Supporting Information

Detection of the Antimicrobial Triclosan in Environmental Samples by Immunoassay

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Hapten Syntheses

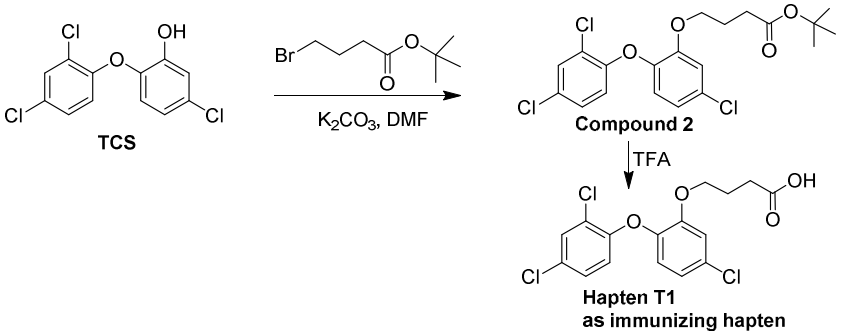
Chemicals and Instruments. All reagents were analytical grade from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO). Precoated silica gel 60 F254 glass plates (0.25 mm, EMD Chemicals, Temecula, CA) were used for thin layer chromatography (TLC) analysis. Silica gel was used for column chromatography. Proton NMR (^{1}H NMR) spectra were measured with a General Electric QE-300 spectrometer (Bruker NMR, Billerica, ME) using tetramethylsilane as an internal standard. Electrospray mass spectra of haptens in positive (MS-ESI$^+$) or negative (MS-ESI$^-$) mode were recorded by a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK). R_f values refer to TLC on the silica gel plates with visualization under exposure to either ultraviolet light (254 nm) or iodine vapor.

Nomenclature. The nomenclature of haptens was designated with the aid of Chemdraw Ultra 11.0 (CambridgeSoft, Cambridge, MA).

Immunizing haptens

Synthesis of 4-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)butanoic acid (Hapten T1, BDH 382-02, Scheme 1): The mixture of triclosan (400 mg, 1.38 mmol), 4-bromo-butyric acid *tert*-butyl ester (399 mg, 1.79 mmol), and potassium carbonate (286 mg, 2.07 mmol) in 2 mL of anhydrous DMF was reacted at 100 ^{o}C for 3 h. The resulting mixture was filtered to remove excess K_2CO_3 and HBr generated in the reaction. The filtrate diluted with 20 mL of ethyl acetate was washed twice with 20 mL of distilled water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by rotary evaporation. The residue was chromatographed on silica gel eluting with a mixture of ethyl acetate/hexane (1:2, v/v). Fractions containing pure product by TLC were stripped under high vacuum to obtain 179 mg of Compound **2** (**BDH 382-01)** as a transparent oil. TLC (ethyl acetate/hexane=1:10, v/v) R_f, 0.54.

Trifluoroacetic acid (TFA) (0.5 mL) was added to the ester intermediate **(BDH 382-01)** and the mixture was allowed to stand at ambient temperature for 30 min. After the addition of 50 mL of distilled water and acidification with 6 N HCl to pH 2, the mixture was extracted twice with 50 mL of ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by rotary evaporation. The concentrate was recrystallized from a mixture of ethyl acetate and hexane to give **Hapten T1** (391 mg, yield: 62%) as a white solid: mp 85-88 ^{o}C; TLC (ethyl acetate/hexane/acetic acid, 5:15:0.1, v/v/v) R_f, 0.28; MS-ESI$^-$ *m/z* calcd for $[M - H]^- = C_{16}H_{13}Cl_3O_4$, 373.99; observed, 373.05. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.43 (dd, *J* = 2.4, 1.4 Hz, 1H), 7.11 – 7.09 (m, 1H), 7.08 – 7.07 (m, 1H), 6.96 (s, 1H), 6.95 (d, *J* = 3.0 Hz 1H), 6.64 (d, *J* = 8.8 Hz, 1H), 3.99 (t, *J* = 5.9 Hz, 2H), 2.32 (t, *J* = 7.2 Hz, 2H), 1.96 (p, *J* = 6.7 Hz, 2H).

