CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200701232

Disulfide Symmetric Dimers as Stable Pre-Hapten Forms for Bioconjugation: A Strategy to Prepare Immunoreagents for the Detection of Sulfophenyl Carboxylate Residues in Environmental Samples

M.-Carmen Estévez, Roger Galve, Francisco Sánchez-Baeza, and M.-Pilar Marco*[a]

Abstract: A convenient, generic synthesis of bioconjugates from haptens with a thiol group has been established. The corresponding haptens are synthesized as stable symmetric dimmers through a disulfide bond that is reduced immediately before conjugation with the aid of a di(*n*-butyl)phenylphosphine polystyrene (DBPP) resin. This strategy was used to prepare haptenized biomolecules and to raise antibodies against short-alkyl-chain sulfophenyl carboxylates (*X*-C_z-SPCs; *X* is the position of the benzylic group and *z* is the alkyl-chain length) formed after degradation of the widely used domestic and industrial linear alkylbenzene sulfonates (LASs) surfactants. Because of the complexity of the LASs technical mixture, homologous and pseudo-heterologous immunization strategies have been studied with the aim of broadening antibody recognition of the SPC family. With this purpose, two types of immunizing haptens have been synthesized and used to prepare bioconjugates and raise antibodies. Type-A bioconjugates (SPC_A–protein) were prepared by synthesizing type-A haptens as stable symmetric dimers, generically 2,2′-dithiobis[5-{4-(*N*-ethylsulfamoyl)}phenylalkanoic acids] (*X*-C_z-S-SPC). On the other hand, type-B bioconjugates (SPC_B–protein) were prepared by treating the carboxylic groups of the corresponding 4-sulfophenylalkanoic acids (*X*-C_z-SPC) with the amino groups of the lysine residues by using classical carbodiimide procedures. Type-A haptens produced antibodies with a much higher avidity for the target analyte. Under competitive immunochemical configurations (As112/2-C_5–ovalbumin), these antibodies can reach a limit of detection (LOD) of 40 ng L^{-1} with an IC_{50} value of 200 ng L^{-1} for 3-C_6-SPC, which opens up the possibility of trace contamination of edible waters by surfactants with 3-C_6-SPC as a marker of LAS pollution. A comparative study of the properties of the three families of polyclonal antibodies produced revealed that antibodies raised through pseudo-heterologous immunization strategies produced antibodies with a broader specificity versus the SPC family. These results indicate that this approach could be useful in avoiding synthetic difficulties associated with preparing haptens that preserve all the most important chemical functionalities of the molecule.

Keywords: antibodies · immunization · immunoassays · resins · solid-phase synthesis · sulfophenyl carboxylates

Introduction

The preparation of bioconjugates with small organic molecules often faces the problem that important functional (and/or antigenic) chemical groups can be blocked by the biomolecule if used as conjugation sites.[1,2] Hapten derivatives can be designed and synthetically prepared to respect these particular groups while introducing other chemical functionalities that allow covalent attachment to the desired biomolecule. However, the reactivity of such new chemical functionalities must be orthogonal to the reactivities of the other chemical groups, which may give additional difficulties in hapten synthesis and further covalent coupling of highly functionalized small organic molecules to biomacromolecules. Such a circumstance was confronted within the European Project aimed at developing an immunosensor for the detection of industrial pollutants, such as the sulfophenyl carboxylates (SPCs).[3]

SPCs are the main metabolites of the degradation of linear alkylbenzene sulfonates (LASs),[4,5] which are used as surfactants in domestic and industrial purposes in high amounts worldwide. Their frequent detection in the environ-

