecule. Based on this information and the structure-activity relationship analysis of PA-XY1's anti-HBV activity, we designed the fourth-generation small-molecule probes of PA-XY1. Using PA-XY1 as the active fragment, we constructed additional probe molecular segments at its C-10 phenolic hydroxyl group. The specific design is illustrated in Figure 5.

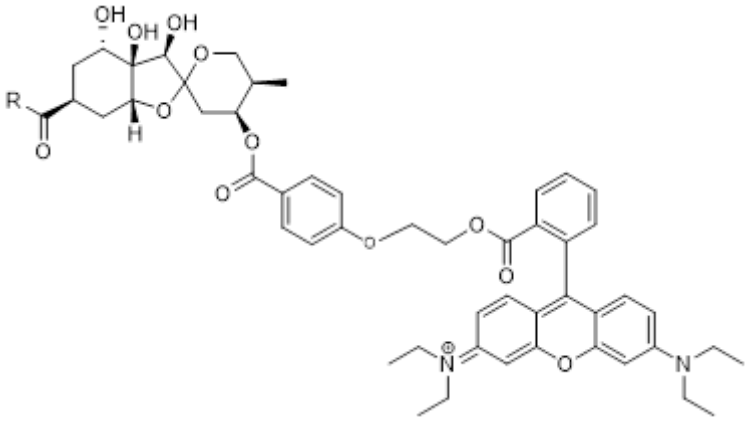

Figure 5: Schematic Diagram of the Fourth-Generation PA-XY1 Molecular Probe.

4. Synthesis of PA-XY1 Molecular Probes

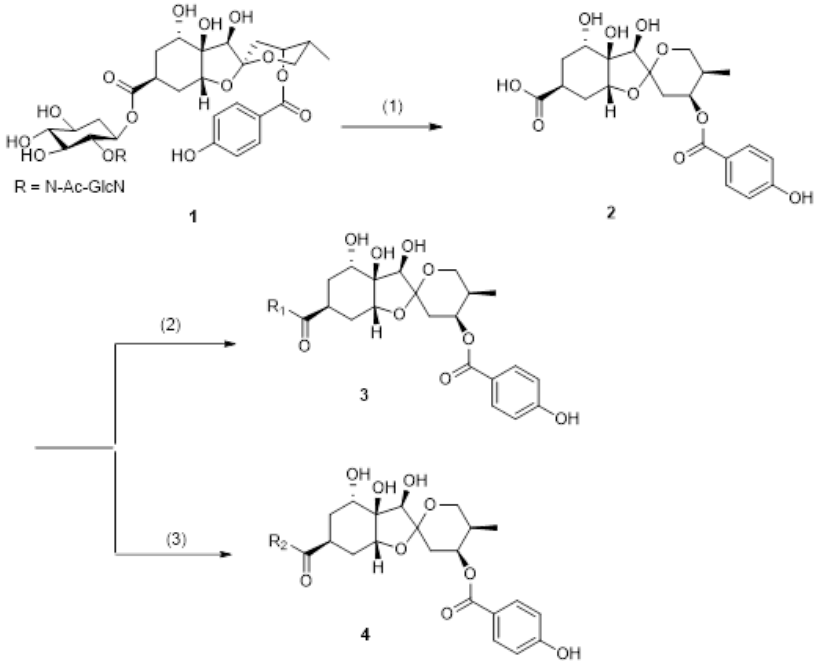

Figure 6: Schematic diagram of the synthesis route for PA-XY1 and PA-N.

Small-molecule probes interact with proteins not only specifically but also potentially nonspecifically within cells or cell lysates. Since probe molecules are composed of an active fragment, a linker segment, a labeling group, and a photoaffinity group, each component has the potential to interact with proteins. Therefore, for each class of probes, we synthesized three types of probe molecules: blank small-molecule probes, positive small-molecule probes, and negative small-molecule probes. This was done to eliminate background protein interference caused by non-active segments, such as linker segments, labeling groups, and photoaffinity groups. Specifically, the blank small-molecule probes consist of the linker segment,

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labeling group, and photoaffinity group; the positive small-molecule probes are composed of the active fragment, linker segment, labeling group, and photoaffinity group; while the negative small-molecule probes consist of the active fragment, linker segment, labeling group, and photoaffinity group. Compound 1 undergoes alkaline hydrolysis in 10% K_2CO_3 solution to yield Compound 2. Compound 2 reacts with an amine under the conditions of EDCI and HOSu as coupling agents and DMAP as a catalyst, resulting in Compound 3 (PA-XY1). Similarly, Compound 2 reacts with an amine under the same conditions (EDCI, HOSu, DMAP) to yield Compound 4 (PA-N). The detailed synthesis route is shown in Figure 6.

