Development of An Enzyme-Linked Immunosorbent Assay (ELISA) for the Determination of the Linear Alkylbenzene Sulfonates (LAS) and long chain Sulfophenyl Carboxylates (SPCs) using Antibodies Generated by pseudo-Heterologous Immunization

SUPPORTING INFORMATION

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ORGANIC CHEMISTRY METHODS: EXPERIMENTAL SECTION

**General Methods and Instrument:** Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). Unless otherwise indicated, purification of the reaction mixtures was accomplished by “flash” chromatography using silica gel as the stationary phase. ¹H and ¹³C NMR spectra were obtained with a Varian Unity-300 (Varian Inc., Palo Alto, CA) spectrometer (300 MHz for ¹H and 75 MHz for ¹³C). The chemical reagents used in this synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI). The technical LAS and the mixture of alkylbenzene synthetic precursors were kindly provided by PETRESA S.A. (San Roque, Cádiz, España). The exact percentage weight of each LAS homologue was 0.4% for <5 phenyl C10, 11.8% phenyl C10, 34% phenyl C11, 30.3% phenyl C12, 22.5% phenyl C13, 0.4% phenyl C14 and 0.1% of parafines.

**Synthesis of the SFA hapten** (see figure 1, scheme I).

**N-(4-alkylphenyl)sulfonyl-3-aminopropanoic acid (SFA).** Chlorosulfonic acid (1.7 ml, 25 mmol, 3 eq) was placed in a two-neck round-bottom flask provided with magnetic stirring and under Ar atmosphere. One of the necks was connected to a trap with 1M NaOH to neutralize the HCl formed. The other neck was used to add slowly the alkylbenzene technical mixture (1g, 4.2 mmol) at room temperature and the initial pale yellow colour of the reaction mixture became intense red. The reaction was finished after 1:30 h as observed by TLC (hexane as eluent). The crude was poured over a water-ice mixture (100 mL) and the formation of a white precipitate was observed. This white precipitated was extracted with hexane washed with sat. NaHCO₃, dried with anh.MgSO₄, filtered and evaporated. The crude residue was purified by silicagel flash chromatography using hexane as mobile phase to obtain the corresponding mixture of 4-alkylphenylsulfonyl chlorides as a pale yellow oil (400 mg 30% yield). ¹H NMR (300MHz, CDCl₃); δ: 0.85 (m, 6H, -CH₃), 1.21 (m, 15H, -CH₂-), 1.57 (m, 2H, -CH₂-), 1.67 (m, 2H, -CH₂-), 2.62 (m, 1H, PhCH=), 7.37 (d, J=8Hz, 1HAr meta), 7.95 (d, J=8Hz, 1HAr orto). ¹³C NMR (75MHz, CDCl₃); δ: 12.0-14.0 (t), 20.6-38.8 (d), 46.1-46.4 (s), 127.1 (s), 128.8 (s), 141.8 (s), 155.5 (s). A solution of the mixture of 4-alkylphenylsulfonyl chlorides (200 mg, 0.6 mmol) in anhydrous CH₂Cl₂ (2 mL) was slowly added under Ar atmosphere to a solution of triethylamine (300 mg, 3 eq) and the chlorhydrate of methy 3-aminoproanoate (107 mg, 0.77 mmol, 1.3 eq.) in the same solvent (3 mL) placed in a three-necks round bottom flask with magnetic stirring. The mixture was left to react at RT under stirring until the starting material had disappeared according to TLC (hexane: ethyl acetate 1:1). The solvent was evaporated, suspended in saturated NaHCO₃ and extracted with AcOEt. The organic layer was dried with...
MgSO₄, filtered and evaporated to dryness to obtain a mixture of methyl N-(4-alkylphenyl)sulfonyl-3-aminopropanoates (140 mg, 60% yield) as a yellow oil. Finally the esters were hydrolyzed in MeOH (1.3 mL) with 1M KOH (1mL, 1 mmol, 3 eq) for 2 h. The solvent was then evaporated and the residue dissolved with 1N HCl and extracted with AcOEt to obtain SFA (90 mg, 67% yield) as a yellow oil. ¹H NMR (300MHz, CD₂OD); δ: 0.85 (m, 6H, -CH₃), 1.22 (m, 15H, -CH₂-), 1.62 (m, 4H, -CH₂-), 2.44 (t, J=14Hz, 2H, -CH₂COOH), 2.62 (m, 1H, PhCH=), 3.11 (t, J=14Hz, J=6.5Hz, 2H, NHCH₂-), 7.36 (d, J=8.5Hz, 1H Ar meta), 7.78 (d, J=8.5Hz, 1H Ar ortho). ¹³C NMR (75MHz, CD₂OD); δ: 14.3-14.4 (-CH₃), 21.7-40.1 (-CH₂-), 47.0-47.2 (PhCH=), 128.1 (C ortho), 129.6 (C meta), 139.1 (C ipso), 153.0 (C para), 174.8 (CO).IR, ν (KBr, cm⁻¹); 3278 (-OH st, -NH- st), 2856-2956 (C-H st), 1716 (C=O st), 1598 (Ar-C-C), 1326 (-SO₂NH st as), 1159 (-SO₂NH st si).