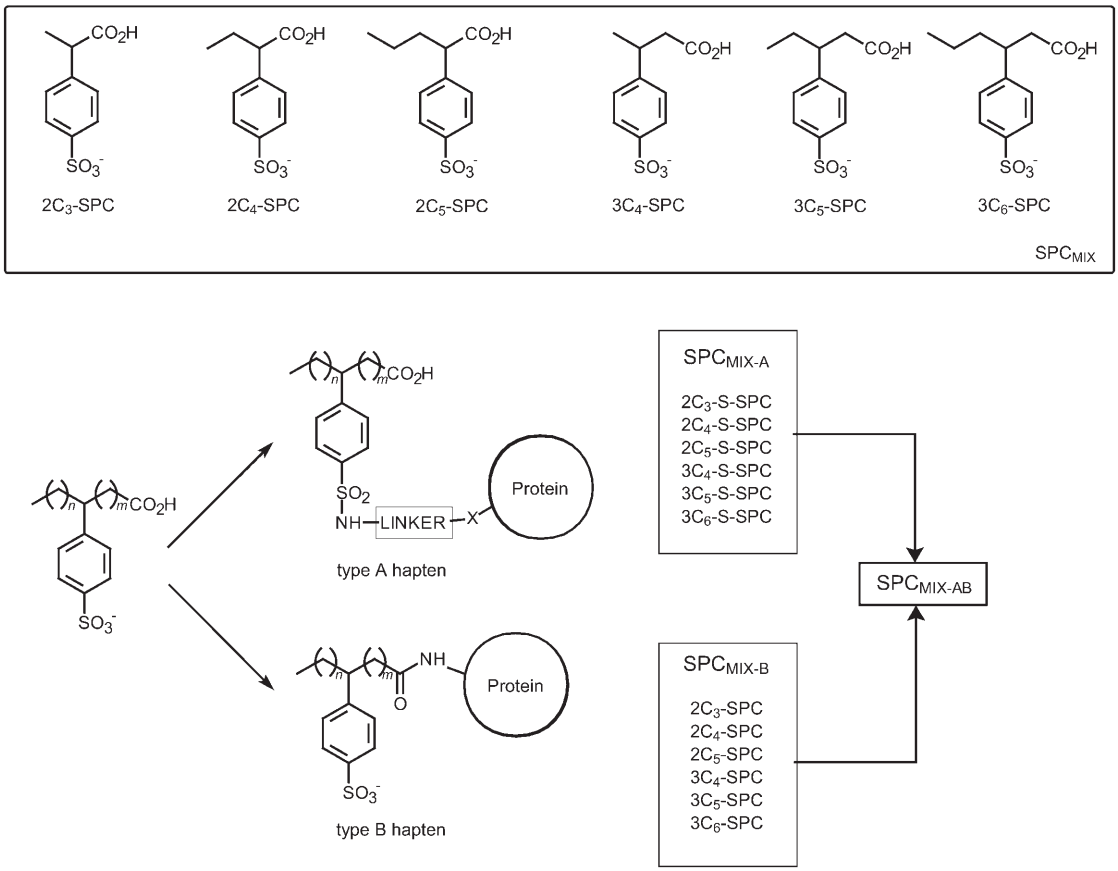
[a] Dr. M.-C. Estévez, Dr. R. Galve, Dr. F. Sánchez-Baeza, M.-P. Marco
Applied Molecular Receptors Group (AMRg)
CSIC, CIBER of Bioengineering, Biomaterials and Nanomedicine
IIQAB-CSIC, Jorge Girona, 18—26, 08034-Barcelona (Spain)
Fax: (+34) 93-204-5904
E-mail: mpmqob@iiqab.csic.es

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

ment at significant concentration levels[6–8] has raised concern over their potential synergistic role, which favors the permeation of other toxicants through biological membranes thanks to their amphoteric and surfactant properties.[9,10] Microbial degradation[11] is very fast and can be considered to be the major elimination pathway, which involves the oxidation of the terminal methyl group of the alkyl chain (ω oxidation) followed by the successive loss of two carbon units as a result of β-oxidation processes.[4] The complexity of the LAS mixture (i.e., alkyl chains of 10–14 carbon units and different positional isomers at the benzylic position) determines the consequent complexity of the SPC mixture formed after degradation of LASs. Thus, the first compounds generated after the degradation of LAS are SPCs with long alkyl chains (>10 carbon units), but the relatively rapid β-oxidation processes lead to SPCs with short alkyl chains (see Scheme 1). Particularly, those SPC isomeric mixtures with alkyl chains of five and six carbon units (*X*-C_5-SPC and *X*-C_6-SPC, *X* indicates that the aromatic ring can be placed at any position) have been considered by many authors as the more likely key intermediates of the degradation pathway.[12–14] Nevertheless, certain analytical limitations may have prevented the proper detection of shorter-alkyl-chain SPC congeners. Thus, the high polarity of these short-alkyl-chain SPCs, as a result of the presence of the carboxylic acid and sulfonate groups, causes significant difficulties in extracting them efficiently from the aqueous media before chromatographic analysis.[12,15–17]

The preparation of immunoreagents able to detect a wide range of short-alkyl-chain SPCs was one of the goals of this investigation. However, the production of antibodies with class-directed specificity is an uncertain goal that has been persecuted by many research groups with the purpose of developing immunochemical screening procedures able to detect, to a similar extent, different congeners of structurally related families of substances (i.e., sulfonamide antibiotics,[18–20] sulfonyl urea herbicides,[21,22] and so forth). The strategies employed frequently consist of preparing hapten–protein immunoconjugates that expose common features of the chemical structures to the immune system. However, this approach, which may require long and intricate synthetic procedures to keep important antigenic epitopes free, has often failed.

We recently reported our attempts to produce generic antibodies for SPCs through using an equimolar mixture of immunogens prepared by coupling six short-alkyl-chain SPC haptens, thus maximizing the recognition of the common sulfonic group (type-B haptens) to horseshoe crab hemocyanin (HC; $SPC_{mix\text{-}B}$; see Scheme 1).[23] However, these antibodies only recognized *X*-C_5-SPCs and *X*-C_6-SPCs preferentially, while other SPCs were poorly recognized, thus indicating that the different lengths and branching of the alkyl-



Scheme 1. Chemical structures of the six representative SPCs and haptens.

