Supporting Information
for DOI: 10.1055/s-0030-1258479
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Synthesis of Novel Haptens and Conjugates for Antibody Production against Kainoid Family

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(2S,3R)-3-[2-(benzylxylo)-2-oxoethyl]-4-oxopyrrolidine-1,2-dibenzylester (6):

Synthesis according to the protocol described by Baldwin. 1 chromatography on silica gel, eluting with diethyl ether / petroleum ether = 40/60, yielded the compound as a yellow oil (Rf: 0.45, 35%).

\[ \alpha_D = +0.367. \]

\[ \text{[1H NMR (200 MHz, CDCl}_3, \delta \text{ ppm), 55°C, (mixture of conformers):} \]

- 2.17-2.38 (m, 1H x 0.5); 2.75-2.99 (m, 2H); 3.32-3.46 (m, 1H x 0.5); 3.98-4.07 (m, 2H); 4.53 (d, \(J = 5.76 \text{ Hz, } 0.5\text{H}\)); 4.95-5.32 (m, 6.5H); 7.21-7.34 (m, 15H).

\[ \text{[13C NMR (62 MHz, CDCl}_3, \delta \text{ ppm), (mixture of conformers):} \]

- 29.7, 33.6 (CH\text{2}); 46.8, 47.3 (CH); 52.0, 52.1 (CH\text{2}); 59.5, 59.6 (CH); 66.6, 68.1 (CH\text{2}); 127.4, 129.5 (CH); 134.6, 135.9 (C); 154.0, 154.8 (C); 169.5-171.0 (C); 206.6, 207.2 (C).

\[ \text{HRMS (ES) [M+Na}^+\text{]:calcd. for } C_{29}H_{27}NO_{11}Na. 524,1679, \text{found: } 524,1673. \]

2S,3S)-dibenzyl 3-(2(2benzyloxy)-2-oxoethyl)-4-(2-tert-butoxy-2-oxoethylidene)pyrrolidin-1,2-dicarboxylate (7):

\[ \text{tert-Butyldiethylphosphonoacetate (1.13 mL, 4.79 mmol) was added to a solution of LiHMDS (1.06 M) (3.77 mL, 4 mmol) in dry THF (15 mL) at -78°C. After 30 mn., a solution of ketone (6) (2 g, 4 mmol) in dry THF (15 mL) was added dropwise to the solution of the phosphonate anion. The reaction mixture was allowed to warm to room temperature and after 12 hours, it was quenched with 10%HCl (10 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (3 x 30 mL). The combined organic layers were washed with brine (15 mL), dried over Na\text{2SO}_4 and concentrated to give 3.25 g of the crude product. This one was purified by flash chromatography on silica gel (eluant diethyl ether / petroleum ether = 40/60) to afford the product as a yellow oil (Rf: 0.7, 720 mg, 28%).} \]

\[ \text{[1H NMR (300 MHz, CDCl}_3, \delta \text{ ppm), (mixture of stereoisomers and conformers):} \]

- 1.33, 1.38 (2 s, 9H); 2.40-2.74 (m, 2H); 3.27-3.32 (m, 1H x 0.5); 4.04-4.09 (m, 1H x 0.5); 4.20-4.24 (m, 1H); 4.30-4.55 (m, 1H); 4.56-4.59 (m, 1H); 4.89-5.14 (m, 6H); 5.60, 5.67 (2s, 1H); 7.14-7.26 (m, 15H).} \]

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$^{13}$C NMR (75 MHz, CDCl$_3$, $\delta$ ppm), (mixture of stereoisomers and conformers): 27.1 (CH$_3$); 36.7, 38.3 (CH$_2$); 41.4, 42.3, 44.0, 45.0 (CH); 49.2, 49.7, 50.1, 50.7 (CH$_2$); 61.8, 62.1, 63.0, 63.3 (CH); 65.7-66.5 (CH$_2$); 79.9 (C); 115.8, 116.2 (CH); 125.9-128.5 (CH); 134.2, 135.2 (C), 140.1 (C); 153.3-155.9 (C); 163.3-163.7 (C); 168.9-170.1 (C).

HRMS (ES) [M+Na$^+$]: calcd. for C$_{35}$H$_{37}$NO$_8$Na: 622,2411; found: 622,2441.

BSA-immunoconjugate (9):

![Diagram](image)

A solution of NHS (3.9 mg, 0.335 mmol), EDC (5.8 mg, 0.320 mmol), and acid (8) (12.4 mg, 0.265 mmol) was prepared in DMF (1 mL), at 0°C, under inert atmosphere and stirring control during 1 hour. This mixture was added slowly to a solution of BSA (34.5 mg, 0.0005 mmol) and borate buffer pH=8.7 (15 mL) in DMF (1 mL), with constant stirring. This preparation was kept at room temperature 30 mn, and then at 4°C over a night. After reaction, the mixture was dialyzed against 2L of 0.01M PBS, (pH 7.5) for 72 h with two exchanges of buffer, and against 2 L of distilled water and then lyophilized. A white powder (13 mg) was obtained.

The coupling efficiency for BSA immunoconjugate (9) was estimated by Mass Spectrometry MALDI-TOF.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Change in mass (Da)</th>
<th>MW hapten (Da)</th>
<th>Ratio Conjugate/BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-(9)</td>
<td>9414 ± 117</td>
<td>526</td>
<td>17.9 ± 0.2</td>
</tr>
</tbody>
</table>

BSA- immunoconjugate (10):

![Diagram](image)

The BSA-immunoconjugate (9) (13 mg) was dissolved in EtOH / H$_2$O (2 mL/ 2 mL), and added to a flask containing 10 mol % of Pd-C (10 mg). An H$_2$ atmosphere was then applied during 5 hours. The catalyst was again added (10 mg), and the mixture was again subjected to an H$_2$ atmosphere, for another 2 hours. After filtration, the crude product was dialyzed and lyophilized in the same conditions as described above and obtained as a grey powder (30 mg).

