SUPPORTING INFORMATION

Analysis of Nonylphenol: Advances and Improvements in the Immunochemical Determination using Antibodies Raised Against the Technical Mixture and Hydrophilic Immunoreagents

M.- Carmen Estevez, Mark Kreuzer, Francisco Sanchez-Baeza and M.-Pilar Marco*

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

*To whom correspondence should be sent:

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

Phone: +34 93 4006171
FAX: +34 93 2045904
E-mail: mpmqob@iiqab.csic.es

General Methods: ^{1}H and ^{13}C NMR spectra were obtained with a Varian Unity-300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) or a Varian Inova 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) (Varian Inc. Palo Alto, CA). Infrared (IR) spectra were measured on a Bomen MB 120 FTIR spectrophotometer (Hartmann & Braun, Québec, Canada). Gas chromatography-mass spectrometry (GC-MS) was performed on an MD-800 capillary gas chromatograph with an MS quadrupole detector (Fisons Instruments, VG, Manchester, UK) and the data are reported as *m/z* (relative intensity). The ion source temperature was set at 200ºC, a 15m × 0.25mm i.d × 0.15 μm (film thickness) DB-225 fused capillary column (J&W, Folsom, CA) was used; Helium (He) was the carrier gas employed at 1mL min^{-1}. GC conditions were as follows: temperature program , 100ºC (1min) (7ºC/min), 300ºC (0min); injector temperature, 250ºC. High Resolution Mass Spectrometry (HRMS) by electronic impact (EI) was performed on a Micromass Autospec spectrometer by the Unity of Mass Spectrometry of the Universidad de Santiago de Compostela (Spain).

***1-Methoxy-4-nonylbenzene,* NPa.** Iodomethane (11.2 mL, 180 mmol) was added dropwise to a stirred suspension of NP (10 g, 45 mmol) and anhydrous K_2CO_3 (25 g, 181 mmol) in dry acetone (150 mL) according to standard procedures to obtain ***1-methoxy-4-nonylbenzene,* NPa,** (9.79g, 92% yield) which was characterized according to their spectroscopic data. ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) 0.50-1.66 (bs, 19H, isomeric nonyl chain), 3.79 (s, 3H, OCH_3) 6.83 (d, J=9 Hz, $2H_{Ar}$ *ortho*), 7.13-7.28 (m, $2H_{Ar}$ *meta*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 8.6-41.4 (isomeric nonyl chain), 55.1 (OCH_3) 113.1 (C-2', C-6'), 126.8 (C-3', C-5'), 140.0 (C-4'), 157.0 (C-1'). IR, ν (KBr, cm^{-1}): 2961 (-C-H st), 1513 (ar C-C) 1251 (C-O-C st as), 1039 (C-O-C st si), 827 (ar C-H δoop).

5-(2-Methoxy-5-nonylphenyl)-5-oxo-pentanoic acid (NP2). Following, **NPa** (2 g, 8.5 mmol) in nitromethane (10 mL) was added dropwise to a suspension of anhydrous $AlCl_3$ (2.27 g, 17 mmol) and glutaric anhydride (0.97 g, 8.5 mmol) in nitromethane (30 mL) placed in a round-bottom flask under argon atmosphere and cooled to -20ºC. The temperature of the mixture was kept for 90 min at -20ºC and then allowed to reach room temperature under stirring for 3h, until no evolution was observed by TLC. The reaction mixture was poured into an ice-cooled solution of concentrated HCl and extracted with CH_2Cl_2. The organic phase was then consecutively washed with saturated NaCl and H_2O and finally dried with $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The product obtained was then purified by "flash" chromatography using a hexane/EtOAc gradient as mobile phase to finally isolate the desired product **NP2** as a yellow oil (1.2 g, 40% yield). The product was characterized by spectroscopic and spectrometric data ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) 0.50-1.66 (bs,