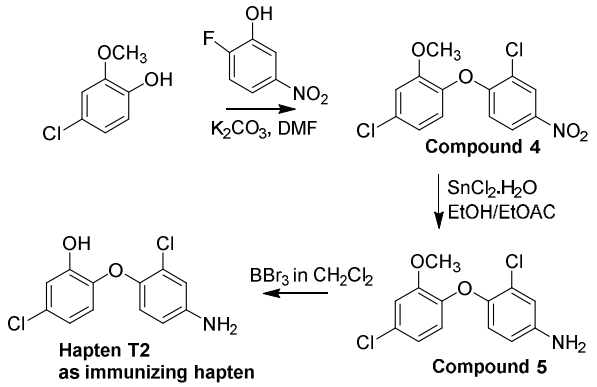


Scheme 1

Synthesis of 2-(4-amino-2-chlorophenoxy)-5-chlorophenol (Hapten T2, BDH 382-42, Scheme 2):
This hapten was synthesized according to the method described (Freundlich et al. 2005). To a solution of 4-chloro-2-methoxyphenol (1085 mg, 6.84 mmol), suspended K_2CO_3 (945 mg, 6.84 mmol) in dimethylformamide (DMF, 5 mL) was added 3-chloro-4-fluoronitrobenzene (1000 mg, 5.7 mmol). The reaction mixture was refluxed overnight. The mixture was diluted with ethyl acetate (200 mL) and 1 N NaOH (200 mL). The organic layer was separated, washed with water (100 mL), and concentrated. The residue was recrystallized from a mixture of ethyl acetate and hexane to give a white solid, **Compound 4** (**BDH-382-26)** (1539 mg, yield 86%). ^{1}H NMR (300 MHz, chloroform-*d*) δ 8.36 (d, *J* = 2.7 Hz, 1H), 8.00 (dd, *J* = 9.1, 2.7 Hz, 1H), 7.07 (d, *J* = 8.4 Hz, 1H), 7.03 (d, *J* = 2.1 Hz, 1H), 7.02 – 6.98 (m, 1H), 6.67 (d, *J* = 9.1 Hz, 1H), 3.78 (s, 3H).

The mixture of **Compound 4** (500 mg, 1.60 mmol) and stannous chloride dihydrate (3610 mg, 16 mmol) in 10 mL of ethanol was stirred at 70 °C in an oil bath for 1 h and at room temperature for 2 h. The mixture was cooled and poured into the slurry of water, ethyl acetate, and Celite. $NaHCO_3$ was added in portions. The neutral mixture was filtered, and the solids on the filter and in the flask were washed with water and ethyl acetate. The ethyl acetate phase was separated and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The oil residue was purified by using silica gel chromatography with a mixture of methanol/methylene chloride (1:15, v/v) as an eluent to give **Compound 5** (**BDH-382-36)** (411 mg, yield: 91%) as a brown solid. TLC (methanol/methylene chloride, 1:15, v/v) R_f , 0.78. ^{1}H NMR (300 MHz, chloroform-*d*) δ 6.94 (dd, *J* = 2.3, 1.2 Hz, 1H), 6.83 – 6.81 (m, 1H), 6.80 – 6.78 (m, 0H), 6.77 (dd, *J* = 2.7, 1.2 Hz, 1H), 6.59 – 6.54 (m, 1H), 6.52 (dd, *J* = 2.8, 1.2 Hz, 0H), 3.89 (d, *J* = 1.2 Hz, 4H), 3.65 (s, 3H).

A solution of **Compound 5** (350 mg, 1.24 mmol) in 20 mL CH_2Cl_2 was treated dropwise with 25 mL BBr_3 in CH_2Cl_2 (1 M). The mixture was stirred at 0°C for 30 min and at room temperature overnight. Water (200 mL) and CH_2Cl_2 (twice, 100 mL) was added into the mixture. The organic layer was separated, combined, evaporated and recrystallized from H_2O to get a brown solid of **Compound 6** (**BDH-382-42)** (206 mg, yield: 62%): TLC (methanol/methylene chloride, 1:15, v/v) R_f, 0.53; MS-ESI+ *m/z* calcd for $[M + H]^+ = C_{12}H_9Cl_2NO_2$, 269.00; observed, 270.03. ^{1}H NMR (300 MHz, chloroform-*d*) δ 6.73 (d, *J* = 2.3 Hz, 1H), 6.61 (d, *J* = 4.7 Hz, 1H), 6.49 (dd, J1 = 3 Hz, J2 = 9 Hz, 1H), 6.39 (dd, J1 = 3 Hz, J2 = 9 Hz, 1H), 6.33 (dd, J1 = 3 Hz, J2 = 6 Hz, 1H), 6.26 (dd, J1= 1.8 Hz, J2 = 8.4 Hz, 1H).



Scheme 2

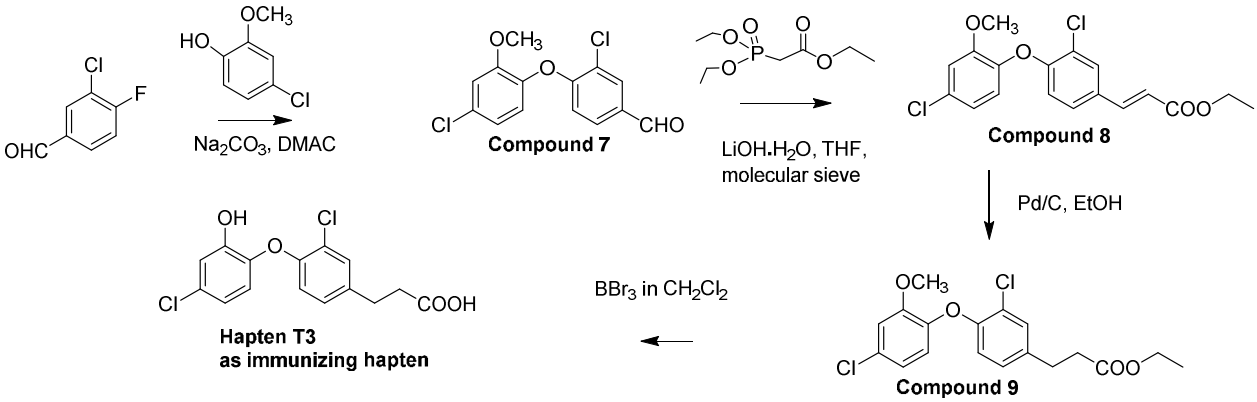
Synthesis of 3-(3-chloro-4-(4-chloro-2-hydroxyphenoxy)phenyl)propanoic acid (Hapten T3, BDH 382-48, Scheme 3):

