Immunochromical Determination of 2,4,6-Trichloroanisole as Responsible Agent of the Musty Odor in Foods. Part 1: Molecular Modeling Studies for Antibody Production.

SUPPORTING INFORMATION

Nuria Sanvicens, Francisco Sánchez-Baeza and M.-Pilar Marco

1Department of Biological Organic Chemistry, IIQAB-CSIC, Jordi Girona, 18-26. 08034-Barcelona, Spain.

*Corresponding author:
Dr. M.-Pilar Marco
Department of Biological Organic Chemistry
IIQAB-CSIC
Jorge Girona, 18-26
08034-Barcelona
Spain

Phone: +34 93 400 6171
FAX: +34 93 204 5904
Email: mpmqob@iiqab.csic.es
General Methods and Instruments. Thin Layer Chromatography (TLC) was performed on 0.25mm, pre-coated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). Unless otherwise indicated, purification of the reaction mixtures was accomplished by “flash” chromatography using silicagel as stationary phase. $^1$H and $^{13}$C NMR spectra were obtained with a Varian Unity-300 (Varian Inc., Palo Alto, CA, USA) spectrometer (300MHz for $^1$H and 75MHz for 13C) or on a Gemini 200 (199.975 MHz for $^1$H and 50.289 for $^{13}$C). Infrared spectra were measured on a Bomen MB 120 FTIR spectrophotometer (Hartmann & Braun, Québec, Canada). (GC-MS) was performed on a MD-800 capillary gas chromatograph with MS quadrupole detector (Fison Instrumentes, VG, Manchester, UK) and the data are reported as m/z (relative intensity). The ion-source temperature was set at 200°C, a 15m x 0.25mm i.d. x 0.15m (film thickness) DB-225 fused capillary column (J&W, Folsom, CA, USA) was used; He was the carrier gas employed at 1mL/min. GC conditions were as follows: temperature program, 80-220°C (10°C/min), 220°C (10min); injector temperature 250°C. The MALDI-TOF-MS (matrix assisted laser desorption ionization time-of-flight mass spectrometer) used for analyzing the protein conjugates was a Perspective BioSpectrometry™ Workstation provided with the software Voyager-DE™-RP (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA, USA) and Grams/386™ (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corporation (Salem, NH, USA).

Synthesis of the haptens. Haptens C9, C10, C12, C18-C20 and C22 as methyl ethers were synthesized from the corresponding phenolic compounds as methyl esters, according to the following general protocol. A mixture of CH3I (4 mmol) and dry K2CO3 (4 mmol) was added to a solution of the corresponding phenol (1mmol) in anhydrous DMF (4 mL) at room temperature under Ar atmosphere. The mixture was stirred for 12 hours at room temperature, washed with H2O to eliminate the excess of CH3I and extracted with Et2O. The organic layer was then washed again with water and aq. 1N HCl to eliminate the remained DMF. Finally the resulting organic phase was dried with MgSO4, filtered and evaporated under reduced pressure. The crude was purified by column chromatography (silicagel, CH2Cl2: hexane 1:1) to obtain the resultant pure anisole. Following the methyl ester was hydrolyzed by dissolving the anisol (1 mmol) in THF (18 mL), in a round bottom flask provided with a condenser and a magnetic stirrer. A solution of aqueous 1N NaOH (2.6 mmol) was added and the mixture heated at 80°C during 3h. The THF was evaporated and the crude redissolved with NaHCO3 saturated solution (30 mL) and washed with Et2O. The aqueous phase was then acidified and extracted with AcOEt, dried with MgSO4, filtered and evaporated under reduced pressure to obtain the hapten.
3-(2-Methoxy-3,5,6-trichlorophenyl)-2-propenoic acid 12 (hapten C9). From methyl 3-(2-hydroxy-3,5,6-trichlorophenyl)-2-propenoate 10 (41) methyl 3-(2-methoxy-3,5,6-trichlorophenyl)-2-propenoate 11 was obtained as a white solid (0.37g 88% yield). $^1$H NMR (200MHz, CDCl$_3$); δ (ppm): 3.71 (s, 3H, -OCH$_3$ ester), 3.77 (s, 3H, -OCH$_3$ anisole), 6.75 (d, J=16.2Hz, 1H, =CHCOO-), 7.45 (s, 1H$_{Ar}$) 7.77 (d, J=16.2Hz, 1H, PhCH=). The hydrolysis of the ester yielded C9 as white solid (0.29 g, 83% yield). $^1$H NMR (200MHz, CDCl$_3$); δ (ppm): 3.74 (s, 3H, -OCH$_3$), 6.80 (d, J=16.2Hz, 1H, =CHCOO-), 7.48 (s, 1H$_{Ar}$), 7.89 (d, J=16.2Hz, 1H, PhCH=).