19H, isomeric nonyl chain),2.03 (m, 2H, J=7.2 Hz, -CH_2-), 2.46 (t, 2H, J=7.2 Hz, -CH_2COO-), 3.07 (t, 2H, J=7.2 Hz, -$COCH_2$-), 3.87 (s, 3H, OCH_3), 6.76 (d, J=8.7, H_{Ar}-3), 7.30-7.44 (bs, H_{Ar}-4), 7.58-7.68 (bs, H_{Ar}-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 8.6-40.4 (isomeric nonyl chain), 19.3 (C-3), 33.3 (C-2), 42.6 (C-4), 55.4 (OCH_3), 110.9 (C-3'), 127.8 (C-1'), 131.3 (C-4',C-6'), 140.1-141.5 (C-5'), 156.4 (C-2'), 179.3 (C-1), 201.9 (C-5). IR, ν (KBr, cm^{-1}): 2961 (C-H st), 2931 (COO-H st), 1711 (C=O st *acid*), 1644 (C=O *ketone*), 1505-1487 (ar C-C), 1255 (C-O-C st as), 1025 (C-O-C st si), 820 (arC-H δoop). EI-MS *m/z* (%):348 (M^+, 12), 319 (13), 291 (13), 277 (35), 263 (100), 261 (39), 259 (26), 248 (22), 244 (50), 231 (26), 217 (16), 203 (56), 189 (18), 175 (21). HRMS *m/z* (calculated for $C_{21}H_{32}O_4$): 348.230060; *m/z* (observed): 348.229181. Error (ppm): 2.5. As a by-product of the reaction, ***5-(4-methoxyphenyl)-5-oxo-pentanoic acid,*** **NPc,** was also obtained (95 mg, 5.5% yield) as could be identified by its spectroscopic data (see below).

Ethyl 5-(2-methoxy-5-nonylphenyl)-pentanoate, NPb. Palladium on activated carbon (5%, 72 mg of Pd/C 10%) and three drops of $HClO_4$ were added to a solution of **NP2** (470 mg, 1.35 mmol) in absolute EtOH (6.75 mL) under H_2 at atmospheric pressure and was stirred at room temperature overnight to obtain the reduced compound as a pale yellow liquid corresponding to the desired product **NPb,** (460 mg, 94% yield) according to spectroscopic data. ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) 0.53-1.66 (bs, 19H, isomeric nonyl chain; 4H, -$(CH_2)_2$-, 3H, -CH_3), 2.33 (t, 2H, J=7.2 Hz, -CH_2COO-), 2.63 (t, 2H, J=7.2 Hz, -CH_2Ph), 3.79 (s, 3H, OCH_3), 4.12 (q, 2H, J=7.2 Hz, -$COOCH_2$-), 6.76 (bs, H_{Ar}-3), 7.02 (bs, H_{Ar}-4), 7.07 (bs, H_{Ar}-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 8.6-40.4 (isomeric nonyl chain), 24.8 (C-3), 29.5 (C-2), 30.2 (C-4), 34.3 (C-5), 55.1 (OCH_3 *anisole*), 60.1 (OCH_2- *ester*), 109.4 (C-3'), 124.7-128.6 (C-1',C-4',C-6'), 139.2-142.2 (C-5'), 154.9 (C-1'), 154.9 (C-2'), 173.8 (C-1).

5-(2-Methoxy-5-nonylphenyl)-pentanoic acid, **NP3.** The subsequent hydrolysis of the ester **NPb** (110 mg, 0.3 mmol) with 1M KOH led to a yellow oil (97 mg, 97% yield) identified as the desired product **NP3** by spectroscopic and spectrometric data. ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) 0.53-1.66 (bs, 19H, isomeric nonyl chain; 4H, -$(CH_2)_2$-), 2.39 (t, 2H, J=6.5 Hz, -CH_2COO-), 2.63 (t, 2H, J=6.9 Hz, -CH_2Ph), 3.80 (s, 3H, OCH_3), 6.76 (d, J= 8.4 Hz, H_{Ar}-3), 7.03 (bs, H_{Ar}-4), 7.07 (bs, 1H_{Ar}-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 8.6-40.4 (isomeric nonyl chain), 24.4 (C-3), 29.4 (C-2), 30.1 (C-4), 33.9 (C-5), 55.1 (OCH_3), 109.4 (C-3'), 124.7-128.6 (C-1',C-4', C-6'), 139.3-142.0 (C-5'), 154.9 (C-2'), 180.4 (C-1). IR, ν (KBr, cm^{-1}): 2961 (C-H st), 2933 (COO-H st), 1715 (C=O st), 1487 (arC-C), 1244 (C-O-C st as), 1040 (C-O-C st si), 815 (ar C-H δ oop). EI-MS *m/z* (%):334 (M^+, 13), 305 (19), 291 (13), 277 (24), 263 (18), 249 (100), 242 (21), 233 (23), 231 (32), 221 (16), 217 (16), 201 (11), 187 (18), 175

(14), 161 (22). HRMS *m/z* (calculated for $C_{21}H_{34}O_3$): 334.250795; *m/z* (observed): 334.249492. Error (ppm): 3.9.