4.1. Synthesis of the First-Generation Molecular Probes of PA-XY1

Biotin undergoes a substitution reaction with 1,4-diiodobutane in the presence of K_2CO_3 to yield Compound 5. PA-XY1 then reacts with Compound 5 under the same K_2CO_3 conditions to produce Compound 6. Similarly, PA-N reacts with Compound 5 in the presence of K_2CO_3 to yield Compound 7. The detailed synthesis process is shown in Figure 7.

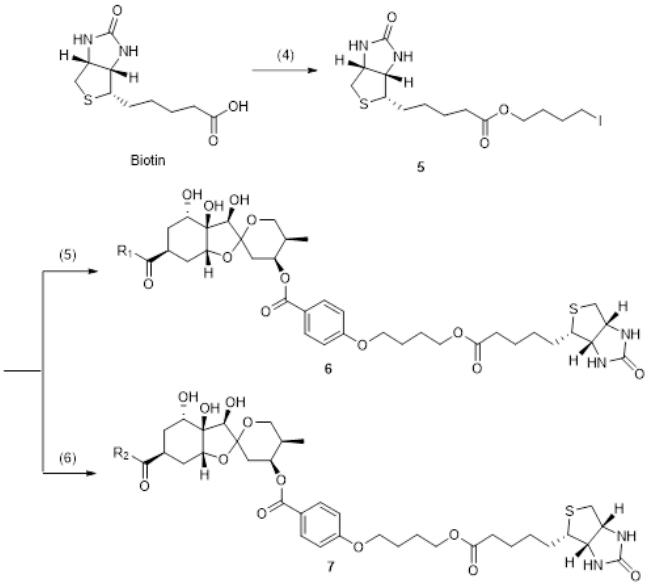

Figure 7: Schematic diagram of the synthesis of the first-generation molecular probes of PA-XY1.

4.2. Synthesis of the Second-Generation Molecular Probes of PA-XY1

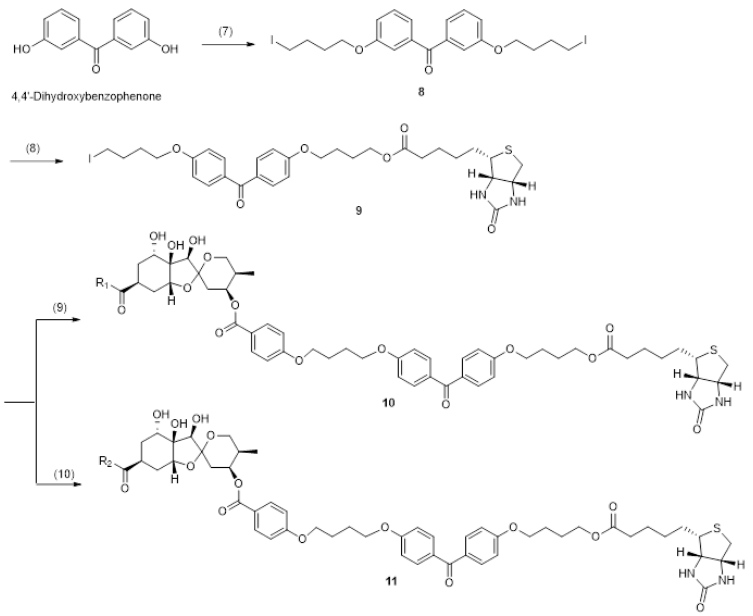

Figure 8: Schematic diagram of the synthesis of the second-generation molecular probes of PA-XY1.

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4,4′-Dihydroxybenzophenone reacts with 1,4-diiodobutane in the presence of K_2CO_3 to produce Compound 8. Biotin then undergoes a substitution reaction with Compound 8 under the action of K_2CO_3 to yield Compound 9. PA-XY1 reacts with Compound 9 in the presence of K_2CO_3 to produce Compound 10. Similarly, PA-N reacts with Compound 9 under identical conditions to yield Compound 11. The detailed synthesis process is illustrated in Figure 8.

4.3. Synthesis of the Third-Generation Molecular Probes of PA-XY1

Under the action of K_2CO_3, 4,4′-Dihydroxybenzophenone underwent a substitution reaction with 3-bromopropyne to yield compound 12. Subsequently, compound 12 reacted with 1,2-dibromoethane in the presence of K_2CO_3, affording compound 13. Compound 13 further underwent a substitution reaction with PA-XY1 under the same conditions, leading to the formation of compound 14. Similarly, compound 13 reacted with PA-N under the action of K_2CO_3, yielding compound 15. In a parallel synthesis, biotin reacted with 1,4-diiodobutane under the action of K_2CO_3 to form compound 16, which subsequently underwent a substitution reaction with sodium azide (NaN_3) to produce compound 17. The detailed synthetic pathway is illustrated in Figure 9.

