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Design and efficient synthesis of novel haptens and complete antigens for the AOZ, a toxic metabolite of furazolidone

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Abstract

A good strategy was brought forward for designing efficient haptens and complete antigens for 3-amino-2-oxazolidinone (AOZ). Haptens designed newly were achieved facilely in good yield by using LiCl–$N(Et)_3$ as new catalysis system, the structure of which was elucidated by spectroscopy analysis, such as NMR and MS. Novel antigens for AOZ were prepared successfully by convenient active ester method. The ratios of haptens **3** and **4** to carrier proteins were proven respectively as 41:1 (**5a**), 39:1 (**6a**), 11:1 (**5b**) and 9:1 (**6b**) by trinitrobenzene sulfonic acid (TNBS) method. The results of indirect competitive ELISA (ic-ELISA) of antiserums indicated that the haptens with a short unsaturated side chain can evoke specific immune response effectively.

Keywords: Furazolidone; AOZ; Antigen; Hapten; Immunoassay

Furazolidone (*N*-(5-nitro-2-furfurylidenenamino)-2-oxazolidinone) is a member of the nitrofuran antibiotics family, which was commonly employed as a veterinary drug for its obvious growth promotion effect and good antibacterial activity. Since 1995, the use of furazolidone has been prohibited completely in food animal production in many countries because of its carcinogenicity and mutagenicity [1,2]. However, as a result of the low costs of furazolidone and its treatment benefits, it has been proven by detecting AOZ, a toxic metabolite derived from furazolidone, in animal origin food that illegal abuse of furazolidone is still under way [2,3].

Among analytical methods of AOZ residue, immunoassay is an efficient screening technique for monitoring of illegal furazolidone. However, immunoassay formats reported are mainly based on detecting AOZ's derivative NPAOZ (3-(2-nitrobenzylideneamino)oxazolidin-2-one), which must undergo a troublesome derivatization step [2,4]. To our best knowledge, few reports have surfaced on the successful development of specific antibody to AOZ and immunoassay method for detecting AOZ itself directly [5]. Generally, Organic molecules less than 1000 Da possess no antigenicity. Thus, they must be covalently conjugated to carrier protein by introducing a linker with an active terminal group on organic molecular structure to form complete antigens to elicit specific immune response smoothly.

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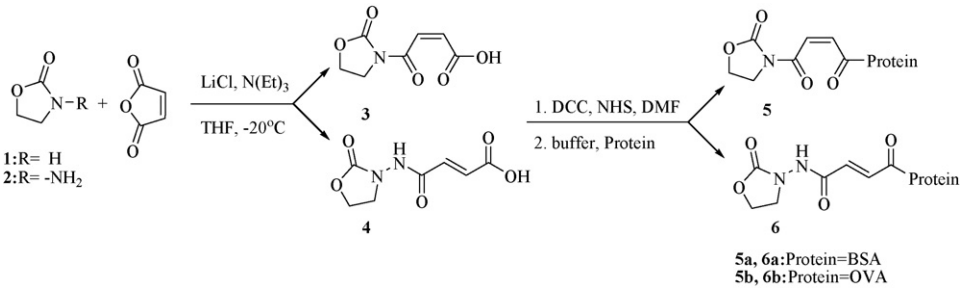

Scheme 1. Synthesis of haptens and artificial antigens.

However, AOZ has so small molecular weight and no aromaticity that it is difficult to elicit specific antibody, even though it were bound to carrier through common saturated linear aliphatic side chains [5]. Fortunately, a short unsaturated linear chain attached to this kind of small molecules can remarkably improve the capability of eliciting specific antibody [6]. So, our strategy is to attach a short unsaturated aliphatic arm to AOZ and its mimic 2-oxazolidinone to form haptens **3** and **4** which were further coupled with carrier protein (BSA and OVA) to give novel complete antigens **5** and **6** for production of specific antibody to AOZ (Scheme 1). It's notable that so far no reports have been found about our novel strategy.

First, hapten **3** was prepared smoothly in good yield by the previous method reported by us [7]. Then, our efforts focused on synthesizing hapten **4**. It's well known that pyridine (and/or DMAP) was a typic catalyst for acylation reaction of many active groups such as amino and hydroxyl group with aliphatic anhydride as acylating agent [8,9]. Our first attempt was to acylate AOZ with maleic anhydride into hapten **4** in pyridine. However, the reaction failed to give the desired products in satisfactory yield. Interestingly, after an in-depth screening of reaction strategies, we found that the reaction can give the purpose hapten **4** in a good yield by using LiCl–$N(Et)_3$ as catalyst in THF solvent at $-20\ ^\circ C$, as shown in Scheme 1. Specially, the unexpected result on synthesis of hapten **4** with LiCl–$N(Et)_3$ as catalyst afforded a novel and efficient catalysis strategy for the acylation reaction of amino group of hydrazine type with aliphatic anhydride, on which no report has been found.