3-Chloro-4-(4-chloro-2-methoxyphenoxy)benzaldehyde (**Compound 7, BDH-382-44**) was synthesized according to the method described (Ahn et al. 2009) using 3-chloro-4-fluorobenzdehyde (1000 mg, 6.3 mmol) and 4-chloro-2-methoxyphenol (999 mg, 6.3 mmol). ^{1}H NMR (300 MHz, chloroform-*d*) δ 9.87 (s, 1H), 7.97 (s, 1H), 7.64 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.03 – 7.01 (m, 2H), 7.00 (d, *J* = 2.3 Hz, 1H), 6.72 (d, *J* = 8.5 Hz, 1H), 3.78 (s, 3H).

To a solution of **Compound 7** (500 mg, 1.69 mmol) and triethyl 4-phosphonoacetate (417 mg, 1.86 mmol) in dry tetrahydrofuran (THF, 10 mL) was added $LiOH{\cdot}H_2O$ (78 mg, 1.86 mmol) and molecular sieve 4 Å (2.5 g). The mixture was refluxed overnight under N_2 conditions. Ethyl acetate (50 mL), water (50 mL) and NaCl (1 g) were added and the organic layer was separated. After removing the organic solvent, the crude residue was purified by silica gel chromatography using a mixture of ethyl acetate and hexane (1:10, v/v) to give **Compound 8** (**BDH-382-45)** as a white solid (575 mg, yield: 93%): TLC (ethyl acetate/hexane=1:5, v/v) R_f, 0.51; ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.66 – 7.52 (m, 1H), 6.99 (q, *J* = 1.4 Hz, 1H), 6.97 – 6.90 (m, 1H), 6.67 (dd, *J* = 8.5, 1.8 Hz, 1H), 6.37 – 6.30 (m, 1H), 4.26 (dd, *J* = 7.0, 1.8 Hz, 2H), 3.80 (d, *J* = 1.8 Hz, 3H), 1.33 (td, *J* = 7.2, 1.9 Hz, 3H).

Compound 8 (130 mg, 0.36 mmol) was converted to **Compound 9** (**BDH-382-46)** using Pd/C in ethanol (7 mL) at 70 °C under H_2 condition. Pd/C was removed by filtration. The mixture was concentrated and the crude residue was purified by silica gel chromatography using a mixture of ethyl acetate and hexane (1:5, v/v) to give **Compound 9** (50 mg, yield: 38%) as a transparent oil. TLC (ethyl acetate/hexane, 1:5, v/v, developed twice) R_f, 0.50. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.28 (d, *J* = 2.1 Hz, 1H), 7.13 (s, 1H), 7.03 – 6.91 (m, 1H), 6.89 (dd, *J* = 5.1, 1.9 Hz, 2H), 6.86 (d, *J* = 1.9 Hz, 1H), 6.73 (d, *J* = 8.4 Hz, 1H), 4.13 (dd, *J* = 7.1, 2.2 Hz, 2H), 3.85 (d, *J* = 2.6 Hz, 3H), 2.90 (td, *J* = 7.7, 3.7 Hz, 2H), 2.60 (td, *J* = 7.7, 1.4 Hz, 2H), 1.24 (td, *J* = 7.2, 1.7 Hz, 3H).

To a cooled solution of **Compound 9** (50 mg, 0.13 mmol) in CH_2Cl_2 was added a 1 M solution of BBr_3 in CH_2Cl_2 (0.26 mL, 0.26 mmol). After stirring at 0 °C for 4 h, an additional 2 mL BBr_3 in CH_2Cl_2 was added. The reaction mixture was stirred for another 30 min and then water (50 mL) and 1 N NaOH (3 mL) were added. The mixture was diluted with ethyl acetate (twice, 50 mL) and water (50 mL). The organic layers were separated, combined, and concentrated. The crude residue was recrystallized from a mixture of ethyl acetate and hexane to give a white solid of **Compound 10** (**BDH 382-48)** (43 mg, yield 90%): TLC (methanol/methylene chloride/acetic acid, 2:18:0.02, v/v/v) R_f, 0.32; MS-ESI$^-$ *m/z* calcd for $[M - H]^- = C_{15}H_{12}Cl_2O_4$, 326.01; observed, 325.05. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.92 (d, *J* = 44.9 Hz, 1H), 7.66 (s, 1H), 7.04 (d, *J* = 7.8 Hz, 1H), 6.89 (s, 2H), 6.75 (d, *J* = 8.2 Hz, 2H), 6.08 (s, 1H), 2.78 (s, 1H).