Synthesis of SPC haptens.

Different SPCs (5C₅, 6C₆, 7C₇, 9C₉, 12C₁₂ and 13C₁₃) were synthesized from the corresponding ω-phenylalkylcarboxylic acids 1b-6b by sulfonation of the aromatic ring. The 5-phenylpentanoic acid 1b and the (4-carboxybutyl)triphenylphosphonium bromide used to prepare 2a were obtained from commercial sources. The phenylcarboxylic acids 2b-6b were prepared through a Wittig reaction using benzaldehyde and the respective phosphonium bromide salts 2-6, followed by reduction of the double bond formed with H₂ using Pd/C as catalyst (see figure 1, scheme II).

Preparation of (ω-Carboxyalkyl)triphenylphosphine bromides 3-6. General Protocol. The corresponding ω-bromoalkanoic acid (9 mmol, 1eq) and the triphenylphosphine (9 mmol, 1eq) were placed in a round bottom flask provided of a refrigerant and a magnetic bar. The mixture was heated at 100 ºC under Ar atmosphere. After 4 h, a total conversion of the acid was observed by ¹H NMR. The reaction was left overnight at the vacuum line at 100 ºC. The yield was quantitative.

(5-carboxypentyl)triphenylphosphonium bromide 2. ¹H NMR (300MHz, CDCl₃); δ: 1.42-1.85 (m, 2H, -CH₂-), 1.85-2.09 (m, 2H, -CH₂-), 2.71 (t, 2H, CH₂COOH), 3.64 (ba, 2H, -CH₂PPh₃), 7.68-7.84 (m, 15H, Ph₃P).

(6-carboxyhexyl)triphenylphosphonium bromide 3. ¹H NMR (300MHz, CDCl₃); δ: 1.58-1.80 (m, 6H, -CH₂-), 2.27 (t, 2H, CH₂COOH), 3.64 (ba, 2H, -CH₂PPh₃), 7.68-7.84 (m, 15H, Ph₃P).
(8-carboxyoctyl)triphenylphosphonium bromide 4. $^1$H NMR (300MHz, CDCl$_3$); δ: 1.29 (m, 4H, -CH$_2$-), 1.60 (m, 6H, -CH$_2$-), 2.41 (t, J=14Hz, 2H, CH$_2$COOH), 3.64 (ba, 2H, -CH$_2$PPh$_3$), 7.68-7.84 (m, 15H, Ph$_3$P).

(11-carboxyundecyl) triphenylphosphonium bromide 5. $^1$H NMR (300MHz, CDCl$_3$); δ: 1.20 (m, 10H, -CH$_2$-), 1.60 (m, 6H, -CH$_2$-), 2.37 (t, J=15Hz, 2H, CH$_2$COOH), 3.68 (ba, 2H, -CH$_2$PPh$_3$), 7.67-7.85 (m, 15H, Ph$_3$P).

(12-carboxydodecyl) triphenylphosphonium bromide 6 $^1$H NMR (300MHz, CDCl$_3$); δ: 1.20 (m, 10H, -CH$_2$-), 1.60 (m, 6H, -CH$_2$-), 2.37 (t, J=15Hz, 2H, CH$_2$COOH), 3.68 (ba, 2H, -CH$_2$PPh$_3$), 7.67-7.85 (m, 15H, Ph$_3$P).