2-Methoxy-3,5,6-trichlorobenzoic acid 15 (hapten C10). As described before from commercially available 2-hydroxy-3,5,6-trichlorobenzoic acid 13, methyl 2-methoxy-3,5,6-trichlorobenzoate 14 was obtained as a white solid (79 mg 69% yield). $^1$H NMR (200MHz, CDCl$_3$); δ (ppm): 3.89 (s, 3H, -OCH$_3$ ester), 3.97 (s, 3H, -OCH$_3$ anisole), 7.54 (s, 1H$_{Ar}$ para). $^{13}$C NMR (75MHz, CDCl$_3$); δ (ppm): 53.1 (-OCH$_3$, ester), 62.4 (-OCH$_3$, anisole), 127.2 (C-3'), 128.2 (C-1'), 128.9 (C-5'), 131.5 (C-6'), 131.8 (C-4'), 152.3 (C-2'), 164.3 (C-1). Following the ester was hydrolyzed to obtain C10 as a white solid (68 mg, 87% yield). $^1$H NMR (200MHz, CDCl$_3$); δ (ppm): 3.90 (s, 3H), 7.52 (s, 1H$_{Ar}$ para), 9.15 (bs, 1H, COOH). $^{13}$C NMR (75MHz, CDCl$_3$); δ (ppm): 62.6 (-OCH$_3$), 127.4 (C-3'), 128.1 (C-1'), 129.2 (C-5'), 130.6 (C-6'), 132.3 (C-4'), 152.3 (C-2'), 178.2 (C-1).