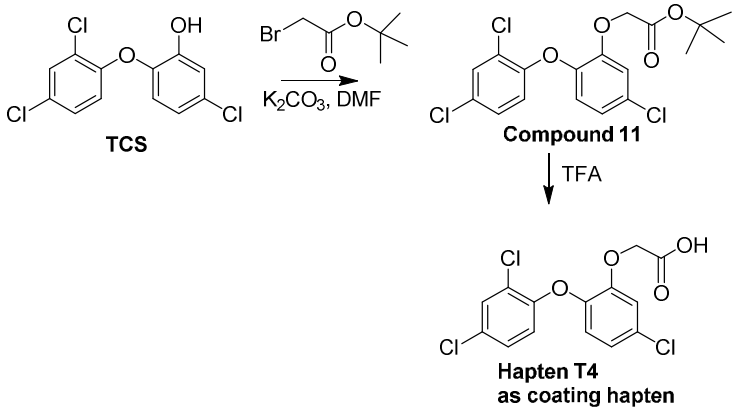


Scheme 3

Coating haptens

Synthesis of 2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)acetic acid (Hapten T4, BDH 382-16, Scheme 4): **Compound 11 (BDH 382-15)** was synthesized using *tert*-butyl bromoacetate using the same synthetic method as **Compound 3**. That is, the mixture of triclosan (400 mg, 1.38 mmol), tert-butyl bromoacetate (349 mg, 1.79 mmol), and potassium carbonate (286 mg, 2.07 mmol) in 2 mL of anhydrous DMF was reacted at 100 ^{o}C for 3 h. The resulting mixture was filtered to remove excess K_2CO_3 and HBr produced in the reaction. The filtrate, diluted with 20 mL of ethyl acetate was washed twice with 20 mL of distilled water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:2, v/v). Fractions containing pure product by TLC were stripped under high vacuum to obtain **Compound 11** as a transparent oil. TLC (ethyl acetate/hexane=1:10, v/v) R_f, 0.47.

Trifluoroacetic acid (TFA) (0.5 mL) was added to the ester intermediate **(Compound 11)** and the mixture was allowed to stand at ambient temperature for 30 min. After the addition of 50 mL of distilled water and acidification with 6 N HCl to pH 2, the mixture was extracted twice with 50 mL of ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The concentrate was recrystallized from a mixture of ethyl acetate and hexane to give **Hapten T4** (**BDH 382-16)** (436 mg, yield: 90%) as a white solid. mp 85-88 ^{o}C. TLC (ethyl acetate/hexane/acetic acid, 5:15:0.1, v/v/v) R_f, 0.20. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.45 (d, *J* = 2.5 Hz, 1H), 7.14 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.01 (d, *J* = 2.3 Hz, 1H), 7.00 – 6.98 (m, 1H), 6.89 – 6.82 (m, 1H), 6.79 (d, *J* = 8.8 Hz, 1H), 4.71 (s, 2H).

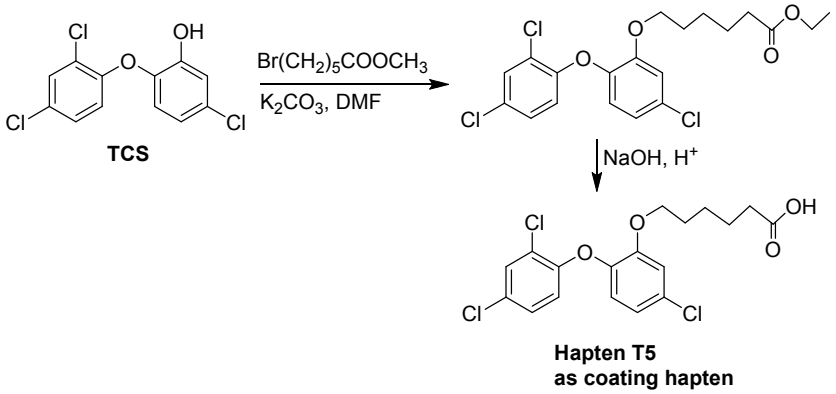


Scheme 4

Synthesis of 6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexanoic acid (Hapten T5, BDH 382-17, Scheme 5): The mixture of triclosan (824 mg, 2.76 mmol), ethyl bromohexanoate (800 mg, 3.58 mmol), and potassium carbonate (572 mg, 4.14 mmol) in 2 mL of anhydrous DMF was reacted at 100 ^{o}C overnight. The resulting mixture was filtered to remove excess K_2CO_3 and HBr produced in the reaction. The filtrate diluted with 20 mL of ethyl acetate was washed twice with 20 mL of distilled water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:2, v/v). Fractions containing pure product by TLC were stripped under high vacuum to the bromoester as a transparent oil. TLC (ethyl acetate/hexane, 1:10, v/v) R_f, 0.75.