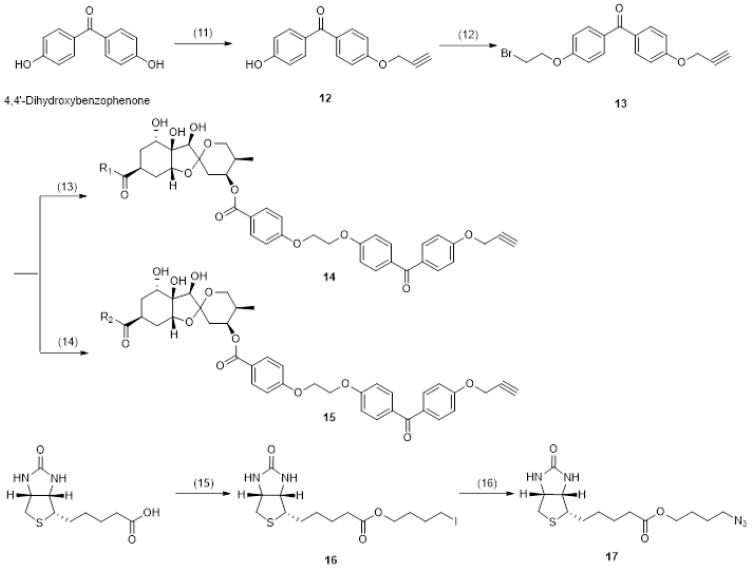

Figure 9: Schematic diagram of the synthesis of the third-generation molecular probes of PA-XY1.

4.4. Synthesis of the Fourth-Generation Molecular Probes of PA-XY1

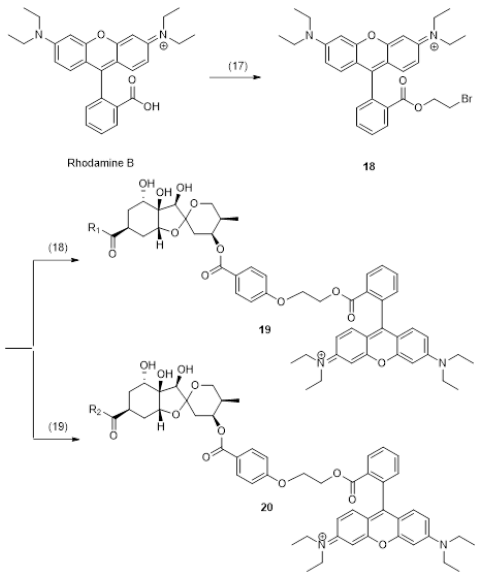

Figure 10: Schematic diagram of the synthesis of the fourth-generation molecular probes of PA-XY1.

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Rhodamine B was dissolved in dichloromethane (CH_2Cl_2) and reacted with 2-bromoethanol using EDCI as a coupling agent and DMAP as a catalyst to produce compound 18. Subsequently, compound 18 reacted with PA-XY1 in the presence of K_2CO_3, resulting in compound 19. Similarly, compound 18 reacted with PA-N under identical conditions to yield compound 20. The detailed synthetic pathway is shown in Figure 10.

5. Conclusion

For each generation of molecular probes targeting PA-XY1, three types of probes—blank, positive, and negative—were synthesized. This design ensures that in subsequent biological experiments aimed at identifying target proteins, background proteins can be effectively eliminated using blank and negative probes, thereby ensuring that the identified proteins exhibit high specificity. First-generation probes were designed to identify target proteins that bind to the active group via covalent bonds. Second-generation probes targeted proteins that bind to the active group via non-covalent interactions. Third-generation probes also focused on identifying target proteins that bind through non-covalent interactions. These probes were optimized based on the second-generation probes by introducing an alkyne group as a potential reporting moiety. After the probe molecule binds to the target protein, the actual reporter group is introduced via Click Chemistry, thereby avoiding interference caused by oversized reporter groups that could affect the labeling efficiency of the probe. Fourth-generation probes were developed for intracellular localization of target proteins to observe their distribution within cells. The HBV activity tests demonstrated that all four generations of probes retained anti-HBV activity comparable to the active group PA-XY1. This provides an effective research tool for the study of target proteins with anti-HBV activity associated with the active molecule PA-XY1.

Acknowledgements

The authors would like to express their sincere gratitude to the Jiangsu Health Vocational College School level Scientific Research Project (Grant No. JKC2021002) for providing funds to support this study. Also, we thank Home for Researchers editorial team (www.home-for-researchers.com) for language editing service.

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