6-Phenylhexanoic acid 2b. Anhydrous DMSO (6.1 mL, 0.1 mol, 3.5 eq.) was added to a suspension of 60% NaH (1.24 g, 0.3 mol, 2.1 eq.; previously been washed with anh. pentane (3 x 15 mL) and dried), placed in a round-bottom flask with a refrigerant, under magnetic stirring and Ar atmosphere, and the mixture was heated at 65°C for 30 min until no more formation of H$_2$ was observed and the solution took a pale green-yellow colour. At that moment the solution was left to reach room temperature and the phosphonium salt 2 (2.8 g, 6 mmol, 1 eq) dissolved in anh. DMSO (5 mL) was added dropwise. A characteristic colour shift to an intense red was produced indicating the formation of the ilure. After 15 min, freshly distilled benzaldehyde (0.65 mL, 6 mmol, 1eq.) was added and left to react for 2 h at RT under stirring until the starting material had disappeared according to TLC (hexane: ethyl acetate: AcOH, 1:1:0.1). The crude of the reaction was acidified with 1N HCl and extracted with ethyl ether. The organic phase was washed with H$_2$O, dried with MgSO$_4$ and filtered. The solvent was evaporated to dryness to obtain 2.54 g of a brown solid containing the desired product Z/E 6-phenyl-5-hexenoic acid. The crude mixture was esterified in MeOH with few drops of conc. H$_2$SO$_4$ at RT for 12 h until observing the total disappearance of the starting material by TLC. The solvent was evaporated and the residue dissolved in ethyl ether and washed with sat. NaHCO$_3$. The organic phase was dried with MgSO$_4$, filtered and evaporated. The crude mixture was purified by silicagel flash chromatography using hexane:ethyl ether as mobile phase to obtain a 6:4 Z:E mixture of 6-phenyl-5-hexenoic methyl ester 2a (510 mg, 44% yield). Z-isomer $^1$H NMR (300 MHz, CDCl$_3$): δ(ppm) 1.65-1.84 (m, 2H, CH$_2$), 2.20 (dt, J=14 Hz, J=7 Hz, 2H, CHCH$_2$), 2.30 (t, J=15 Hz, 2H, CH$_2$COOCH$_3$), 3.64 (s, 3H COOCH$_3$), 5.56 (dt, J=14.5 Hz, J=12 Hz, 1H, CHCH$_2$), 6.39 (d, J=13 Hz, 1H, PhCH), 7.13-7.35 (m, 5H, PhCH). $^{13}$C NMR (75 MHz, CDCl$_3$): δ(ppm) 24.4 (d), 27.7 (d), 33.29 (d), 51.4 (s), 125.8 (t), 126.5 (t), 128.0 (t), 128.4 (t), 129.4 (t), 130.7 (t), 137.3 (q), 173.8 (q). E-isomer $^1$H NMR (300 MHz, CDCl$_3$): δ(ppm) 1.75-1.84 (m, 2H, CH$_2$), 2.31 (dt, J=15 Hz, J=7.5 Hz, 2H, CHCH$_2$), 2.33 (t, J=15 Hz, 2H, CH$_2$COOCH$_3$), 3.64
(s, 3H, COOCH₃), 6.11 (dt, J=15.5 Hz, J=14 Hz, 1H, CHCH₂), 6.34 (d, J=19 Hz, 1H, PhCH), 7.13-7.35 (m, 5H, PhCH). ¹³C NMR (75 MHz, CDCl₃): δ(ppm) 24.4 (d), 27.7 (d), 33.4 (d), 51.4 (s), 125.8 (t), 126.9 (t), 128.0 (t), 128.6 (t), 129.7 (t), 131.4 (t), 137.4 (q), 173.9 (q). The double bond of the ester (800 mg, 3.92 mmol) was reduced with H₂ using Pd/C (10% Pd, 208.4 mg, 0.19 mmol Pd) as catalyst in MeOH (10 mL). The suspension was purged several times with vacuum/H₂ cycles to remove the O₂ present in the media and finally was kept under H₂ at atmospheric pressure. The reaction mixture was stirred for 2h at RT, until the disappearance of the starting material was observed by TLC (hexane:ethyl ether, 1:1). The suspension was then purged again with vacuum/N₂ cycles to eliminate the H₂. The catalyst was removed by filtration and the MeOH was evaporated to dryness to obtain an oil corresponding to the desired reduced product methyl 6-phenylhexanoate (784 g, 97% yield). The ester was hydrolyzed in THF (18 mL) and with 0.5N NaOH (18 mL) at RT until the disappearance of the ester by TLC. The THF was removed under vacuum and the aqueous solution acidified with conc. HCl and extracted with Et₂O. The organic phase was dried with MgSO₄, filtered and evaporated to dryness to obtain 6-phenylhexenoic acid 2b (541 mg, overall yield 35 % as white solid ¹H NMR (300 MHz, CDCl₃): δ(ppm) 1.35 (tt, J= 19 Hz, J= 15 Hz, 2H, CH₂), 1.60-1.68 (m, 4H, CH₂), 2.30 (t, J= 15 Hz, 2H, CH₂), 2.60 (t, J= 15 Hz, 2H, CH₂), 7.14-7.28 (m, 5H, Ph). ¹³C NMR (75 MHz, CDCl₃): δ: 24.7 (d), 28.6 (d), 31.0 (d), 33.9 (d), 35.6 (d), 125.5 (s), 128.1 (s), 128.2 (s), 142.4 (q), 174.1 (q).