6N NaOH (5 mL) and methanol (10 mL) was added to the ester intermediate and the mixture was allowed to stand at 70 ^{o}C for 2 d. After the addition of 50 mL of distilled water and acidification with 6 N HCl to pH 2, the mixture was extracted twice with 50 mL of ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The concentrate was recrystallized from a mixture of ethyl acetate and hexane to give **Hapten T5** (1022 mg, yield: 92%) as a white solid. TLC (methylene

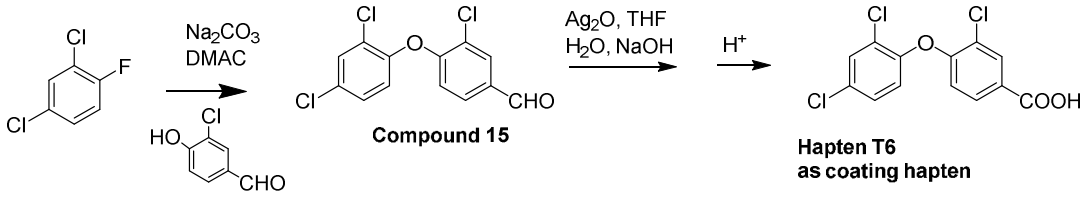
chloride/MeOH/acetic acid=2:18:0.02, v/v/v) R_f, 0.54. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.42 (d, *J* = 2.5 Hz, 1H), 7.09 (dd, *J* = 2.5, 0.9 Hz, 1H), 7.06 (dd, *J* = 2.5, 0.9 Hz, 1H), 6.96 – 6.95 (m, 1H), 6.94 (s, 4H), 6.93 (d, *J* = 1.0 Hz, 1H), 3.91 (t, *J* = 6.1 Hz, 4H), 2.29 (t, *J* = 7.4 Hz, 3H), 1.61 (dp, *J* = 22.8, 7.7, 7.1 Hz, 10H), 1.28 (q, *J* = 7.0, 5.6 Hz, 4H).



Scheme 5

Synthesis of 3-chloro-4-(2,4-dichlorophenoxy)benzoic acid (Hapten T6, BDH 382-63, Scheme 6):
2,4-Dichlorophenol (616 mg, 3.78 mmol) and 3-chloro-4-fluorobenzaldehyde (500 mg, 3.15 mmol) were dissolved in N,N-dimethylacetamide (DMAC, 10 mL). Anhydrous Na_2CO_3 and molecular sieve 4 Å were added and the reaction mixture was refluxed overnight under a stream of nitrogen gas. The mixture was diluted with ethyl acetate (200 mL) and 1 N NaOH (200 mL). The organic layer was separated, washed with water (100 mL), and concentrated. The crude residues were purified by silica gel chromatography using a mixture of ethyl acetate and hexane (1:20, v/v) to give **Compound 15** (**BDH-382-56)** as a transparent oil (770 mg, yield: 82%). TLC (ethyl acetate/hexane, 1:5, v/v) R_f, 0.51.

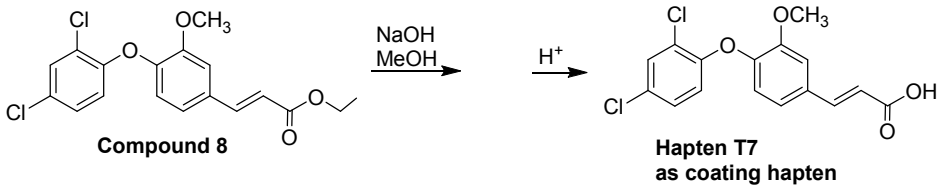
Compound 15 (770 mg, 2.57 mmol) was dissolved in dimethyl sulfoxide (5 mL) and tetrahydrofuran (5 mL). Silver oxide (1200 mg, 5.14 mmol) and water (5 mL) were added and the mixture was reacted at 100 °C for 2 h. The mixture was made alkaline with 1 N NaOH (50 mL) and washed with ethyl acetate to remove unreacted **Compound 15**. The alkaline solution was acidified with 6 N HCl and extracted with ethyl acetate. The organic layer was separated and concentrated. The residue was recrystallized from ethyl acetate and hexane to give **Compound 16** (**BDH-382-63)** (522 mg, yield: 64%): TLC (methanol/methylene chloride/acetic acid, 2:18:0.02, v/v/v) R_f, 0.46; MS-ESI- *m/z* calcd for $[M - H]^-$ =C13H7Cl3O3, 315.95; observed, 314.9; ^{1}H NMR (300 MHz, DMSO-*d6*) δ 13.25 (s, 1H), 8.04 (d, *J* = 2.1 Hz, 1H), 7.84 (dd, *J* = 8.4, 2.7 Hz, 1H), 7.50 (dd, *J* = 8.7, 2.4 Hz, 1H), 7.27 (d, *J* = 9 Hz, 1H), 6.93 (d, *J* = 8.7 Hz, 1H).