***5-(2-Hydroxy-5-nonylphenyl)-pentanoic acid,* NPVA.** A 1M solution of BBr_3 in hexane was added dropwise (1.25 mL, 1.25 mmol) to a solution of **NPb** (125 mg, 0.35 mmol) in anhydrous CH_2Cl_2 (2 mL) placed in a round-bottom flask under argon atmosphere and cooled at -78ºC. The reaction mixture was allowed to reach room temperature and stirred for 4h until the reaction was completed according to the TLC analysis. The reaction mixture was then poured into an ice/water solution and extracted with CH_2Cl_2. The organic phase was washed with saturated NaCl, dried with $MgSO_4$, filtered and evaporated to obtain an oil corresponding to a mixture of the desire product NPVA and their corresponding ethyl ester (100 mg, NPVA/ethyl ester, 33:67) according to its spectroscopic data. Without further purification the oil obtained was dissolved in MeOH (0.7 mL) and a solution of 1M KOH was added (0.5 mL). The reaction was stirred at room temperature until the total disappearance of the ester, according to the analysis by TLC. The reaction was then stopped and treated as described before obtaining a yellow oil identified as the desired product **NPVA** (78 mg, 70% yield) according to its spectroscopic and spectrometric data. ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) 0.53-1.70 (bs, 19H, isomeric nonyl chain; 4H, -$(CH_2)_2$-), 2.41 (t, 2H, J=6.3 Hz, -CH_2COO-), 2.63 (t, 2H, J=6.9 Hz, -CH_2Ph), 6.67 (d, J= 8.1 Hz, 1H_{Ar}-3), 6.98 (bs, H_{Ar}-4), 7.03 (bs, 1H_{Ar}-6). ^{13}C NMR (75 MHz, $CDCl_3$) δ(ppm): 8.6-40.4 (isomeric nonyl chain), 24.2 (C-3), 29.2 (C-2), 29.8 (C-4), 33.7 (C-5), 114.5 (C-3'), 124.7-128.6 (C-1', C-4', C-6'), 140.0-142.8 (C-5'), 150.7 (C-2'), 179.7 (C-1). IR, ν (KBr, cm^{-1}): 3413 (-OH st), 2958 (-C-H st), 2933 (COO-H st), 1710 (C=O st), 1505-1488 (ar C-C), 1259 (C-O st *phenol*), 822 (ar C-H δ oop). MS, *m/z* (%): 320 (M+, 9), 291 (15), 249 (20), 235 (80), 217 (100), 203 (28), 189 (20), 171 (15), 147 (35). EI-MS *m/z* (%):320 (M^+, 16), 291 (22), 277 (14), 263 (25), 249 (59), 235 (100), 231 (25), 221 (11), 217 (86), 203 (29), 189 (17). HRMS *m/z* (calculated for $C_{20}H_{32}O_3$): 320.235145; *m/z* (observed): 320.234293. Error (ppm): 2.7.

***5-(2-Hydroxy-5-nonylphenyl)-5-oxo-pentanoic acid,* NP1.** As described above, a solution of **NP2** (100 mg, 0.29 mmol) in anhydrous CH_2Cl_2 was cooled to -78ºC and reacted with BBr_3 (0.9 mL, 0.9 mmol) until no more evolution was observed in the reaction by TLC. The reaction mixture was treated as before obtaining 83 mg of a crude oil. This residue was purified by "flash" chromatography with a hexane/EtOAc gradient as mobile phase, to finally isolate the desired product **NP1** (50 mg, 52% yield) identified according to its spectroscopic and spectrometric data. ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) 0.5-1.8 (bs, 19H, isomeric nonyl chain), 2.09 (m, 2H, J=7.2 Hz, -CH_2-), 2.54 (t, 2H, J=7.2 Hz, -CH_2COO-), 3.11 (t, 2H, J=7.2

Hz, -$COCH_2$-), 6.92 (d, J= 9 Hz, $1H_{Ar}$-3), 7.45 (bs, $1H_{Ar}$-4), 7.60 (bs, $1H_{Ar}$-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 8.6-40.3 (isomeric nonyl chain), 19.2 (C-3), 32.9 (C-2), 37.0 (C-4), 118.0 (C-3'), 126.6 (C-1'), 135.0 (C-6'), 138.5(C-4'), 140.1(C-5'), 160.1 (C-2'), 179.2 (C-1), 205.6 (C-5). IR, ν (KBr, cm^{-1}): 2961 (-C-H st), 2931 (COO-H st), 1711 (C=O st *acid*), 1644 (C=O st *ketone*), 1485 (COO^- st). EI-MS *m/z* (%):334 (M^+, 12), 301 (20), 291 (14), 277 (26), 263 (53), 259 (17), 249 (100), 242 (43), 232 (18), 231 (11), 217 (36), 203 (37), 189 (16), 175 (22), 161 (21). HRMS *m/z* (calculated for $C_{20}H_{30}O_4$): 334.214410; *m/z* (observed): 334.214332. Error (ppm): 0.2.

