Bioorganic & Medicinal Chemistry Letters 20 (2010) 1792–1795

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Use of polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline for the preparation of a hapten–protein conjugate for antibody development

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ARTICLE INFO

Article history:
Received 27 October 2009
Revised 17 December 2009
Accepted 18 December 2009
Available online 7 January 2010

Keywords:
Polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline
PS-IIDQ
Phytanic acid
Immunogen
Bioconjugation
ELISA

ABSTRACT

Polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (PS-IIDQ), a polymer-supported covalent coupling reagent, was successfully employed for the first time in the bioconjugation of an example hapten (phytanic acid derivative) to a carrier protein (bovine serum albumin (BSA)) within the context of immunogen preparation for antibody development. The ability of the prepared example phytanic acid derivative–BSA conjugate to bind an anti-phytanic acid antibody was confirmed using an enzyme-linked immunosorbent assay (ELISA).

Small molecules (haptens < 1000 Da) generally do not trigger an immune response since their low molecular weight is not sufficient to elicit a response from the recipient's immune system. However, it is possible to trigger antibody production against such haptens by conjugating the haptens to large carrier proteins, resulting in the production of an immunogen that is of sufficient size to be recognised by an immune system.[1] Chemistries used for covalent attachment of haptens to carrier proteins are well established for many functional group types available on both carrier proteins and haptens. Covalent attachment may be accomplished either by direct conjugation between an existing functional group on the hapten and the protein carrier[2] or by more complex methods involving modification of the hapten to introduce suitable coupling groups and/or insertion of linker arms to present additional molecular features.[3] A fundamental characteristic of an immunogen is the number of hapten molecules covalently attached to the carrier protein. The optimal number of hapten attached to the carrier protein has been debated concerning relevance to immunogenicity.[4]

Often, bioconjugates intended for use as immunogens are prepared by directly coupling carboxylic acid-containing haptens or hapten derivatives to primary amine groups of lysine residues on proteins using ethyl dimethylaminopropylcarbodiimide (EDC) or dicyclohexylcarbodiimide (DCC).[5] However, this process can lead to undesirable side reactions such as crosslinking of the protein and *N*-acylurea formation on the protein.[6]

Over the past decade, interest in the development of new polymer-supported reagents has increased,[7] predominantly because these reagents combine the traditional advantages of solution-phase chemistry with the convenience of solid-phase handling. Thus using polymer-supported materials, unreacted reagents and by-products remain on the resin and can be easily removed by filtration at the end of the reaction. Polymer supported coupling reagents that are currently available include several immobilised carbodiimides such as PS-EDC, PS-DCC, PS-TBTU, and PS-BOP.[7,8] However, the two latter reagents imply the release of uronium or phosphonium salts into solution during coupling, clearly an undesirable occurrence for an immobilised reagent, as these by-products have to be removed.

Recently, Bradley et al. reported[9] polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (PS-IIDQ) as an efficient coupling reagent for amide bond formation. Encouraged by its features and advantages, including that it does not require any pre-activation, addition of any other additives or formation of volatile by-products and high stability under general laboratory conditions, the object of the present study is to demonstrate the application of PS-IIDQ for the first time in the bioconjugation of a hapten to protein to form an immunogen. This study

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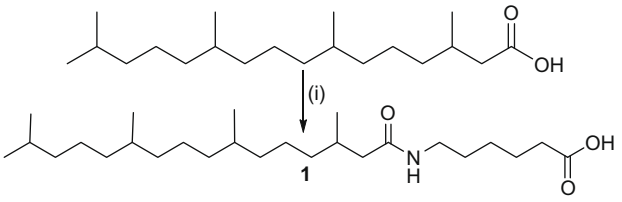
doi:10.1016/j.bmcl.2009.12.078



Scheme 1. (i) $NH_2(CH_2)_5COOH$, EDC, NHS and DMF at room temperature with overnight stirring.