The coupling efficiency for BSA immunoconjugate (10) was estimated by Mass Spectrometry MALDI-TOF.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Change in mass (Da)</th>
<th>MW hapten (Da)</th>
<th>Ratio Conjugate/BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-(10)</td>
<td>4225 ± 246</td>
<td>214</td>
<td>19.7 ± 1.1</td>
</tr>
</tbody>
</table>

(2S,3S,Z)-dibenzyl 3-(2-(benzyloxy)-2-oxoethyl)-4-(tert-butoxy-4-oxobut-2-enylidene)-pyrrolidine-1,2dicarboxylate (11):

![Diagram](image)

Same procedure described for compound (7), with tert-butylphosphonocrotonate (1.34 g, 4.79 mmol). The reaction mixture was heated at reflux during 2h 30. The crude product was purified by chromatography HPLC (column proteo Jupiter 4 µm), eluant
(H2O/acetonitrile: 85/15), flow: 5 mL·min⁻¹, and then afforded the product as an orange oil (650 mg, 22%).

1H NMR (300 MHz, CDCl₃, δ ppm), (mixture of stereoisomers and conformers): 1.42 (s, 9H); 2.44-2.65 (m, 2H); 3.27-3.30 (m, 1H x 0.8); 3.55-3.65 (m, 1H x 0.2); 4.17-4.53 (m, 3H); 4.95-5.10 (m, 6H); 5.60 (d, J = 15.3 Hz, 1H); 5.91 (d, J = 11.5 Hz, 1H); 6.92-7.03 (m, 1H); 7.15-7.24 (m; 15H).

13C NMR (75 MHz, CDCl₃, δ ppm), (mixture of stereoisomers and conformers): 28.2 (CH₃); 39.2 (CH₂); 41.6, 42.5, 44.8, 45.8 (CH); 48.1, 48.7 (CH₂); 63.8 (CH); 66.9-67.5 (CH₂); 80.7 (C); 122.0 (CH); 124.4 (CH); 127.8-129.2 (CH); 135.5, 136.2 (C); 138.0 (CH); 145.5, 146.7 (C); 154.6, 155.1 (C); 165.8 (C); 170.2, 170.8 (C).

HRMS (ES) [M+Na⁺]: calcd. for C₃₇H₃₉NO₈Na 648.2567, found: 648.2543.

BSA- Immunoconjugate (13):

Same procedure described above for BSA-immunoconjugate (9), from acid (12) (29.4 mg, 0.516 mmol). After dialyze and lyophilization, the product was obtained as a white powder (50 mg). The coupling efficiency for BSA immunoconjugate (13) was estimated by Mass Spectrometry MALDI-TOF.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Change in mass (Da)</th>
<th>MW conjugate-MW BSA</th>
<th>MW hapten (Da)</th>
<th>Ratio Conjugate/BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-(13)</td>
<td>1659 ± 221</td>
<td>553</td>
<td>3 ± 0.4</td>
<td></td>
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</table>

BSA- Immunoconjugate (14):

Same procedure described above for BSA-immunoconjugate (10). After dialyze and lyophilization, a grey powder (30 mg) was obtained. The coupling efficiency for BSA immunoconjugate (14) was estimated by Mass Spectrometry MALDI-TOF.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Change in mass (Da)</th>
<th>MW conjugate-MW BSA</th>
<th>MW hapten (Da)</th>
<th>Ratio Conjugate/BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-(14)</td>
<td>900 ± 146</td>
<td>243</td>
<td>3.7 ± 0.4</td>
<td></td>
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</table>

Immunization Protocol:

A 90 days immunization protocol with two New Zealand White rabbits was conducted for each antigen 10 and 14 (2 rabbits for each hapten). Immunisations were achieved by Proteogenix (Oberhausenbergen, France). One intradermal and seven subcutaneous injections were performed with 150 µg of each hapten conjugated to BSA and mixed with Freund-like adjuvant. To follow up on the immune response (antibody titre), blood samples were collected from ear vein at days 49 and 90. Total bleeding of rabbits was performed at day 90. Final sera were supplemented with 0.02% NaN₃ for preservation. Samples were assayed using the ELISA procedure described below.

Antibody titers:
Antigens used for immunization (haptens conjugated to BSA) were coated on 96-well microplate. Coating was performed overnight at 4°C with 100 ng of antigen per well in 10 mM sodium carbonate buffer pH 9. After blocking with 10% non-fat dry milk and washing, sera samples diluted from 1:100 to 1:100 000 in PBS with 0.05% Tween20 were incubated for 1 h at 37°C. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody was finally used for detection, and coloration was developed with substrate solution containing 1 mg/ml of nitrophenyl phosphate in diethanolamine buffer. The reaction was stopped by adding 3N NaOH and the absorbance at 405 nm (OD405) was determined with a microplate reader. Pre-immune rabbit sera were used as negative controls. Antibody titer was defined as the maximum serum dilution for which OD405 > 2x OD405 of the pre-immune serum.

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>Immunogens</th>
<th>Titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(10)</td>
<td>1/65 000</td>
</tr>
<tr>
<td>2</td>
<td>(10)</td>
<td>1/65 000</td>
</tr>
<tr>
<td>3</td>
<td>(14)</td>
<td>1/30 000</td>
</tr>
<tr>
<td>4</td>
<td>(14)</td>
<td>1/16 000</td>
</tr>
</tbody>
</table>

Reference