3-(2-Methoxy-3,5,6-trichlorophenyl)-propanoic acid 18 (hapten C12). As described above from methyl 3-(2-hydroxy-3,5,6-trichlorophenyl)-propanoate 16 (41) methyl 3-(2-methoxy-3,5,6-trichlorophenyl)-propanoate 17 was obtained as pale yellow oil (20 mg, 75% yield). $^1$H NMR (200MHz, CDCl$_3$); δ (ppm): 2.74 (t, J=6.9Hz, 2H, -CH$_2$COO-), 3.14 (t, J=6.9Hz, 2H, PhCH$_2$), 3.71 (s, 3H, -OCH$_3$ ester), 3.86 (s, 3H, -OCH$_3$ anisole), 7.40 (s, 1H$_{Ar}$ meta). $^{13}$C NMR (75MHz, CDCl$_3$); δ (ppm): 23.9 (-OCH$_3$, ester), 32.4 (C-3), 52.4 (C-2) 61.4 (-OCH$_3$, anisole), 120.3 (C-3'), 125.3 (C-1'), 128.2 (C-4'), 128.4 (C-5'), 131.8 (C-6'), 150.2 (C-2'), 175.2 (C-1). Following the methyl ester was hydrolyzed to obtain C12 (16 mg, 85% yield). $^1$H NMR (200MHz, DMSO-d$_6$); δ (ppm): 2.38 (t, J=6.9Hz, 2H, -CH$_2$COO-), 3.01 (t, J=6.9Hz, 2H, PhCH$_2$), 3.83 (s, 3H, -OCH$_3$), 7.62 (s, 1H$_{Ar}$ meta). $^{13}$C-NMR (75MHz, DMSO-d$_6$); δ (ppm): 24.3 (C-3), 32.1 (C-2), 61.0 (-OCH$_3$), 120.3 (C-3'), 122.3 (C-1'), 128.0 (C-4'), 130.0 (C-5'), 130.6 (C-6'), 150.8 (C-2'), 173.3 (C-1).
3-(3,6-Dichloro-2-methoxyphenyl)-propanoic acid 21 (hapten C18). As described above from 3-(2-hydroxy-3,6-dichlorophenyl)-propanoic acid 19 (41) methyl 3-(3,6-dichloro-2-methoxyphenyl)-propanoate 20 was obtained as a white solid (21 mg, 84% yield). 1H NMR (200MHz, DMSO-d6); δ (ppm): 2.55 (t, J=7.4Hz, 2H, -CH₂COO-), 2.88 (t, J=7.4Hz, 2H, PhCH₂-), 3.62 (s, 3H, -OCH₃ ester), 3.77 (s, 3H, -OCH₃ anisole), 7.01 (d, J=2.6Hz, 1H₆, meta), 7.19 (d, J=2.6Hz, 1H₆, para). Following the methyl ester was hydrolyzed by treating 20 (21 mg 0.08 mmol) in MeOH (3 mL) with 1N KOH (2 mL) for 12h to render C18 as a white solid (17 mg, 89% yield). 1H NMR (200MHz, CDCl₃); δ (ppm): 2.68 (t, J=7.5Hz, 2H, -CH₂COO-), 2.95 (t, J=7.5Hz, 2H, PhCH₂-), 3.85 (s, 3H, -OCH₃), 7.10 (d, J=2.7Hz, 1H₆, meta), 7.27 (d, J=2.7Hz, 1H₆, para).

2,6-Dichloro-3-methoxycetic acid 23 (hapten C19). As previously described from methyl 2,6-dichloro-3-hydroxycetate 21 (37) methyl 2,6-dichloro-3-methoxycetate 22 was obtained as a white solid (100 mg, 87% yield). 1H NMR (200MHz, CDCl₃); δ (ppm): 3.65 (s, 3H, -OCH₃ ester), 3.82 (s, 2H, -CH₂-), 3.96 (s, 3H, -OCH₃ anisole), 6.99 (d, J=9Hz, 1H₆, para), 7.27 (d, J=9Hz, 1H₆, meta). 13C NMR (75MHz, CDCl₃); δ (ppm): 37.0 (C-2), 52.3 (-OCH₃ ester), 56.4 (-OCH₃ anisole), 111.5 (C-4'), 115.4 (C-2'), 127.0 (C-6'), 127.7 (C-5'), 132.2 (C-1'), 154.1 (C-3'), 169.9 (C-1). Following the methyl ester was hydrolyzed to obtain C19 as a white solid (104mg 96% yield). 1H NMR (200MHz, CDCl₃); δ (ppm): 3.79 (s, 2H, -CH₂-), 3.89 (s, 3H, -OCH₃), 6.92 (d, J=9Hz, 1H₆, para), 7.25 (d, J=9Hz, 1H₆, meta).

2-(2,4-Dichloro-5-methoxyphenyl)-acetic acid 26 (hapten C20). As described above, from methyl 2-(2,4-dichloro-5-hydroxyphenyl)-acetate 24 (37) methyl 2-(2,4-dichloro-5-methoxyphenyl)-acetate 25 was obtained as white solid (0.41g, 86% yield). 1H-NMR (200MHz, CDCl₃); δ(ppm): 3.73 (s, 3H, -OCH₃ ester), 3.74 (s, 2H, -CH₂-), 3.89 (s, 3H, -OCH₃ anisole), 6.86 (s, 1H₆, ortho), 7.40 (s, 1H₆, meta). 13C-NMR (75MHz, CDCl₃); δ(ppm): 38.8 (-OCH₃ ester), 52.3 (C-2), 56.4 (-OCH₃ anisole), 114.5 (C-6'), 122.2 (C-4'), 125.8 (C-2'), 130.4 (C-3'), 131.6 (C-1'), 153.8 (C-5'), 170.6 (C-1). Following the methyl ester was hydrolyzed to obtain C20 as a white solid (0.28 g, 75% yield). 1H NMR (200MHz, CDCl₃); δ (ppm): 3.72 (s, 2H, -CH₂-), 3.92 (s, 3H, -OCH₃), 6.84 (s, 1H₆, ortho), 7.36 (s, 1H₆, meta).