Table 1
Different initial molar ratios of protein and hapten used to prepare conjugates PA-1 to PA-4

Code	Protein quantity	Hapten quantity	Protein–hapten molar ratio
PA-1	0.14 μmol (9.4 mg, 940 μL)	3 μmol (60 μL)	1:21
PA-2	0.13 μmol (8.8 mg, 880 μL)	6 μmol (120 μL)	1:46
PA-3	0.12 μmol (8.2 mg, 820 L)	9 μmol (180 μL)	1:75
PA-4	0.11 μmol (7.6 mg, 760 μL)	12 μmol (240 μL)	1:109

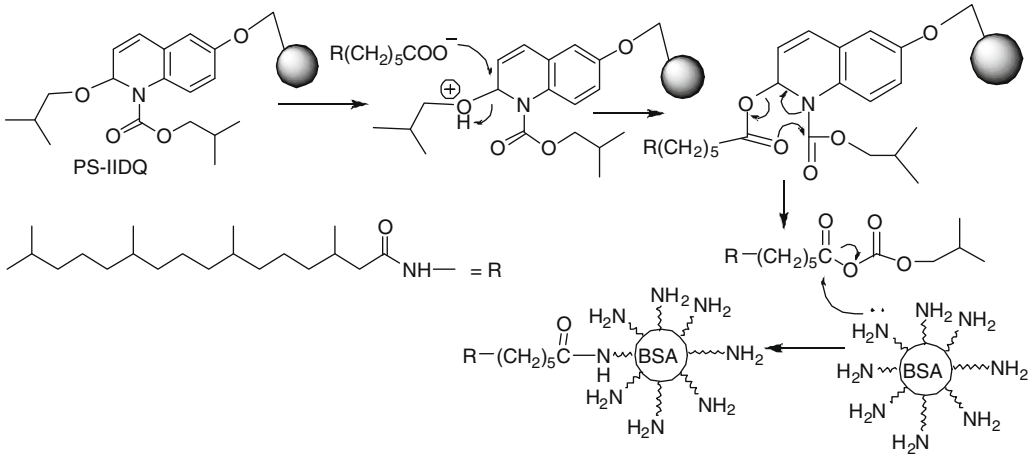
will use a phytanic acid derivative[10] as a representative example of a hapten and BSA as the protein carrier to assess its performance compared to existing supported carbodiimide approaches—specifically PS-DCC and PS-EDC.

PS-IIDQ was synthesised according to a previously reported method.[9a] Briefly, hydroxyquinoline was coupled onto Merrifield's resin using excess potassium carbonate in dimethyl acetamide (DMA) under reflux for 6 h to form PS-quinoline. The product was treated with isobutyl chloroformate in the presence of Hunig's base at 0 °C for 3 h to yield a highly reactive intermediate that was quenched by the addition of isobutanol. The reagent was characterised by NMR and FT-IR. The results were in agreement with reported data.[9a] For a single batch of PS-IIDQ, the loading was determined to be 1.6 mmol/g. This was based on the loading of Merrifield's resin used to prepare PS-IIDQ and on the assumption that 100% conversion of Merrifield's resin to PS-IIDQ was achieved. The phytanic acid derivative (**1**) was synthesised as shown in Scheme 1 and was characterised by IR, NMR, and MS.[11] The synthesis of the hapten–protein conjugates using PS-IIDQ is shown schematically in Scheme 2. The quantity of PS-IIDQ required for the bioconjugation was 2 equiv. To determine the optimal conditions for effective hapten–protein conjugation, conjugates were prepared[12] at four different molar ratios (1:21, 1:46, 1:75, and 1:109 and coded PA-1 to PA-4) as shown in Table 1. In order to assess the coupling performance of PS-IIDQ, PS-DCC and PS-EDC were also used separately as coupling reagents, that is, as controls. The free amino groups in the protein before and after the conjugation were determined by reaction with TNBSA.[13] Reaction of primary amines with TNBSA forms a highly chromogenic trinitrophenyl derivative that can easily be quantified by colorimetric read-out at 335 nm. The result of the TNBSA analyses of the conjugates is shown in Table 2. Degree of hapten conjugation to carrier protein was calculated from the absorbance values at 335 nm (which is the characteristic absorption peak of the TNP group) using the following equation: substitution (%) = $[A_{control}-A_{conjugate})/A_{control}] \times 100$. Control experiments were performed in parallel and consisted of protein alone and protein mixed with hapten without PS-IIDQ. These controls were also reacted with TNBSA, and confirmed that no conjugation occurred.