7-Phenylheptanoic acid 3b. As described before for 2b, benzaldehyde (0.8 ml, 8.05 mmol) was reacted with the phosphonium bromide 3 (3.7 g, 8.05 mmol) to obtain the Z/E 7-phenyl-6-heptenoic acid. The crude reaction mixture was esterified with MeOH/H₂SO₄ and purified to obtain a 6:4 mixture of Z/E methyl 7-phenyl-6-heptenoate 3a (340 mg, 17 % yield) as a solid. Z-isomer ¹H NMR (300 MHz, CDCl₃): δ(ppm) 1.44-1.53 (m, 2H, CH₂), 1.60-1.69 (m, 2H, CH₂), 2.22 (dt, J=13.5 Hz, J=7 Hz, 2H, CHCH₂), 2.32 (t, J=12, 2H, CH₂COO), 3.67 (s, 3H COOCH₃), 5.66 (dt, J=12 Hz, J=12 Hz, 1H, CHCH₂), 6.37 (d, J=12 Hz, 1H, PhCH), 7.15-7.35(m, 5H, PhCH). ¹³C NMR (75 MHz, CDCl₃): δ: 24.4 (d), 28.7 (d), 32.5 (d), 34.0 (d), 51.4 (s), 125.9 (t), 126.7 (t), 128.1 (t), 128.7 (t), 130.08 (t), 137.6 (q), 174.0 (q). E-isomer ¹H NMR (300 MHz, CDCl₃): δ(ppm) 1.48-1.53 (m, 2H, CH₂), 1.60-1.69 (m, 2H, CH₂), 2.24 (dt, J=13.5 Hz, J=7 Hz, 2H, CHCH₂), 2.34 (t, J=12 Hz, 2H, CH₂COO), 3.67 (s, 3H COOCH₃), 6.2 (dt, J=12 Hz, J=12 Hz, 1H, CHCH₂), 6.43 (d, J=12 Hz, 1H, PhCH), 7.15-7.35(m, 5H, PhCH). ¹³C NMR (75 MHz, CDCl₃): δ(ppm): 24.4 (d), 28.7 (d), 32.9 (d), 34.0 (d), 51.4 (s), 125.9 (t), 126.4 (t), 128.4 (t), 128.8 (t), 129.8 (t), 137.6 (q), 174.0 (q). As before, the double bond (300 mg, 1.21 mmol) was reduced with H₂ and Pd/C ((10% Pd, 63.8 mg, 0.06 mmol Pd) to obtain the corresponding methyl 7-phenylheptanoic acid (270 mg, 89 % yield). Finally, the hydrolysis of the ester gave the desired compound 3b (262 mg, 97 % yield). ¹H NMR (300 MHz, CDCl₃): δ(ppm) 1.33-1.36
9-phenylnonanoic acid 4b. As described before for 2b, benzaldehyde (0.4 ml, 3.93 mmol) was reacted with the phosphonium bromide 4 (1.9 g, 3.93 mmol) to obtain the Z/E 7-phenyl-6-heptenoic acid. The crude reaction mixture was esterified with MeOH/H$_2$SO$_4$ and purified to obtain a 6:4 mixture of Z/E methyl 7-phenyl-6-heptenoate 4a (443 mg, 46 % yield). Z-isomer $^1$H NMR (300 MHz, CDCl$_3$): $\delta$(ppm) 1.25-1.4 (m, 4H, CH$_2$), 1.44-1.52 (m, 2H, CH$_2$), 1.59 (m, 2H, CH$_2$), 2.18 (dt, J=13.5 Hz, J=7 Hz, 2H, CH=CH$_2$), 2.28 (t, J=15 Hz, 2H, CH$_2$COO), 3.64 (s, 3H COOCH$_3$), 5.65 (dt, J=12 Hz, J=12 Hz, 1H, CH=CH$_2$), 6.41 (d, J=12 Hz, 1H, PhCH)$_2$. 13C NMR (75 MHz, CDCl$_3$): $\delta$(ppm) 24.6 (d), 28.5 (d), 28.9 (d), 29.1 (d), 32.9 (d), 34.0 (d), 51.4 (s), 125.9 (t), 126.7 (t), 128.1 (t), 128.7 (t), 130.08 (t), 137.7 (q), 174.3 (q). E-isomer $^1$H NMR (300 MHz, CDCl$_3$): $\delta$(ppm) 1.25-1.4 (m, 4H, CH$_2$), 1.44-1.55 (m, 2H, CH$_2$), 1.59 (m, 2H, CH$_2$), 2.28 (t, J=15 Hz, 2H, CH$_2$COO), 2.30 (dt, J=15 Hz, J=7.5 Hz, 2H, CH=CH$_2$), 3.64 (s, 3H COOCH$_3$), 6.21 (dt, J=15 Hz, J=13.5 Hz, 1H, CH=CH$_2$), 6.37 (d, J=13 Hz, 1H, PhCH)$_2$. 13C NMR (75 MHz, CDCl$_3$): $\delta$(ppm) 24.6 (d), 28.8 (d), 28.9 (d), 29.7 (d), 34.0 (d), 51.4 (s), 125.9 (t), 126.4 (t), 128.4 (t), 128.8 (t), 129.8 (t), 137.8 (q), 174.3 (q). As before, the double bond (443 mg, 1.79 mmol) was reduced with H$_2$ and Pd/C ((10% Pd, 95 mg, 0.08 mmol Pd) to obtain the corresponding methyl 9-phenylheptanoic acid (270 mg, 89 % yield). Finally, the hydrolysis of the ester gave the desired compound 3b (354 mg, 80 % yield) as pale yellow oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$(ppm) 1.29 (m, 6H, CH$_2$), 1.61 (m, 4H, CH$_2$), 2.29 (t, J=15 Hz, 2H, CH=CH$_2$), 2.59 (t, J=15 Hz, 2H, CH=CH$_2$), 7.16-7.35 (m, 5H, Ph). 13C NMR (75 MHz, CDCl$_3$): $\delta$(ppm) 24.8 (d), 29.04 (d), 29.09 (d), 29.1 (d), 31.4 (d), 34.0 (d), 35.8 (d), 125.4 (s), 128.1 (s), 128.3 (s), 142.7 (q), 174.2 (q).

