

Synthesis of Green Emitting Coumarin Bioconjugate for the Selective Determination of Flu Antigen

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Fluorescent bioconjugates have paramount importance in biomedical research and clinical diagnosis¹ with proper selectivity and sensitivity.^{2,3} Many organic dyes have been used extensively over long period of time for developing fluorescent probes but at the same time new label technologies have been continuously gaining interests because commonly used radioactive, colorimetric, luminescent, or fluorescent methods are not fulfilling the ideal label requirements, including cost, stability, sensitivity and detection. The appropriate organic dyes for bioconjugation must possess long term stable reactive functionality, should have high labeling efficiency, minimum side reactions and form a stable covalent bond with the antibody.

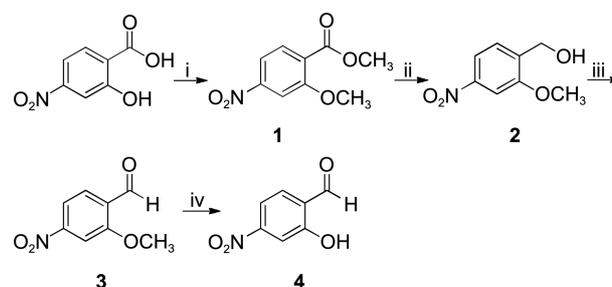
Regarding the tremendous fluorescence ability, coumarins have been used in biology and medicine as fluorescent probes.⁴ The heterocyclic coumarin derivative with sufficient fluorescent characteristic⁵ has been used in this experiment to synthesize the fluorescent bioconjugate for the selective detection of the flu antigen. The choice of the benzothiazolyl coumarin bioconjugate over other synthetic dyes is influenced by its easy synthetic mode, the superiority of green fluorescence and its wide application in laser dye.⁶ The challenging part of the synthesis was functionalization of the coumarin derivative for appropriate coupling with antibody, which was delivered by introducing the isothiocyanate group that could couple to the amino group present in the antibody.³ Bioconjugation with the isothiocyanate have advantage over other functional groups as it reacts with amine by addition reaction and no any side products at all which make the purification process simple.⁷

In this paper, we synthesized the benzothiazolyl coumarin with isothiocyanate functionality starting from commercially available 2-hydroxy-4-nitro benzoic acid. The first step was protection of both carboxylic acid and hydroxyl group of the starting material by methylation to give compound **1** with 99% yield.

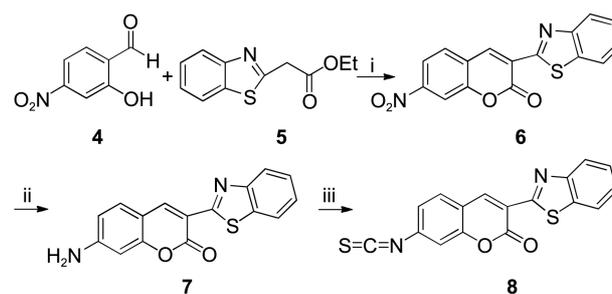
The second step was the reduction of the methyl ester to give the corresponding alcohol **2** with 95% yield by treating with diisobutylaluminium hydride. Then the primary alcohol **2** was oxidized to aldehyde in 94% yield to give 2-methoxy-4-nitrobenzaldehyde **3**. The O-methyl group of compound **3**

was deprotected by boron tribromide at low temperature with 96% yield to give 2-hydroxy-4-nitrobenzaldehyde **4** (Scheme 1).

The ethyl 2-(benzo[d]thiazol-2-yl)acetate **5** was obtained according to literature procedure⁸ in 80% yield by condensation between ethyl cyanoacetate and 2-aminophenol, under the oxygen and moisture free condition, at 120 °C. The synthesis of benzothiazolyl coumarin **6**,⁹ was done according to the literature procedure^{10,11} by condensation of the compound **4** and **5** in the presence of piperidine in ethanol with 82% yield. The nitro group of the compound **6** was reduced to amine by tin(II)chloride in presence of tetrabutyl ammonium bromide with 30% yield. Finally the benzothiazolyl coumarin with isothiocyanate functionality **8**,¹² was synthesized with 60% yield in CH₂Cl₂ using 1,1-



Scheme 1. i) K₂CO₃, (CH₃)₂SO₄, reflux, 4 h, 99%. ii) DIBAL, THF, 0 °C, 1 h, 95%. iii) PDC, DCM, 40 °C, 3 h, 94%. iv) BBr₃, DCM, -78 °C → rt, 4 h, 96%.