2-(Methoxy-2,4,6-trichlorophenyl)-acetic acid 29 (hapten C22). As before, from methyl 3-(3-hydroxy-2,4,6-trichlorophenyl)-acetate 27 (37) pure methyl 3-(3-methoxy-2,4,6-trichlorophenyl)-
acetate 28 was obtained as white solid (0.37 g, 72% yield). $^1$H NMR (200MHz, CDCl₃); δ (ppm): 3.66 (s, 3H, -OCH₃ ester), 3.82 (s, 2H, -CH₂-), 3.92 (s, 3H, -OCH₃ anisole), 7.33 (s, 1H, meta). Following the methyl ester was hydrolyzed to yield C12 as white solid (71 mg, 95% yield). $^1$H NMR (200MHz, CDCl₃); δ (ppm): 3.81 (s, 2H, CH₂), 3.96 (s, 3H, -OCH₃), 7.34 (s, 1H, meta). $^{13}$C-NMR (75MHz, CDCl₃); δ(ppm): 36.7 (C-2), 60.8 (-OCH₃), 128.85 (C-4'), 130.5 (C-2'), 130.7 (C-6'), 130.7 (C-5'), 131.5 (C-1'), 151.5 (C-3'), 175.3 (C-1).

**Hapten Density analysis.** Hapten densities of the BSA conjugates were determined by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) by comparing the molecular weight of the standard BSA and that of the conjugates. MALDI spectra were obtained by mixing 1 μL of the matrix (E-3,5-dimethoxy-4-hydroxycinnamic acid, 10mg mL⁻¹ in CH₃CN/H₂O 70:30, 0.1% TFA) and 1 μL of a solution of the conjugates or proteins (5mg mL⁻¹ in CH₃CN/H₂O 70:30, 0.1% TFA).

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>δ¹</th>
<th>Conjugate</th>
<th>δ</th>
<th>Conjugate</th>
<th>δ</th>
<th>Conjugate</th>
<th>δ</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-BSA</td>
<td>26</td>
<td>C7-BSA</td>
<td>7</td>
<td>C13-BSA</td>
<td>12</td>
<td>C19-BSA</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C2-BSA</td>
<td>49</td>
<td>C8-BSA</td>
<td>10</td>
<td>C14-BSA</td>
<td>18</td>
<td>C20-BSA</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>C3-BSA</td>
<td>25</td>
<td>C9-BSA</td>
<td>7</td>
<td>C15-BSA</td>
<td>13</td>
<td>C21-BSA</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C4-BSA</td>
<td>24</td>
<td>C10-BSA</td>
<td>4</td>
<td>C16-BSA</td>
<td>8</td>
<td>C22-BSA</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>C5-BSA</td>
<td>8</td>
<td>A-BSA (C11)</td>
<td>4</td>
<td>C-BSA (C17)</td>
<td>7</td>
<td>B-BSA (C23)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>C6-BSA</td>
<td>7</td>
<td>C12-BSA</td>
<td></td>
<td>C18-BSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Hapten densities (δ) of the BSA conjugates measured y MALDI-TOF-MS.

¹Hapten densities were calculated according to the following formula:

(Mw conjugate - Mw intact BSA)/Mw hapten.

Only the BSA conjugates are shown for comparison of the conjugation rates achieved.

The same pattern was observed on each hapten for the other protein conjugates. All the conjugates were prepared by the mixed anhydride method.