The resulting conjugates were additionally characterised by MALDI-MS.[14] As shown in Figure 1, spectra were successfully obtained for all four conjugates (PA-1 to PA-4). The molecular weight of native BSA was determined to be 66,451, while that determined on the basis of the published protein sequence was 66,432.9.[15] The relative increase in the molecular weight of the conjugates was manifested as a gradual mass peak shift as a function of hapten to protein ratio. This is interpreted as an increase in the hapten density of the conjugates and therefore provides a method for determining the number of haptens per protein molecule. The observed value for BSA was used in the following equation to determine the hapten density: number of haptens = (conjugate MW-BSA MW)/(hapten MW-18); the value of 18 refers to the loss of a water molecule from the phytanic acid derivative in the formation of the amide bond. The molecular weight of each conjugate was calculated from the peak centroid using the software provided with the MS instrument.

The findings of MALDI-MS analysis in terms of hapten density were in close agreement with the data obtained from the TNBSA spectrophotometric method of analysis. In the following consideration only the MALDI-MS data will be considered.

Comparing the results obtained with PS-IIDQ to those obtained from two other polymer-supported carbodiimides, that is, PS-EDC and PS-DCC, under identical conditions, and using a hapten–protein concentration ratio of 46:1, the PS-IIDQ exhibited the highest hapten density of 12:1 compared to 9:1 for PS-EDC and 7:1 for PS-DCC. The highest hapten density of 22:1 occurred using a hapten–protein concentration ratio of 109:1 and PS-IIDQ. Considering the three-dimensional structure of BSA, only 26 ε-NH_2 groups of the total 59 lysines residues in BSA are present at the protein



Scheme 2. PS-IIDQ-supported preparation of a hapten (phytanic acid derivative)-protein (BSA) conjugate.

Table 2
Determination of the hapten density on hapten–protein conjugates using chemical (TNBSA) and physical (MALDI-MS) methods

Conjugate	Carrier to hapten molar ratio	Chemical TNBSA method[a]		MALDI-MS		
		% of amino groups consumed	Average no. of amino groups used	Observed mass (Da)	Change in mass (ΔM) (Da)	$\Delta M/M_n$[b] (hapten density)[c]
PA-1	1:21	32	8.3	70,955	4504	11
PA-2	1:46	40	10.4	71,454	5003	12
PA-3	1:75	52	13.5	72,714	6263	15
PA-4	1:109	73	18.9	75,621	9170	22
PS-EDC	1:46	34	8.8	70,263	3812	9
PS-DCC	1:46	25	6.5	69,339	2888	7
native BSA	n/a	0	0.0	66,451	n/a	n/a

[a] The number of lysine groups on the protein that were utilized in conjugates PA-1 to PA-4 before and after conjugation.
[b] Mass of the hapten (phytanic acid derivative) $M_n = 425$.
[c] Number of hapten molecules per BSA molecule.