Scheme 6

Synthesis of (E)-3-(4-(2,4-dichlorophenoxy)-3-methoxyphenyl)acrylic acid (Hapten T7, BDH-382-68, Scheme 7)
Compound 8 was dissolved in MeOH and hydrolyzed under 10% alkaline conditions at 70 °C overnight. The reaction mixture was acidified with 6 N HCl and extracted with ethyl acetate. The organic layer was concentrated. The residue was recrystallized from a mixture of ethyl acetate and hexane to give **Hapten T7** as a white solid (20

mg, yield: 8%). TLC (methanol/methylene chloride/acetic acid, 2:18:0.02, v/v/v) R_f, 0.43. ^{1}H NMR (300 MHz, DMSO-*d6*) δ 12.40 (s, 1H), 7.93 (d, *J* = 1.5 Hz, 1H), 7.57 (d, *J* = 2.1 Hz, 1H), 7.51 (d, *J* = 15.9 Hz, 1H), 7.29 (d, *J* = 2.1 Hz, 1H), 7.10 (d, *J* = 9 Hz, 1H), 7.03 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.67 (d, *J* = 8.7 Hz, 1H), 6.48 (d, *J* = 16.2 Hz, 1H), 3.76 (s, 3H).

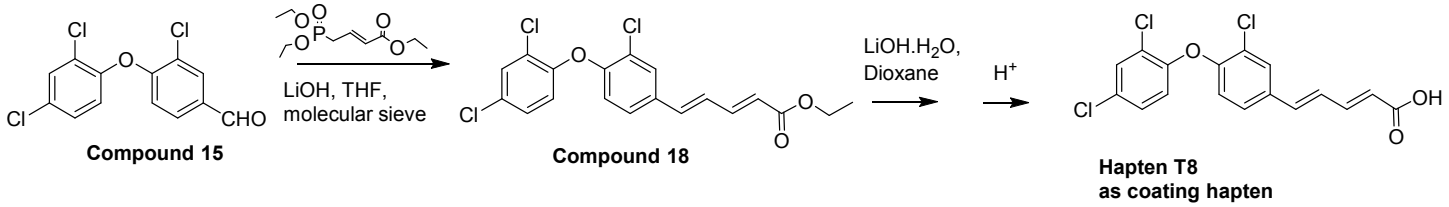


Scheme 7

Synthesis of (2E,4E)-5-(3-chloro-4-(2,4-dichlorophenoxy)phenyl)penta-2,4-dienoic acid (Hapten T8, BDH 382-70, Scheme 8):

A suspension of the aldehyde (**Compound 15**, 345 mg, 1.15 mmol), triethyl 4-phosphonocrotonate (350 mg, 1.27 mmol), LiOH·H_2O (54 mg, 1.27 mmol), and activated 4 Å molecular sieve (1.9 g) in tetrahydrofuran and under a stream of nitrogen gas was refluxed overnight. The crude reaction mixture was filtered through silica gel, eluting with ethyl acetate. The mixture was concentrated. The residue was purified with silica gel using a mixture of ethyl acetate and hexane (1:10, v/v) to give **Compound 18** (**BDH-382-67)** as a white solid (331 mg, yield: 73%). TLC (ethyl acetate/hexane, 1:5, v/v) R_f, 0.68. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.58 (d, *J* = 1.8 Hz, 1H), 7.49 (d, *J* = 2.4 Hz, 1H), 7.45 (d, *J* = 5.4 Hz, 1H), 7.40 (dd, *J* = 9.6, 5.1 Hz, 1H), 7.28 (dd, *J* = 5.1, 2.7 Hz, 1H), 6.85 (d, *J* = 9.0 Hz, 1H), 6.80 (dd, *J* = 6.6, 1.8 Hz, 1H), 6.76 (d, *J* = 3.9 Hz), 6.00 (d, *J* = 15.0 Hz, 1H), 4.23 (q, *J* = 7.2 Hz, 2H), 1.32 (t, *J* = 6.9 Hz, 3H).

Compound 18 (250 mg, 0.63 mmol) was dissolved in 1,4-dioxane (5 mL) and hydrolyzed under alkaline conditions using LiOH·H_2O (127 mg, 3.02 mmole) at 40 ^{o}C overnight. The reaction mixture was acidified with 6 N HCl and extracted with ethyl acetate. The organic layer was concentrated. The residue was recrystallized from a mixture of ethyl acetate and hexane to give **Hapten T8** as a white solid (150 mg, yield: 65%). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 12.31 (s, 1H), 7.83 (dd, *J* = 28.0, 2.3 Hz, 1H), 7.52 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.44 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.31 (dd, *J* = 15.2, 10.9 Hz, 1H), 7.15 (dd, *J* = 15.6, 10.9 Hz, 1H), 7.06 - 7.01 (m, 1H), 6.01 (d, *J* = 15.2 Hz, 1H).