***5-(4-Nonylphenoxy)-pentanoic acid,* NP4**. NP (1 g, 4.53 mmol) dissolved dry acetone (20 mL) with anhydrous K_2CO_3 (1.87 g, 13.6 mmol) was reacted with a solution of methyl 5-bromopentanoate (1.38 g, 7.1 mmol) in dry acetone (5 mL). After heating the reaction at 60ºC for 48h, the mixture was filtered, evaporated and the residue redissolved in Et_2O. The organic layer was consecutively washed with 1N HCl and saturated NaCl, dried with $MgSO_4$, filtered and the solvent evaporated under vacuum. The residue was then purified by column chromatography packed with silica gel using 9:1 hexane/EtOAc as mobile phase to finally obtain the desired product ***methyl 5-(4-nonylphenoxy)-pentanoate,* NPe** as a yellow oil (1.2 g, 81% yield) identified according to its spectroscopic and spectrometric data ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.50-1.66 (bs, 19H, isomeric nonyl chain), 1.84 (m, 4H, -$(CH_2)_2$-), 2.41 (t, J=6.9 Hz, 2H, -CH_2COO-), 3.67 (s, 3H, -OCH_3), 3.96 (t, J=5.7 Hz, 2H, $PhOCH_2$-), 6.81 (d, J=8.7 Hz, $2H_{Ar}$ *ortho*), 7.13-7.26 (m, $2H_{Ar}$ *meta*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 8.6-40.2 (isomeric nonyl chain), 21.6 (C-3), 28.7 (C-4), 33.7 (C-2), 51.5 (OCH_3), 67.1 (C-5), 113.5 (C-2', C-6'), 127.1 (C-3', C-5'), 139.9 (C-4'), 156.4 (C-1'), 173.9 (C-1). MS, *m/z* (%): 334 (M+, 10), 305 (28), 263 (36), 249 (40), 135 (35), 115(50), 107 (55), 83 (75), 73 (84), 55 (100). The ester **NPe** (1.2 g, 3.6 mmol) was hydrolyzed with 1M KOH as described before and a yellow oil (1.1 g, 95% yield) was obtained, identified as the desired product **NP4** according to its spectroscopic and spectrometric data. ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.50-1.66 (bs, 19H, isomeric nonyl chain), 1.84 (m, 4H, -$(CH_2)_2$-), 2.46 (t, J=6.6 Hz, 2H, -CH_2COO-), 3.97 (t, J=5.7 Hz, 2H, $PhOCH_2$-), 6.81 (d, J=9 Hz, $2H_{Ar}$ *ortho*), 7.13-7.26 (m, $2H_{Ar}$ *meta*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 8.6-40.2 (isomeric nonyl chain), 21.4 (C-3), 28.6 (C-4), 33.6 (C-2), 67.1 (C-5), 113.6 (C-2', C-6'), 127.0 (C-3', C-5'), 140.0 (C-4'), 156.4 (C-1'), 179.6 (C-1). IR, ν (KBr, cm^{-1}): 2963 (-C-H st), 1715 (C=O st), 1515 (arC-C), 1244 (C-O-C st as), 825 (ar C-H δ oop). EI-MS *m/z* (%):320 (M^+, 19), 291 (25), 277 (16), 263 (21), 249 (55), 235 (100), 221 (11), 217 (86), 149 (36), 135 (80), 134 (16), 121 (28), 107 (25). HRMS *m/z* (calculated for $C_{20}H_{32}O_3$): 320.235145; *m/z* (observed): 320.235484. Error (ppm): -1.1.