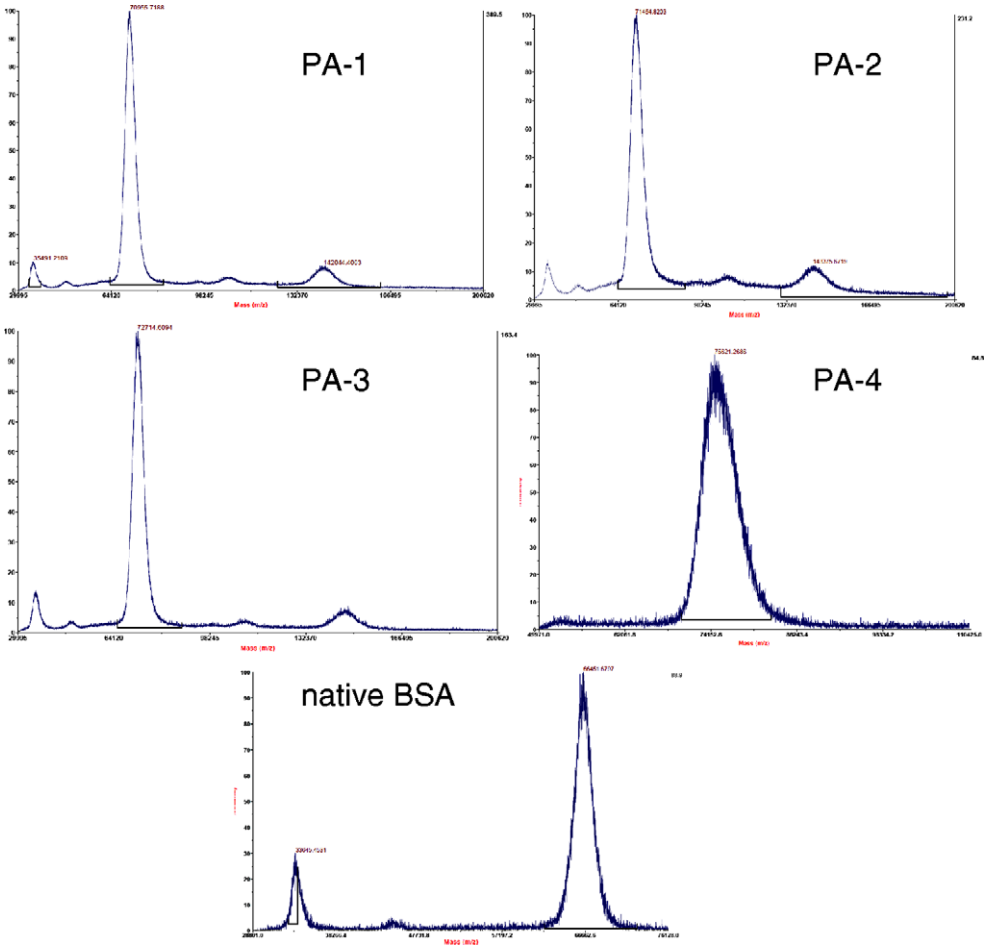


Figure 1. MALDI-MS spectra of hapten–protein conjugates PA-1 to PA-4 and native BSA. On each graph the X-axis represents the mass (MW) and Y-axis measures intensity (%). Each spectrum contains only one major peak that corresponds to the singly charged peak (M+H)+ and which is used to determine the MW of the conjugate or protein (note that the spectra are plotted with different X-axis ranges).

surface and therefore readily available for coupling to haptens. Therefore the calculated hapten density for PA-4 was close to the theoretical maximum for a non-denatured protein and reflects the high efficiency of the conjugation method.

Enzyme-linked immunosorbent assays (ELISA)[16] were carried out in order to test the binding efficiencies of the conjugates (PA-1 to PA-4) to a commercially available polyclonal antibody raised against a phytanic acid–BSA immunogen (Abcam plc, Cambridge, UK, cat. no. ab51309). As shown in Figure 2, the four conjugates exhibited a gradual increase in binding efficiency as the hapten to protein ratio was increased.

The results indicate that firstly all the conjugates synthesised with PS-IIDQ were able to bind to a relevant antibody via an antibody binding assay and secondly that increased hapten density resulted in greater antibody binding.

In conclusion, we successfully demonstrated the application of PS-IIDQ for the first time in hapten protein conjugation suitable for immunogen production. The highest determined number of