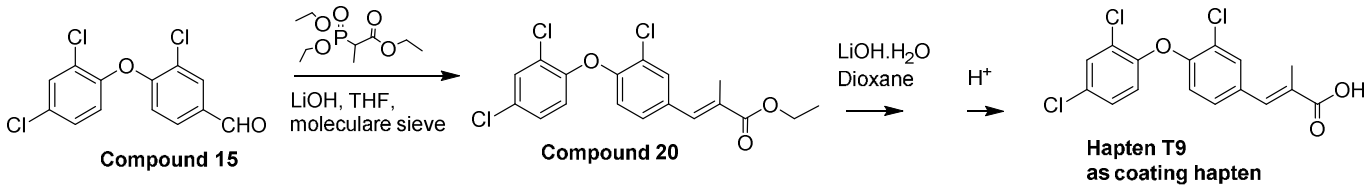
Scheme 8

Synthesis of (E)-3-(3-chloro-4-(2,4-dichlorophenoxy)phenyl)-2-methylacrylic acid (Hapten T9, BDH 382-71, Scheme 9):

A suspension of the aldehyde (**Compound 15**, 500 mg, 1.67 mmol), triethyl 2-phosphonopropionate (438 mg, 1.84 mmol), LiOH·H_2O (77 mg, 1.84 mmol) and activated 4 Å molecular sieve (2.76 g) in tetrahydrofuran and under a stream of nitrogen gas was refluxed overnight. The crude reaction mixture was filtered through silica gel, eluting with ethyl acetate. The mixture was concentrated. The residue was purified with silica gel using a mixture

of ethyl acetate and hexane (1:10, v/v) to give **Compound 20** (**BDH-382-69)** as a white solid (556 mg, yield: 87%). TLC (ethyl acetate/hexane, 1:5, v/v) R_f, 0.75. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.52 (d, *J* = 2.7 Hz, 1H), 7.49 (d, *J* = 2.7 Hz, 2H), 7.21 (dd, *J* = 8.4, 2.4 Hz, 2H), 6.87 (d, *J* = 8.7 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, 1H), 4.27 (q, *J* = 7.2 Hz, 2H), 2.11 (d, *J* = 1.5 Hz, 3H), 1.35 (t, *J* = 6.9 Hz, 3H).

Compound 20 (400 mg, mmol) was dissolved in 1,4-dioxane (5 mL) and hydrolyzed under alkaline conditions using $LiOH{\cdot}H_2O$ (127 mg, 3.02 mmole) at 40 °C overnight. The reaction mixture was acidified with 6 N HCl and extracted with ethyl acetate. The organic layer was concentrated. The residue was recrystallized from ethyl acetate and hexane to give **Hapten T9** as a white solid (327 mg, yield: 88%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.77 (ddd, *J* = 53.6, 6.1, 2.5 Hz, 1H), 7.55 (d, *J* = 4.9 Hz, 1H), 7.45 (ddq, *J* = 8.5, 5.9, 2.4 Hz, 1H), 7.14 – 7.00 (m, 1H), 2.03 (d, *J* = 3 Hz, 3H).



Scheme 9

Coupling methods

Sulfo-N-hydroxysuccinimide (NHS) Method. Hapten **T1** was coupled covalently with the lysine moieties of the carrier proteins such as thyroglobulin. That is, each hapten (0.02 mmol) was dissolved in 1 mL of dimethylformamide (DMF) with sulfo-NHS (0.024 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 0.024 mmol). After the mixture was stirred overnight, the active ester was added slowly to a solution of thyroglobulin (25 mg of protein in 1 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was stirred gently at 4 °C for 24 h to complete the conjugation. The conjugates were dialyzed against phosphate buffer saline (PBS) for 36 h with buffer changes every 12 h and stored at -20 °C until use.

N-Hydroxysuccinimide (NHS) Active Ester Method. The haptens **T3**, **T4**, and **T5** containing a –COOH group were coupled covalently with the lysine moieties of carrier proteins such as thyroglobulin and bovine serum albumin according to the activated ester method. That is, each hapten (0.02 mmol) was dissolved in 0.2 mL of dry dimethylformamide with equimolar NHS and a 10% molar excess of dicyclohexylcarbodiimde. After the mixture was stirred overnight at 22 °C, the precipitated dicyclohexylurea was removed by filtration, and about 0.2 mL of the active ester was added slowly to a solution of the protein (25 mg of protein in 1 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was stirred gently at 4 °C for 24 h to complete the conjugation and then dialyzed and stored as described above.

Mixed Anhydride Method. Haptens **T7**, **T8** and **T9** containing carboxylic acids were activated by the mixed anhydride method. Haptens (0.03 mmol) were dissolved in dry *p*-dioxane. Isobutyl chloroformate and tri-*n*-butylamine were added in slight molar excess. The solution was stirred at room temperature for 30 min. Fifty milligrams of each protein (bovine serum albumin or LPH, *Limulus polyphemus* hemocyanin) was dissolved in 30 mL of 0.2 M borate buffer, pH 8. The protein solution was ice-cooled. To improve the solubility of the activated hapten in the aqueous protein solution, 2 mL of *p*-dioxane was added to the protein solution. The addition of *p*-dioxane to the protein solution caused a slight cloudiness. The activated hapten solution was then added to the protein solution dropwise with stirring. Stirring was continued on ice for 0.5 – 1 h. To remove unreacted small molecules, the protein conjugates were precipitated with ice cold 100% ethanol. The precipitated protein was pelleted by centrifugation at 4 °C for 10 min, 4500*g*. The supernatant containing unreacted small molecules was decanted. The pellet was resuspended with cold ethanol three times, centrifuging between resuspensions. The

supernatants were discarded, and the pellet was resuspended in distilled water to a concentration of approximately 5 mg of protein/mL. All conjugates were assayed for protein content, aliquoted, and stored at -20 or -80 °C until use.