12-phenylnonanoic acid 5b. As described before for 2b, benzaldehyde (0.3 ml, 2.8 mmol) was reacted with the phosphonium bromide 5 ((750 mg, 2.8 mmol) to obtain the Z/E 7-phenyl-6-heptenoic acid. The crude reaction mixture was esterified with MeOH/H$_2$SO$_4$ and purified to obtain a 6:4 mixture of Z/E methyl 7-phenyl-6-heptenoate 5a (444 mg, 54 % yield). Z-isomer $^1$H NMR (300 MHz, CDCl$_3$): $\delta$(ppm) 1.29 (m, 10H, CH$_2$), 1.42 (m, 2H, CH$_2$), 1.59 (m, 2H, CH$_2$), 2.19 (dt, J=13.5 Hz, J=6.5 Hz, 2H, CH=CH$_2$), 2.28 (t, J=15 Hz, 2H, CH$_2$COO), 3.64 (s, 3H OCH$_3$), 5.66 (dt, J=12 Hz, J=12 Hz, 1H, =CHCH$_2$), 6.40 (d, J=12 Hz, 1H, PhCH=), 7.20-7.35 (m, 5H, Ph). 13C NMR (75 MHz, CDCl$_3$): $\delta$(ppm) 24.8 (d), 29.04 (d), 29.09 (d), 29.1 (d), 31.4 (d), 34.0 (d), 35.8 (d), 125.4 (s), 128.1 (s), 128.3 (s), 142.7 (q), 174.2 (q).
and Pd/C ((10% Pd, 26.5 mg, 0.025 mmol Pd) to obtain the corresponding methyl 12-phenylheptanoic acid (390 mg, 97 % yield). Finally, the hydrolysis of the ester gave the desired compound 4b (920 mg, 91 % yield) as white solid. \(^1H\) NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 1.33 (m, 8H, \(-\text{CH}_2\)), 1.69 (m, 4H, \(-\text{CH}_2\)), 2.42 (t, \(J=15\) Hz, 2H, \(-\text{CH}_2\text{COOH}\)), 2.66 (t, \(J=15\) Hz, 2H, PhCH\(_2\)), 7.24-7.34 (m, 5H, Ph). \(^13\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) (ppm) 24.7 (C3), 29.0 (C4), 29.2 (C11), 29.3 (C5), 29.4 (C10), 29.5 (C6, C7, C8), 29.6 (C9), 31.5 (C12), 33.9 (C2), 36.0 (C13), 125.5 (C4'), 128.1 (C2'), 128.4 (C3'), 142.9 (C1'), 179.7 (C1).