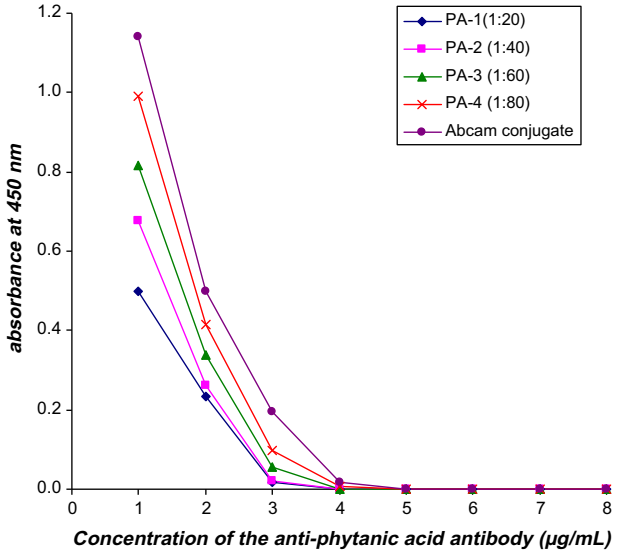


Figure 2. Comparison of the antibody-binding performances of four conjugates (PA-1 to PA-4).

hapten molecules linked to BSA was found to be 22, a number which was close to the theoretical maximum, obtained at an initial hapten to protein molar ratio of 1:109. When comparing the PS-IIDQ reagent to two existing commercial polymer-supported carbodiimides (PS-EDC and PS-DCC) under identical conditions, the PS-IIDQ method resulted to a higher hapten density. The ability of the PS-IIDQ prepared conjugates to successfully bind a relevant antibody was demonstrated via an ELISA, which showed that greater antibody binding was achieved with increase hapten density.

Acknowledgements

The authors would like to thank Dr. Paul Gates, University of Bristol, for the MALDI-MS analysis. This study was funded by the U.K.'s Science and Technology Facilities Council. We would also like to thank the other members of the Life Marker Chip consortium for their support and contributions.