***5-(4-Methoxyphenyl)-pentanoic acid,* NP6.** Similarly as described for **NP2,** anisole (2 g, 18.5 mmol) was reacted with glutaric anhydride (2.1 g, 18.5 mmol) in the presence of $AlCl_3$ (4.91 g, 37 mmol) using nitromethane as solvent (40 mL) to obtain ***5-(4-methoxyphenyl)-5-oxo-pentanoic acid,* NPc,** (1.97 g, 48% yield) as a white powder, identified attending to its spectroscopic and spectrometric data. ^{1}H NMR (300 MHz, CD_3CD): δ (ppm) 1.97 (m, 2H, J=7.2 Hz, -CH_2-), 2.40 (t, 2H, J=7.2 Hz, -CH_2COO-), 3.03 (t, 2H, J=7.2 Hz, -COCH_2-), 3.86 (s, 3H, OCH_3), 6.99 (d, J= 9 Hz, 2H_{Ar}-3,-5), 7.97 (d, J= 9 Hz, 2H_{Ar}-2,-6). ^{13}C NMR (75 MHz, CD_3CD): δ (ppm) 20.9 (C-3), 34.0 (C-2), 38.1 (C-4), 56.0 (OCH_3), 114.8 (C-3', C-5'), 131.0 (C-1'), 131.5 (C-2', C-6'), 165.3 (C-4'), 177.1 (C-1), 200.6 (C-5). IR, ν (KBr, cm^{-1}): 2974 (COO-H st), 1709 (C=O *acid*), 1672 (C=O *ketone*), 1597-1574 (Ar C-C), 1252 (C-O-C as). EI-MS *m/z* (%):222 (M^+, 25), 150 (12), 135 (100). HRMS *m/z* (calculated for $C_{12}H_{14}O_4$): 222.089209; *m/z* (observed): 222.089366. Error (ppm): -0.7. Reduction of the keto group of **NPc** (1.5 g, 6.76 mmol) was similarly performed under H_2 atmosphere with palladium on activated carbon (5%, 309 mg) in MeOH (40 mL) to obtain the ***methyl 5-(4-methoxyphenyl)-pentanoate,* NPd,** (1.6 g, 80% yield) which was identified by spectroscopic and spectrometric data. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.64 (m, 4H, $(CH_2)_2$), 2.31 (t, 2H, J=7.2 Hz, -CH_2COO-), 2.57 (t, J=7.2 Hz, PhCH_2-), 3.66 (s, 3H, $COOCH_3$), 3.78 (s, 3H, -OCH_3) 6.82 (d, J= 8.7 Hz, 2H_{Ar}-3, -5), 7.09 (d, J= 8.7 Hz, 2H_{Ar}-2, -6). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.4 (C-3), 31.1 (C-2), 33.9 (C-4), 34.6 (C-5), 51.4 (OCH_3 *ester*), 55.2 (-OCH_3 *anisole*), 113.6 (C-3', C-5'), 129.2 (C-2', C-6'), 134.1 (C-1'), 157.7 (C-4'), 174.1 (C-1). MS, *m/z* (%): 222 (M+, 40), 191 (14), 147 (23), 134 (40), 121(100), 91(35), 77(32). Finally, the ester (100 mg, 0.45 mmol) was hydrolyzed as described before and a white powder identified as **NP6** (88 mg, 95% yield) was obtained according to its spectroscopic and spectrometric data. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.66 (m, 4H, $(CH_2)_2$), 2.38 (t, 2H, J=6.9 Hz, -CH_2COO-), 2.58 (t, J=7.0 Hz, PhCH_2-), 3.79 (s, 3H, -OCH_3) 6.83 (d, J= 8.7 Hz, 2H_{Ar}-3, -5), 7.09 (d, J= 8.7 Hz, 2H_{Ar}-2, -6). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.2 (C-3), 30.9 (C-2), 33.9 (C-4), 34.6 (C-5), 55.2 (-OCH_3), 113.7(C-3', C-5'), 129.2 (C-2', C-6'), 134.1 (C-1'), 157.7 (C-4'), 179.9 (C-1). IR, ν (KBr, cm^{-1}): 2927 (COO-H st), 1697 (C=O), 1612-1583 (Ar C-C), 1251 (C-O-C as). EI-MS *m/z* (%):208 (M^+, 68), 121 (100). HRMS *m/z* (calculated for $C_{12}H_{16}O_3$): 208.109945; *m/z* (observed): 208.109944. Error (ppm): 0.0.