13-phenylnonanoic acid 6b. As described before for 2b, benzaldehyde (0.728 ml, 7 mmol) was reacted with the phosphonium bromide 6 (1.88 g, 7 mmol) to obtain the \(Z/E\) 7-phenyl-6-heptenoic acid. The crude reaction mixture was esterified with MeOH/H\(_2\)SO\(_4\) and purified to obtain a 6:4 mixture of \(Z/E\) methyl 7-phenyl-6-heptenoate 6a (1.58 g, 50 % yield) as yellow oil. 

\textit{Z}-isomer \(^1H\) NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 1.27 (m, 12H, \(-\text{CH}_2\)), 1.43 (m, 2H, \(-\text{CH}_2\)), 1.61 (m, 2H, \(-\text{CH}_2\)), 2.20 (dt, \(J=13\) Hz, \(J=7\) Hz, 2H, \(-\text{CH}_2\text{CH}\)), 2.30 (t, \(J=15\) Hz, 2H, \(-\text{CH}_2\text{COO}\)), 3.66 (s, 3H, \(-\text{OCH}_3\)), 5.66 (dt, \(J=12\) Hz, \(J=12\) Hz, 1H, \(-\text{CHCH}_2\)), 6.40 (d, \(J=12\) Hz, 1H, PhCH=), 7.18-7.35 (m, 5H, Ph). \(^13\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) (ppm) 25.0 (d, 28.6 (d), 29.1 (d), 29.2-29.4 (d, d, d, d), 29.9 (C10), 33.0 (d), 34.1 (d), 51.4 (t), 125.8 (s), 126.7 (s), 128.0 (s), 128.6 (s), 131.2 (s), 137.8 (q), 174.3 (q). MS, m/z (%): 302 (M+, 25), 270 (21), 131 (46), 117 (96), 104 (100), 91 (74). 

\textit{E}-isomer \(^1H\) NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 1.25 (m, 4H, \(-\text{CH}_2\)), 1.42 (m, 2H, \(-\text{CH}_2\)), 1.59 (m, 2H, \(-\text{CH}_2\)), 2.32 (dt, \(J=15\) Hz, 2.34 (t, \(J=13\) Hz, 2H, 12C12-, 9C9-, 12C12-SPCs. 

Sulfonation of the phenyl carboxylic acids 1b-6b was performed following a similar procedure as described \(^1\), \(^2\). The corresponding phenylcarboxylic acids (2.8 mmol, 1 eq.) were added to conc. H\(_2\)SO\(_4\) (1 mL, 18.22 mmol, 6.5 eq.) placed in a round-bottom flask equipped with a refrigerant and heated at 100ºC. The
reaction mixture was stirred for 2 h and then slowly poured into H2O (80 mL). For the case of 5C5-, 6C6- and 7C7-SPCs, the aqueous solution was washed with Et2O (3 x 25 mL) and then neutralized with CaCO3 (6 g, 75 mmol). The solid formed was removed by filtration and the aqueous solution was evaporated under reduced pressure to dryness to finally obtain the desired SPCs as calcium salts. 9C9-, 12C12- and 13C13-SPCs, were purified from the reaction mixture as sodium salts by extracting the aqueous layer with ethyl acetate. The organic phase was dried with MgSO4, filtered and evaporated to dryness to finally obtain a dark oil that was subsequently washed with a saturated solution of NaHCO3. The yellow precipitate obtained was filtered and washed with ethyl acetate to yield a white solid that was corresponding to the desired compound.