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10. The phytanic acid derivative, as an example hapten derivative, was chosen for convenience as it is one of the potential molecular targets for immunoassay development within an associated project within the authors' laboratory—specifically the development of the immunoassay-based Life Marker Chip instrument for detecting biomarkers of Life on Mars.
11. A solution of ethyl dimethylaminopropylcarbodiimide (EDC) (95 mg, 0.5 mmol) in DMF (200 µL) was added to a solution of phytanic acid (62 mg, 0.2 mmol) in DMF (0.5 mL). This was followed by the addition of a solution of *N*-hydroxysuccinimide (NHS) (57 mg, 0.5 mmol) in DMF (200 µL). After stirring the mixture overnight at room temperature, a solution of six-aminocaproic acid (65.5 mg, 0.5 mmol) in deionised water (0.3 mL) was added, and the reaction mixture was stirred overnight at room temperature. After removing the solvent under reduced pressure, an oily residue was obtained. This residue was re-dissolved in diethyl ether (50 mL) and washed twice with water to remove excess 6-aminocaproic acid. The organic phase was dried over Na_2SO_4, and the solvent was removed by rotary evaporation. The resulting oil was purified by flash chromatography on silica gel using $CHCl_3/CH_3OH$ (8:2) as the eluent to obtain a viscous liquid compound **1** in 42% yield. FT-IR (KBr): ν_{max} 3567, 3341, 2917, 2834, 1756, 1726, 1069, 869 cm^{-1}. ^1H NMR ($CDCl_3$-400 MHz) δ 1.01 (d, J = 6.6 Hz, 6H), 1.06–1.08 (dd, J = 9.2, 6.6 Hz, 9H), 1.16–1.25 (m, 18H), 1.29 (m, 2H), 1.32–1.45 (m, 3H), 1.50–1.53 (m, 4H), 2.26 (m, 1H), 2.32 (m, 2H), 2.50 (t, J = 7.5, 2H), 3.12 (m, 2H), 8.0 (NH, 1 H), 11 (OH, 1H). ^{13}C NMR ($CDCl_3$-400 MHz) δ 20.4, 21.1, 23.2, 24.2, 24.3, 24.4, 24.6, 26.2, 28.1, 29.7, 30.1, 33.2, 34.1, 37.1, 37.7, 39, 39.2, 39.9, 167, 169, 178.4. ESI-MS 426 (M+H); Anal. Calcd for $C_{26}H_{51}NO_3$: C, 73.36; H, 12.08; N, 3.29. Found: C, 73.33; H, 12.05; N, 3.33.
12. *Conjugation of the phytanic acid derivative to BSA using PS-IIDQ.* PS-IIDQ (1.87 mmol/g, 66.6 mg, 124.6 µmol) was added to a stock solution of the phytanic acid derivative (21.25 mg, 50 µmol) in DMF (1 mL). Conjugates of the phytanic acid derivative with the protein (BSA) were prepared at four different protein to hapten molar ratios (1:21, 1:46, 1:75, and 1:109) and were coded PA-1 to PA-4. In the case of the controls, the same micromole quantity of PS-DCC and PS-EDC was used as coupling reagents. The protein stock solution (10 mg/mL; 0.15 µmol/mL) was prepared in borate buffer (pH 9.0), and the final reaction volume of the protein–hapten conjugates was kept constant at 1 mL for each preparation. The hapten was conjugated to the protein by adding different amounts of protein (10 mg/mL) to a final volume of 1 mL to prepare protein–hapten conjugates of different molar ratios, as shown in Table 1. All four conjugates were incubated overnight at rt and centrifuged for 5 min at 10,000 rpm to remove the coupling reagent. They were purified by passing through a P10 gel filtration column (Pharmacia, Sweden). Fractions with the highest protein concentration were determined by absorbance measurements at 280 nm using a molar extinction coefficient 43,824/M/cm on a UV spectrometer. The final protein concentration of the conjugate was determined using a Micro BCA™ protein assay kit (Pierce).
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14. Mass analysis of the hapten–protein conjugates was carried out by MALDI mass-spectrometry (Applied Biosystems MALDI 4700 TOF). Protein solutions were prepared by interaction with C4 resin (3–5 mg, Jupiter 15u C4 300 Å, Phenomenex) in order to desalt and concentrate the protein content in the conjugates. The crystal matrix, 2,5-dihydroxybenzoic acid (Aldrich Chemical Co., Milwaukee, WI), was prepared at a concentration of 15 mg/mL in acetonitrile. Protein samples were typically 10–50 pmol/µL in a 2:1 water/acetonitrile solution. Sample and matrix solutions were mixed in equal volumes (typically 1.5 µL each) directly on the stainless steel probe tip (target) and allowed to dry (~10 min) in a fume hood at room temperature. The crystallised analyte-matrix sample was then rinsed with 0.1% TFA solution by placing approximately 2 µL of the solution on the probe sample at room temperature, allowing it to stand for about 5 s, and then gently drying the crystals with a stream of nitrogen gas. Spectra were recorded at threshold laser irradiance for 50–150 shots in the linear mode at 30 kV. The resulting data were analyzed using the software supplied by Applied Biosystem.
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16. The ELISA plates (96-well polystyrene plates from Nunc) were coated with 100 µL of conjugate (PA-1 to PA-4) at a concentration of 10 µg/mL in phosphate buffer saline (PBS) and incubated for 1 h at room temperature. After coating, any unbound sites were blocked using a blocking buffer of 1 % w/v Marvel skimmed milk in PBS. The plates were incubated for 1 h at room temperature. To test the antibody binding of the conjugate, a primary rabbit polyclonal antibody against phytanic acid–BSA (Abcam plc, Cambridge, UK, cat. no. ab51309) was added in serial dilutions ranging from $1:4 \times 10^2$ to $1:4 \times 10^8$ fold dilution and the plates were incubated for 1 h at room temperature. At the end of the incubation period, the plates were thoroughly washed three times with a washing buffer of 0.05 % v/v Tween 20 in PBS (PBST). The washing step was followed by the addition of a secondary antibody (at a dilution of 1:8000) that was specific to the Fc region of the primary antibody and was HRP-labelled (Novus Biologicals, Littleton, CO, US cat. no. NB7179). The plates were incubated for 1 h at room temperature. This was followed by another washing step in which the plates were washed with PBST as before, and the colour development reaction was carried out by adding 100 µL of TMB in phosphate–citrate with sodium perborate buffer (pH 5.4) to all wells. The reaction was stopped with 50 µL of H_2SO_4 (1 M), and the optical density was measured at 450 nm using a plate reader.