5-(4-Hydroxyphenyl)-pentanoic acid,* NP5.** As described before deprotection of the phenolic group was accomplished by reacting **NPd** (500 mg, 2.25 mmol) in anhydrous CH_2Cl_2 (7 mL) with a 1M solution of BBr_3 in hexane (4.5 mL, 4.5 mmol) at -78 ºC. Treatment of the reaction mixture, afforded and oily product identified by ^{1}H and ^{13}C NMR as ***methyl 5-(4-

hydroxyphenyl)-pentanoate **NPf** (375 mg, 80% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 1.63 (m, 4H, $(CH_2)_2$), 2.34 (t, 2H, J=7.2 Hz, -CH_2COO-), 2.54 (t, J=7.2 Hz, PhCH_2-), 3.67 (s, 3H, -$COOCH_3$), 6.75 (d, J= 8.7 Hz, 2H_{Ar}-3, -5), 7.02 (d, J= 8.7 Hz, 2H_{Ar}-2, -6). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 24.4 (C-3), 31.1 (C-2), 33.9 (C-4), 34.6 (C-5), 51.6 (OCH_3 *ester*), 115.1 (C-3', C-5'), 129.4 (C-2', C-6'), 134.0 (C-1'), 153.7 (C-4'), 174.7 (C-1). Finally, the ester **NPf** (350 mg, 1.68 mmol) was hydrolyzed as described before and a white powder was obtained and identified as **NP5** (300 mg, 92% yield) according to its spectroscopic and spectrometric data. ^{1}H NMR (300 MHz, CD_3OD): δ (ppm) 1.60 (m, 4H, $(CH_2)_2$), 2.29 (t, 2H, J=7.0 Hz, -CH_2COO-), 2.52 (t, J=6.9 Hz, PhCH_2-), 6.68 (d, J= 8.7 Hz, 2H_{Ar}-3, -5), 6.98 (d, J= 8.7 Hz, 2H_{Ar}-2, -6). ^{13}C NMR (75 MHz, CD_3OD): δ (ppm) 25.6 (C-3), 32.4 (C-2), 34.8 (C-4), 35.7 (C-5), 116.0 (C-3', C-5'), 130.2 (C-2', C-6'), 134.3 (C-1'), 156.3 (C-4'), 177.7 (C-1). IR, ν (KBr, cm^{-1}): 3407 (OH st), 2945 (COO-H st), 1693 (C=O), 1610-1597 (Ar C-C). EI-MS *m/z* (%):194 (M^+, 86), 120 (11), 107 (100). HRMS *m/z* (calculated for $C_{11}H_{14}O_3$): 194.094294; *m/z* (observed): 194.095121. Error (ppm): -4.3.

9-(4-Hydroxyphenyl)-nonanoic acid, **NP9.** Oxalyl dichloride (4.3 mL, 49.5 mmol) was added dropwise to the nonanedioic acid monomethyl ester (1 g, 4.95 mmol) and the mixture was stirred under argon atmosphere overnight at RT and then was evaporated to dryness under reduced pressure to obtain **8-chlorocarbonyl-octanoic acid methyl ester** as an oil. The haloester was reacted with anisole (0.49 g, 4.5 mmol) in the presence of $AlCl_3$ (1.2 g, 9.05 mmol) in nitromethane as described before to finally obtain the desired product ***9-(4-methoxyphenyl)-9-oxo-nonanoic acid methyl ester*** **NPc'** (592 mg, 44% yield) identified according to ^{1}H and ^{13}C NMR. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.66 (m, 6H $(CH_2)_3$), 1.62 (m, 2H, -CH_2-), 1.72 (m, 2H, -CH_2-), 2.30 (t, 2H, J=7.5 Hz, -CH_2COO-), 2.90 (t, J=7.5 Hz, -CH_2CO-), 3.66 (s, 3H, -$COOCH_3$), 3. 87 (s, 3H, -OCH_3), 6.93 (d, J= 9 Hz, 2H_{Ar}-3, -5), 7.94 (d, J= 9 Hz, 2H_{Ar} -2, -6). ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 24.6-34.2 (C-2-C-7), 38.3 (C-8), 51.6 (-OCH_3 *ester*), 55.6 (-OCH_3 *anisole*), 113.8 (C-3', C-5'), 130.2 (C-1'), 130.4 (C-2', C-6'), 163.4 (C-4'), 174.4 (C-1), 199.3 (C-9). Reduction of the keto group of **NPc'** (490 mg, 1.65 mmol) was performed as described before under H_2 atmosphere and using Pd on activated carbon (5%, 80 mg) and the compound ***9-(4-methoxyphenyl)-nonanoic acid methyl ester*** **NPd'** (350 mg, 75% yield) was obtained, which was identified by ^{1}H and ^{13}C NMR. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.29 (m, 8H $(CH_2)_4$), 1.57 (m, 2H, -CH_2-), 1.61 (m, 2H, -CH_2-), 2.30 (t, 2H, J=8 Hz, -CH_2COO-), 2.54 (t, J=8 Hz, PhCH_2-), 3.66 (s, 3H, -$COOCH_3$), 3. 78 (s, 3H, -OCH_3), 6.82 (d, J= 8.5 Hz, 2H_{Ar} *meta*), 7.08 (d, J= 8.5 Hz, 2H_{Ar} *ortho*). ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 25.0-35.1 (C-2-C-9), 51.6 (-OCH_3 *ester*), 55.3 (-OCH_3 *anisole*), 113.7 (C-3', C-5'), 129.3 (C-2', C-6'), 135.1 (C-1'), 157.7 (C-4'), 174.5 (C-