½Ca•5C5-SPC (1.5 g, 90 % yield) 1H NMR (300 MHz, CD3OD): δ(ppm) 1.40 (m, 4H, -CH2-), 2.00 (t, J=15Hz, 2H, -CH2COOH), 2.50 (t, J=15Hz, 2H, PhCH2), 7.18 (d, J=8Hz, 2H orto), 7.44 (d, J=8Hz, 2H meta). 13C RMN (75 MHz, CD3OD): δ(ppm) 25.1 (d), 30.7 (d), 34.7 (d), 125.4 (s), 127.3 (s), 142.4 (s), 145.9 (q), 181.3 (q).

½Ca•6C6-SPC (389 mg, 85 % yield) 1H NMR (300 MHz, D2O): δ(ppm) 1.24 (m, 2H, CH2), 1.52 (m, 4H, CH2), 2.28 (t, J= 15 Hz, 2H, CH2), 2.61 (t, J= 15 Hz, 2H, CH2), 7.31 (d, J= 8 Hz, 2H, Ph), 7.62 (d, J= 8 Hz, 2H, Ph). 13C RMN (75 MHz, D2O): δ(ppm) 24.6 (d), 28.4 (d), 30.9 (d), 35.0 (d), 125.8 (s), 127.8 (s), 142.8 (q), 146.1 (q) 174.72 (q). MS, m/z (%): 271.2 (M+, 100), 170.0 (5), 155.1 (6). IR, v (KBr, cm-1); 3561 (-OH st), 1701 (C=O st), 1473 (C-C Ar), 1223 (C-C Ar).

½Ca•7C7-SPC (310 mg, 87 % yield) 1H NMR (300 MHz, DMSO-d6): δ(ppm) 1.22 (m, 4H, CH2), 1.50 (m, 4H, CH2), 2.12 ((t, J= 14.5 Hz, 2H, CH2), 2.65 (t, J= 14.5 Hz, 2H, CH2), 7.35 (d, J= 8 Hz, 2H, Ph), 7.66 (d, J= 8 Hz, 2H, Ph). 13C RMN (75 MHz, DMSO-d6): δ(ppm) 25.4 (d), 26.0 (d), 28.7 (d), 32.9 (d), 31.2 (d), 35.1 (d), 125.8 (s), 127.7 (s), 142.8 (q), 146.4 (q), 174.0 (q). MS, m/z (%): 285.1 (M+, 100), 183.1 (10). IR, v (KBr, cm-1); 3547 (-OH st), 1708 (C=O st), 1448 (C-C Ar), 1132 (Ar-SO3- st).

2Na•9C9-SPC (216 mg, 60 % yield) 1H NMR (300 MHz, D2O): δ(ppm) 1.24 (m, 8H, CH2), 1.49 (m, 4H, CH2), 2.16 (t, J= 14.5 Hz, 2H, CH2), 2.54 (t, J= 15 Hz, 2H, CH2), 7.31 (d, J= 8 Hz, 2H, Ph), 7.62 (d, J= 8 Hz, 2H, Ph). MS, m/z (%): 313.2 (M+, 100), 183.1 (10), 170.1 (5), 156.1 (20). IR, v (KBr, cm-1); 3427 (-OH st), 1797 (C=O st), 1204 (Ar-SO3- st).
2Na •12C_{12}-SPC (200, mg, 56 % yield) $^1$H NMR (300 MHz, DMSO-d$_6$): δ(ppm) 1.22 (m, 14H, -CH$_2$-), 1.45 (m, 4H, -CH$_2$-), 2.02 (t, J=14.5Hz, 2H, -CH$_2$COOH), 2.54 (t, J=15Hz, 2H, PhCH$_2$), 7.10 (d, J=8Hz, 2H ortho), 7.47 (d, J=8Hz, 2H meta).

2Na •13C_{13}-SPC (600, mg, 51 % yield) $^1$H NMR (300 MHz, DMSO-d$_6$): δ(ppm) 1.23 (m, 16H, -CH$_2$-), 1.49 (m, 4H, -CH$_2$-), 2.16 (t, J=15Hz, 2H, -CH$_2$COOH), 2.54 (t, J=15Hz, 2H, PhCH$_2$), 7.10 (d, J=8Hz, 2H ortho), 7.48 (d, J=8Hz, 2H meta).