Diazotization Method. Hapten **T2** was covalently conjugated to tyrosine moieties of the carrier protein. That is, 0.2 N sodium nitrite (0.5 mL) was added dropwise to a solution of the hapten (0.03 mmol) dissolved in a mixture of a few drops of ethanol and 0.2 N HCl (0.5 mL) until a positive starch iodide test was confirmed. The reaction vial was cooled with ice while the solution was stirred. Dimethylformamide (0.3 mL) was added and stirred for 10 min, and the solution was divided into two equal aliquots. One aliquot was added to a solution of thyroglobulin, the other to a solution of bovine serum albumin. The thyroglobulin (25 mg) and the bovine serum albumin (25 mg) were dissolved in 5 mL of ice-cold borate buffer (0.2 M, pH 8.9). The reaction mixtures were cooled in an ice bath and stirred continuously for 30 min. The pH of the yellow solutions was adjusted to 7.0 with 1 N NaOH. Each mixture was dialyzed and stored as described above.

Instrumental Analysis of Water and Biosolid Samples

Analysis of Water samples: Water samples were extracted by solid phase extraction (SPE) using Oasis HLB cartridges (3 cc 60 mg, Waters, Milford, MA) as reported elsewhere (Charles et al., 2011). The HLB cartridges were first washed with 3 mL ethyl acetate, 3 mL methanol twice, and 3 mL 95:5 v/v water/methanol with 0.1% acetic acid. The 1 mL water samples were then loaded onto the cartridges. The samples were spiked with 100 µL 100 ng/mL internal standard (4-phenoxyphenol) and flowed through the sorbent by gravity. They were then washed with 3 mL 95:5 v/v water/methanol with 0.1% acetic acid twice and dried for 20 min with low vacuum. The triclosan was then eluted with 0.5 mL of methanol followed by 1.5 mL of ethyl acetate into tubes containing 6 µL of 30% glycerol in methanol as a trap solution. The volatile solvents were evaporated by using vacuum centrifugation until about 2 µL of trap solution remained in the tube. The residues were dissolved in 1 mL of methanol for LC/MS/MS analysis. All samples were extracted in triplicate.

Analysis of Biosolid samples: Samples of dried biosolid powder (1.0 g) were extracted with a reflux column using methanol (15 mL) at a constant temperature (65°C) and with agitation, on an oil bath for two hours. The agitation process was maintained for another 30 min until the whole solution cooled down and the mixture was then filtered on a vacuum system using filter paper. Biosolid extract samples were purified with a solid phase extraction (SPE) before analysis by LC/MS-MS.

Prior to extraction, 3 cc Waters Oasis®-HLB cartridges were washed with ethyl acetate (3 mL), methanol (2 × 3 mL), and 95:5 v/v water/methanol with 0.1% acetic acid (2 × 3mL). Biosolid extract (3 mL) was then loaded onto the cartridges. Cartridges were washed two times with 3 mL of 95:5 v/v water/methanol with 0.1% acetic acid. The aqueous plug was pulled through the SPE cartridges using high vacuum. The SPE cartridges were further dried with low vacuum about 30 min. SPE cartridges were eluted using 0.5 mL of methanol followed by 1.5 mL of ethyl acetate into 2 mL tubes containing 6 µL of 30% glycerol in methanol as a trap solution. The volatile solvents were removed using a Speed-Vac until only the trap solution of 2 µL of glycerol remained. The residues were reconstituted in 1 mL of methanol containing 10 ng/mL of internal standard (4-phenoxyphenol). The samples were then mixed on a vortex mixer for 1 min, transferred to autosampler vials, and stored at -20 °C until analysis.

The liquid chromatography method followed that used by Ogunyoku and Young (2014). The system used for analysis was an Agilent 1200 SL liquid chromatography series (Agilent Corporation, Palo Alto, CA). The autosampler was kept at 4 °C. Liquid chromatography was performed on a reverse-phase Phenomenex® Luna 3µ C18 (2), 150 x 2.00 mm column. Mobile phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 300 µL/min. Chromatography was optimized to separate all analytes in 15 min, and the injection volume was 10 µL.

The column was connected to a 4000 QTrap tandem mass spectrometer (Applied Biosystems Instrument Corporation, Foster City, CA) equipped with an electrospray source (Turbo V). The instrument was operated in negative multiple reaction monitoring (MRM) mode.

TCS standard was infused into the mass spectrometer and MRM transitions and source parameters optimized. The MRM transitions used to quantify TCS and 4-phenoxyphenol were 289 Da-35 Da and 185 Da -108 Da, respectively.

Peak integration and quantification was performed automatically using the Analyst® v1.6 software. The limit of detection (LOD) and limit of quantification (LOQ) for each of the compounds were determined as 3 and 10 times the signal to noise ratio, and were equaled to 7.67 ng/mL and 24.7 ng/mL, respectively.

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