1). Finally, deprotection of the phenolic group was performed as described above by reacting **NPd'** (300 mg, 1.07 mmol) with BBr_3 (3.2 ml, 3.2 mmol) in anhydrous CH_2Cl_2 (5 mL) at -78ºC. The desired product ***9-(4-hydroxyphenyl)-nonanoic acid methyl ester*** **NPf'** (250 mg, 88% yield) was isolated as described before accompanied by ***9-(4-hydroxyphenyl)-nonanoic acid 4-(8-methoxycarbonyloctyl)-phenyl ester*** in minor amounts.

9-(4-Hydroxyphenyl)-nonanoic acid methyl ester (NPf') ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.29 (m, 8H $(CH_2)_4$), 1.55 (m, 2H, -CH_2-), 1.61 (m, 2H, -CH_2-), 2.31 (t, 2H, J=7.5 Hz, -CH_2COO-), 2.51 (t, J=7.5 Hz, PhCH_2-), 3.68 (s, 3H, -$COOCH_3$), 6.75 (d, J= 8.5 Hz, 2H_{Ar} *meta*), 7.02 (d, J= 8.5 Hz, 2H_{Ar} *ortho*). ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 24.8-35.4 (C-2-C-9), 51.7 (-OCH_3 *ester*), 115.2 (C-3', C-5'), 129.5 (C-2', C-6'), 134.9 (C-1'), 153.7 (C-4'), 174.9 (C-1).

9-(4-Hydroxyphenyl)-nonanoic acid 4-(8-methoxycarbonyloctyl)-phenyl ester. (It is only indicated the differential peaks corresponding to the phenyl ester). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 1.74 (m, 2H, -CH_2-), 2.34 (t, 2H, J=7.5 Hz, -CH_2COOPh), 2.58 (t, J=7.5 Hz, PhCH_2-), 6.97 (d, J= 8.5 Hz, 2H_{Ar} *meta*), 7.16 (d, J= 8.5 Hz, 2H_{Ar} *ortho*). ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 53.5 (OCH_3 *ester*), 121.3 (C-3', C-5'), 129.4 (C-2', C-6'), 140.5 (C-1'), 148.7 (C-4'), 179.4 (-COOPh).

The mixture of esters was hydrolyzed with 1M KOH in MeOH at 50ºC for 24h, obtaining 220 mg of the desired product that was subsequently purified by crystallization to obtain pure brown crystals (125 mg, 53% yield) of ***9-(4-hydroxyphenyl)-nonanoic acid (NP9)*** ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD): δ (ppm) 1.26 (m, 8H $(CH_2)_4$), 1.51 (m, 2H, J=7.5 Hz, -CH_2-), 1.56 (m, 2H, J=7.5 Hz, -CH_2-), 2.25 (t, 2H, J=7.5 Hz, -CH_2COO-), 2.47 (t, J=8 Hz, PhCH_2-), 6.70 (d, J= 8.5 Hz, 2H_{Ar} *meta*), 6.97 (d, J= 8.5 Hz, 2H_{Ar} *ortho*). ^{13}C NMR (125 MHz, $CDCl_3$/ CD_3OD): δ (ppm) 24.9-35.1 (C-2-C-9), 115.1 (C-3', C-5'), 129.4 (C-2', C-6'), 134.2 (C-1'), 154.3 (C-4'), 177.0 (C-1). IR, ν (KBr, cm^{-1}): 3364 (OH st), 2925 (COO-H st), 1719 (C=O), 1611-1597 (Ar C-C). EI-MS *m/z* (%):250 (M^+, 100), 233 (18) 232 (92), 201 (52), 120 (12), 107 (86). HRMS *m/z* (calculated for $C_{15}H_{22}O_3$): 250.156895; *m/z* (observed): 250.156221. Error (ppm): 2.7.