**IMMUNOCHEMISTRY: EXPERIMENTAL PROCEDURES**

**General Methods and Instruments.** 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) and Grams/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corporation (Salem, NH). The pH and the conductivity of all buffers and solutions were measured with a pH meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, DK). Washing steps were performed on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA). The competitive curves were analyzed with a four parameter logistic equation using the software SoftmaxPro v2.6 (Molecular Devices) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

**Chemicals and Immunochemicals.** Immunochemicals and the seasalts used to prepare artificial sea water were obtained from Sigma Chemical Co. (St. Louis, MO). The preparation of the protein conjugates and the antisera is described below. Most of the chemicals used for crossreactivity studies were acquired from Aldrich Chemical Co. (Milwaukee, WI). The technical mixture of LAS was kindly provided by PETRESA S.A. (San Roque, Cádiz, España). The exact percentage weight of each LAS homologue was 0.4% for <5 phenyl C10, 11.8% phenyl C10, 34% phenyl C11, 30.3% phenyl C12, 22.5% phenyl C13, 0.4% phenyl C14 and 0.1% of parafines. The standards used for the calibration of the ELISA experiments were prepared by serial dilutions in DMSO (32 nM - 5000 µM) and diluted 200 times with PBST-I prior the assay was run.
**Direct ELISA. General Protocol.** The plates were coated with the As (100 µL/well in coating buffer) overnight at 4 °C covered with adhesive plate sealers. The day after, the plates were washed four times with PBST (300 µL/well) and different concentrations of the analyte (0.16 nM to 25000 nM, 50 µL/well in PBST; only PBST for zero analyte and in the noncompetitive experiments) followed by the enzyme tracers appropriately diluted (50 µL/well in PBST) were added and incubated for 30 min at room temperature (RT). The plates were washed again as before, and the substrate solution was added (100 µL/well). Color development was stopped after 30 min at RT with 4 N H2SO4 (50 µL/well), and the absorbances were read at 450 nm.

**Noncompetitive Direct ELISA.** The screening of the avidity of the 6 antisera obtained (As93-As98) for the 21 enzyme tracers synthesized was determined by measuring the binding of serial dilutions (1 µg L⁻¹ to 8 ng L⁻¹, 50 µL/well) of each HRP conjugate to the microtiter plates coated with different dilutions (1/1000 to 1/64000, 100 µL/well) of each antisera. From these experiments, immunoreagent concentrations were chosen to produce around 0.7-1 units of absorbance in 30 min.

**Indirect ELISA. General Protocol.** The plates were coated with the antigens (100 µL/well in coating buffer) overnight at 4 °C covered with adhesive plate sealers. The day after, the plates were washed four times with PBST (300 µL/well) and the standard solutions of LAS (0.16 nM to 25000 nM, 50 µL/well in PBST; only PBST for zero analyte and in the noncompetitive experiments) and the antisera (50 µL/well in PBST) were added and incubated for 30 min at room temperature (RT). The plates were washed again as before, and a solution of antiIgG-HRP (1/6000 in PBST) was added to the wells (100 µL/well) and incubated for 30 min more at RT. The plates were washed again, and the substrate solution was added (100 µL/well). Color development was stopped after 30 min at RT with 4 N H2SO4 (50 µL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four parameter equation according to the following formula: \[ Y = \left( \frac{(A - B)}{1 - \left( \frac{x}{C} \right)D} \right) + B, \] where \( A \) is the maximal absorbance, \( B \) is the minimum absorbance, \( C \) is the concentration producing 50% of the maximal absorbance, and \( D \) is the slope at the inflection point of the sigmoid curve.

**Noncompetitive Indirect ELISA** was used for the screening of the avidity of the 6 antisera obtained (As93-As98) versus the 126 coating antigens synthesized, by measuring the binding of serial dilutions (1/1000 to 1/64000, and zero, 50 µL/well) of each antisera to the microtiter plates coated with different dilutions (10 µg mL⁻¹ to 9 ng mL⁻¹, and zero, 100 µL/well) of each of the BSA, CONA, and OVA conjugates. From these experiments, optimum concentrations for
coating antigens and antisera dilutions were chosen to produce around 0.7-1 units of absorbance in 30 min.