Scheme 2. i) piperidine, ethanol, reflux, 2 h, 82%. ii) TBAB, SnCl₂·2H₂O, rt, 24 h, 30%. iii) 1,1-thiocarbonyldi-2(1H)-pyridone, CH₂Cl₂, rt, 2 h, 60%.

Table 1. The antibody concentration in each fraction after the purification of bioconjugate

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
20 eq. of coumarin (Antibody-A)	0.01	0.88	0.31	0.04
40 eq. of coumarin (Antibody-A)	0.01	0.82	0.41	0.03
20 eq. of coumarin (Antibody-B)	0.01	0.54	0.15	0.05
40 eq. of coumarin (Antibody-A)	0.06	0.63	0.19	0.02

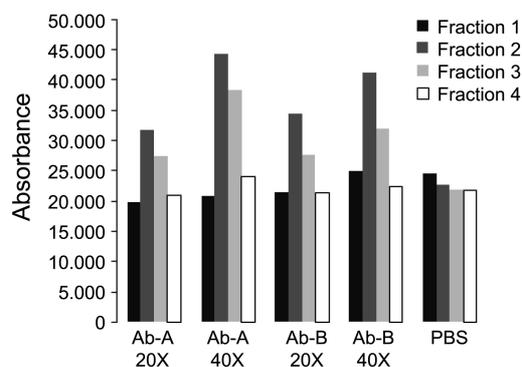
*20 eq. of coumarin (Antibody-A) means a bioconjugate from 20 equivalents of fluorescent coumarin-NCS and Antibody-A. The real concentrations of the two antibodies used in coupling reaction were 2.89 mg/mL (Antibody-A) and 3.8 mg/mL (Antibody-B), respectively.
*Antibody A and B: Anti-Swine Flu 1H7E1 and 3A3H7

thiocarbonyldi-2(1*H*)-pyridone (Scheme 2).

The compound **8** was coupled to the Swine influenza A (H1N1-D) antibody by incubating for 10 hours at 4 °C at the standard pH for conjugation.^{3,7} The above synthesized bioconjugate was purified according to Protein A IgG purification kit (PIERCE). The concentration of the bioconjugate in each eluent was determined by protein analysis by measuring the absorbance at 260 nm (PIERCE). The purified fluorescent antibody was used for the detection of Swine flu virus antigen by fluorescent immunoassay. The antigen antibody binding was determined by measuring the absorbance with 485 nm excitation and 535 nm emission filters using Infinite F200 microplate reader (TECAN, Mannedorf, Switzerland).

For the first time, the coumarin compound with isothiocyanate functionality was used for synthesizing the fluorescent bioconjugate. The protein analysis after the purification of the bioconjugates shows that the fraction 2 and fraction 3 eluents (Table 1) contain the significant amount of monoclonal antibodies that are coupled with fluorescent compounds.

The fluorescence immunoassay results have shown that the detection of the Swine flu virus antigen can be achieved by using the coumarin bioconjugate. The detection of the antigen was optimum which can be clarified by observing

**Figure 1.** Absorbance from fluorescence immuno-assay for each fraction of bioconjugate (Ab-A 20X means a bioconjugate from 20 equivalents of the fluorescent coumarin-NCS and Antibody-A).

the absorbance of the eluent 2 and eluent 3 (Fig. 1). The absorbance was increased with raise in the fluorescent compound concentration that signifies the increment of the fluorescent compound till certain levels do not decrease the affinity of antibody towards the antigen. With two times increase in the fluorescent compound concentration, the absorbance was not increased in the same ratio which indicates the selection of appropriate concentration of the fluorescent compound is required for the optimum antibody-antigen binding and proper detection of the antigen. The presence of the primary amines in the antigen binding site of the antibody can result in the fluorophore conjugation that may change the affinity of antigen antibody binding¹³ but our result shows that with the increase in concentration of fluorophores twice the antigen antibody binding affinity is not affected.

In summary, we have developed the coumarin based fluorescent compound with isothiocyanate group and coupled with the Swine flu antibody. The synthesized bioconjugate detected the flu antigen with specificity. Thus, we can anticipate that coumarin fluorescent compound can be used for the detection of Swine flu virus antigen with selectivity and specificity.

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