IMMUNOCHEMISTRY

Checkerboard titration experiments. The avidity of the As for the coating antigens was determined by measuring the binding (30 min at RT) of serial dilutions of each As (from 1/500 to 1/32000 in PBST, 100 µL/well) to microtiter plates coated (overnight, 4ºC) with 12 different concentrations of each coating antigen (from 1 µg mL^{-1} to 0.9 ng mL^{-1} in coating

buffer, 100 μL/well). The plates were washed and a solution of anti-IgG-HRP (1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min more at RT. Finally, the plates were washed again and the substrate solution was added (100 μL/well). The reaction was incubated for 30 min protected from light at RT and stopped by adding 4N H_2SO_4 (50 μL/well). Optimal concentrations for the CA and As dilutions were chosen to produce absorbances around 0.7-1 units of absorbance incubating 30 min at room temperature corresponding to the 70% of the maximum absorbance under not-saturating conditions.

Table 1. Day- to-day reproducibility of the NP direct ELISAs [a]

Assay	Day	A_{max}	A_{min}	S/N ratio[b]	IC_{50} (mg L^{-1})	slope	R^2
As106/NP5-HRP	**1**	1.430	0.196	7.3	4.6	-0.73	0.967
	2[c]	----	----	----	----	----	----
	3	1.047	0.346	3.0	5.7	-1.35	0.952
	4[d]	*0.789*	*0.248*	*3.2*	*2.9*	*-1.02*	*0.961*
	5	*0.796*	*0.230*	*3.4*	*3.6*	*-1.20*	*0.956*
	6	*0.516*	*0.121*	*4.3*	*1.8*	*-0.72*	*0.946*
	7	*0.345*	*0.090*	*3.8*	*3.3*	*-0.79*	*0.885*
As106/NP9-HRP	**1**	1.496	0.462	3.2	1.5	-0.71	0.952
	2	0.581	0.013	44.7	0.77	-0.44	0.952
	3	0.719	0.270	2.7	0.77	-1.06	0.956
	4	----	----	----	----	----	----
	5	----	----	----	----	----	----
As107/NP5-HRP	**1**	0.844	0.303	2.8	3.5	-1.38	0.820
	2	0.633	0.241	2.6	2.2	-1.72	0.921
	3	0.871	0.294	2.9	2.6	-0.66	0.955
As107/NP9-HRP	**1**	0.909	0.537	1.7	0.20	-1.01	0.939
	2	1.221	0.218	5.6	0.75	-0.72	0.958
	3	0.661	0.437	1.5	0.054	-7.21	0.844
	4	0.572	0.183	3.1	1.0	-1.05	0.944
	5	1.446	0.965	1.5	0.37	-1.25	0.974
	6	0.944	0.197	4.8	0.070	-11.29	0.950
	7[e]	*0.928*	*0.267*	*3.6*	*0.002*	*-1.19*	*0.981*
	8[e]	*0.647*	*0.277*	*2.5*	*0.021*	*-0.80*	*0.971*
	9[e]	----	----	----	----	----	----
As110/NP9-HRP	**1**	0.820	0.374	2.2	4.5	-1.16	0.751
	2	0.517	0.197	2.8	1.7	-1.00	0.683

[a] The analyte is the commercial mixture of technical nonylphenol. The parameters are extracted from the logistic equation used to fit the standard curve.
[b] Signal to noise ratio. It has been calculated by the quotient of $A_{max}/A_{min.}$
[c] The blank rows indicate days in which no competitive assays were obtained using the same conditions.
[d] Rows written in italics indicate assays performed by using PBST with 10% EtOH as the assay buffer in the competition step.
[e] Assays performed with PBST with a 10% of EtOH and 0.01% of Tween 20.

Table 2. Hapten density of BSA conjugates[a]

Hapten	Hapten/Lys	δ hapten[b]	% Conjugation[c]	NP-BSA[d] μg mL^{-1}	NP-CONA μg mL^{-1}
NPVA	1:1	5	14-17	1000	<0.2
NP1	1:1	14	40-47	28	<0.2
NP2	1:1	nd[e]	nd	<0.2	<0.2
NP3	1:1	11	31-37	428	267
NP4	1:1	12	34-40	407	<0.2
NP5	1:1	20	57-67	495	595
NP6	1:1	18	51-60	520	477
NP9	1:1	16	46-53	425	50

[a] All the haptens were conjugated to BSA by the active ester method, except NPVA that was conjugated by the mixed anhydride method.
[b] Hapten density of the BSA conjugates determined by MALDI-TOF-MS.
[c] The degree of conjugation has been calculated considering that the BSA has between 30 and 35 accessible lysine groups.
[d] Stock solutions of 1mg mL^{-1} were prepared in PBS, filtered and the concentration of the soluble fraction determined with the Bradford test.
[e] Not determined due to the poor solubility of the conjugate.