Next, the obtained haptens **3** and **4** which were proved by spectrum analysis [10,11] formed novel immunogens **5a**, **6a** and coating antigens **5b**, **6b** with carrier protein (BSA and OVA) in two steps, as shown in Scheme 1. The ratios of haptens **3** and **4** to carrier proteins were determined successfully as 41:1 (**5a**), 39:1 (**6a**), 11:1 (**5b**) and 9:1 (**6b**), respectively by TNBS method [12]. The achieved immunogens **5a** and **6a** were used to immunize mice for the subsequent production of monoclonal antibodies. The antiserum produced from all four mice had similar titer of higher than 1×10^5 which was defined as the max multiple dilution of antiserum when $A_{450\ nm}$/$CKA_{450\ nm} \geq 2.1$ and determined by standard indirect ELISA method. Finally, taking the antiserum from hapten **3** as an example, preliminary results of ic-ELISA exhibited remarkable inhibition to reaction of antigen with antibody because of addition of hapten **3** and AOZ drug, which proved production of specific antibody to AOZ, as shown in Fig. 1. The above results indicated that the haptens with a short unsaturated side chain can evoke specific immune response effectively. Further studies on preparation of high specific monoclonal antibody to AOZ are well under way.

1. General procedure of synthesis of hapten *3* and *4*

To the solution 2-oxazolidinone (or AOZ) (0.01 mol), lithium chloride (0.011 mol), and triethylamine (0.013 mol) in THF (30 mL) was added maleic anhydride (0.012 mol) at $-20\ ^\circ C$. The mixture was allowed to warm to $10\ ^\circ C$ and stirred for 6 h. After termination of the reaction (TLC monitoring), the solvent THF was removed *in vacuo*, and the residue was partitioned between ethyl acetate and 1 mol/L aqueous $NaHCO_3$. The aqueous layer was subsequently acidified to pH <2 with concentrated HCl and extracted with ethyl acetate. The ethyl acetate layer was washed with saturated brine and then dried over sodium sulfate and filtered. The ethyl acetate was removed *in vacuo*, and the residue was purified by silica gel column chromatography to give target hapten **3** (or **4**) as white solid in higher than 70% yield.

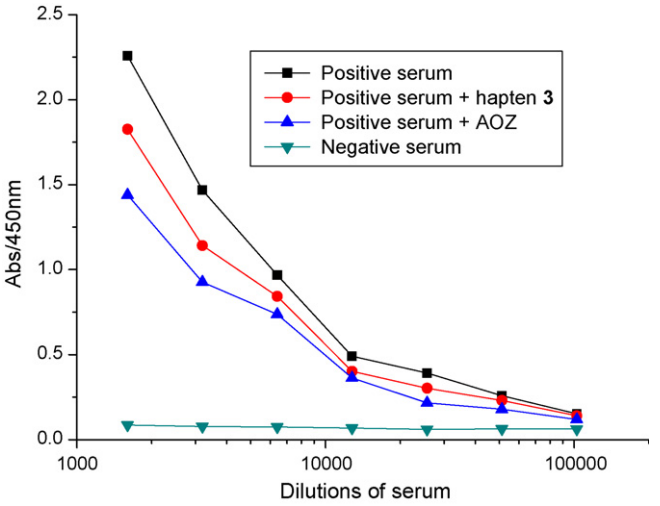

Fig. 1. ic-ELISA for antiserum from hapten 3 with hapten **3**-OVA conjugate as coating antigens (experimental conditions: 1 μg/mL AOZ in phosphate buffer saline (PBS), 1 μg/mL hapten **3a** in the PBS containing 5% methanol, 1 μg/mL coating antigen).

2. General procedure of synthesis of complete antigens

Dry DMF (1 mL) was added to 0.1 mmol of hapten **3** (or **4**) and 0.12 mmol each of *N*-hydroxysuccinimide and DCC. The mixture was stirred at room temperature for 4 h and then centrifuged to remove precipitated urea. The clear supernatant was slowly added to a BSA solution (130 mg of BSA in 5 mL of a 0.2 mol/L borate buffer, pH 8.0). The reaction was stirred overnight at 4 °C and then dialyzed against 9‰ NaCl solution (1000 mL) for 3 days at 4 °C with three buffer changes per day to give immunogens **5a** and **6a** which were diluted to 1 mg/mL by 9‰ NaCl solution, and then stored at −20 °C until used. Similarly, the coating antigens **5b**, **6b** were prepared by the same method with OVA instead of BSA.

Acknowledgments

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[10] Selected data for hapten **3**. MS-ESI *m/z*: 184 $[M-H]^-$. ^{1}H NMR (500 MHz, $CDCl_3$, TMS, δ ppm): 4.14 (t, 2H, J = 8.0 Hz), 4.52 (t, 2H, J = 8.0 Hz), 6.39 (d, 1H, J = 12.5 Hz), 7.32 (d, 1H, J = 12.5 Hz).
[11] Selected data for hapten **4**. MS-APCI *m/z*: 201 $[M+H]^+$. ^{1}H NMR (400 MHz, d_6-DMSO, TMS, δ ppm): 3.68 (t, 2H, J = 8.0 Hz), 4.38 (t, 2H, J = 8.0 Hz), 6.33 (s, 2H), 10.44 (s, br, 1H), 12.87 (s, br, 1H). ^{13}C NMR (75 MHz, d_6-DMSO, TMS, δ ppm): 45.3, 61.7, 128.9, 131.2, 156.8, 163.7, 166.5.
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