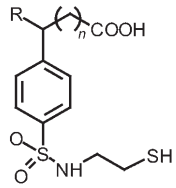
chain patterns played a decisive role in the recognition process. More success has recently been achieved from producing generic antibodies by using antibody engineering methodologies.[24–26] Alternatively, the heterologous immunization approach has been reported a few times with the aim of eliciting antibody responses against more than one epitope, mainly in the context of catalytic antibodies.[27–29] This idea prompted us to investigate the possibility of expanding antibody recognition of the short-alkyl-chain SPC family through this strategy.

The heterologous immunization approach envisaged by Masamune and co-workers[27,28,30] uses a combination of two different, but structurally related, haptens thus maximizing distinct epitopes. With this purpose, we addressed the synthesis of two haptens maximizing recognition of the alkyl carboxylic chains (type-A haptens) and of the sulfonic groups (type-B haptens) to use them in a heterologous immunization protocol. For the type-B haptens, the carboxylic groups of the alkyl chain could be directly used for conjugation. However, the preparation of type-A haptens required the introduction of a spacer arm with an orthogonal functionality. Thus, the SPC molecules could be generically treated with a $H_2N(CH_2)_nX$ linker through the formation of a sulfonamide, where X would be the necessary orthogonal chemical group for bioconjugation. Another carboxylic group would compete with the same group on the SPC alkyl chain at the conjugation step. Similarly, the use of linkers to introduce a free amine group was ruled out since the formation of (a SPC–CONH−)$_n$ species could take place in addition to SPC-NHCO-protein conjugates. The same applied if an alcohol group was introduced to form ester bonds with the protein. Alternatively, thiol groups provided an appropriate reactivity when carboxylic, aldehyde, amine or halogen groups, present in the same small organic molecule, want to be preserved. Moreover, the conjugation reaction could take place through a variety of commercially available bifunctional cross-linkers chosen to react with the amino groups of the lysine residues on one side and with the thiol group on the other side.[32]

We report herein the investigation into the potential of pseudo-heterologous immunization procedures to tailor antibody features. Moreover, we present a simple and generic strategy to prepare bioconjugates with highly functionalized small organic molecules.

Results

Hapten design and synthesis of the SPC dimers: We planned to synthesize the two types of haptens for each of the six short-alkyl-chain SPC representatives. The carboxylic acid groups could directly be used for bioconjugation of the six type-B haptens, although it was necessary to introduce a linker with a thiol group for the type-A haptens (Scheme 2).

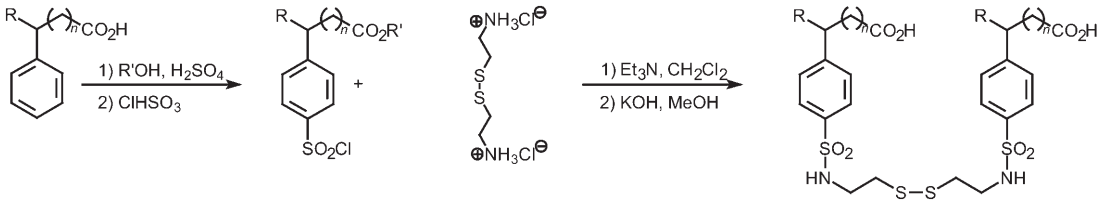


SPC-SH

Scheme 2. Type-A hapten.

To prevent oxidation or avoid intra/intermolecular reactions, free −SH (thiol) groups are commonly protected as thioethers or thioesters, although the formation of thiazolidines, unsymmetrical disulfides, and *S*-sulfenyl derivatives has also been used to a more limited extent.[31] Since oxidation to form the corresponding symmetrical disulfides is also a possible strategy, we envisaged the synthesis of the haptens as disulfide pre-haptens. Finally, cleavage of the disulfide bond could be approached immediately before bioconjugation to a conveniently derivatized protein.

The synthesis of the dimers was performed by preparing the corresponding chlorosulfonic derivatives of phenylcarboxylic acids **1**–**7** followed by the formation of two sulfonamide bonds using cystamine dihydrochloride (Scheme 3). In the first step, it was important to always have an excess of the chlorosulfonic acid to prevent the formation of the corresponding sulfones as by-products.[33] Moreover, the phenylcarboxylic acids were first protected as esters to avoid intramolecular cyclization through Friedel–Crafts acylation reactions. For phenylcarboxylic acids **1**–**5**, the use of a bulky protecting group, such as the butyl group, was required to



R=Me	*n*=0	2-phenylpropionic acid	(**1**)	(**1a**) R'=Bu
R=Et	*n*=0	2-phenylbutyric acid	(**2**)	(**2a**) R'=Bu
R=Pr	*n*=0	2-phenylpentanoic acid	(**3**)	(**3a**) R'=Bu
R=Me	*n*=1	3-phenylbutyric acid	(**4**)	(**4a**) R'=Bu
R=Et	*n*=1	3-phenylpentanoic acid	(**5**)	(**5a**) R'=Bu
R=Pr	*n*=1	3-phenylhexanoic acid	(**6**)	(**6a**) R'=Me
R=H	*n*=4	5-phenypentanoic acid	(**7**)	(**7a**) R'=Me

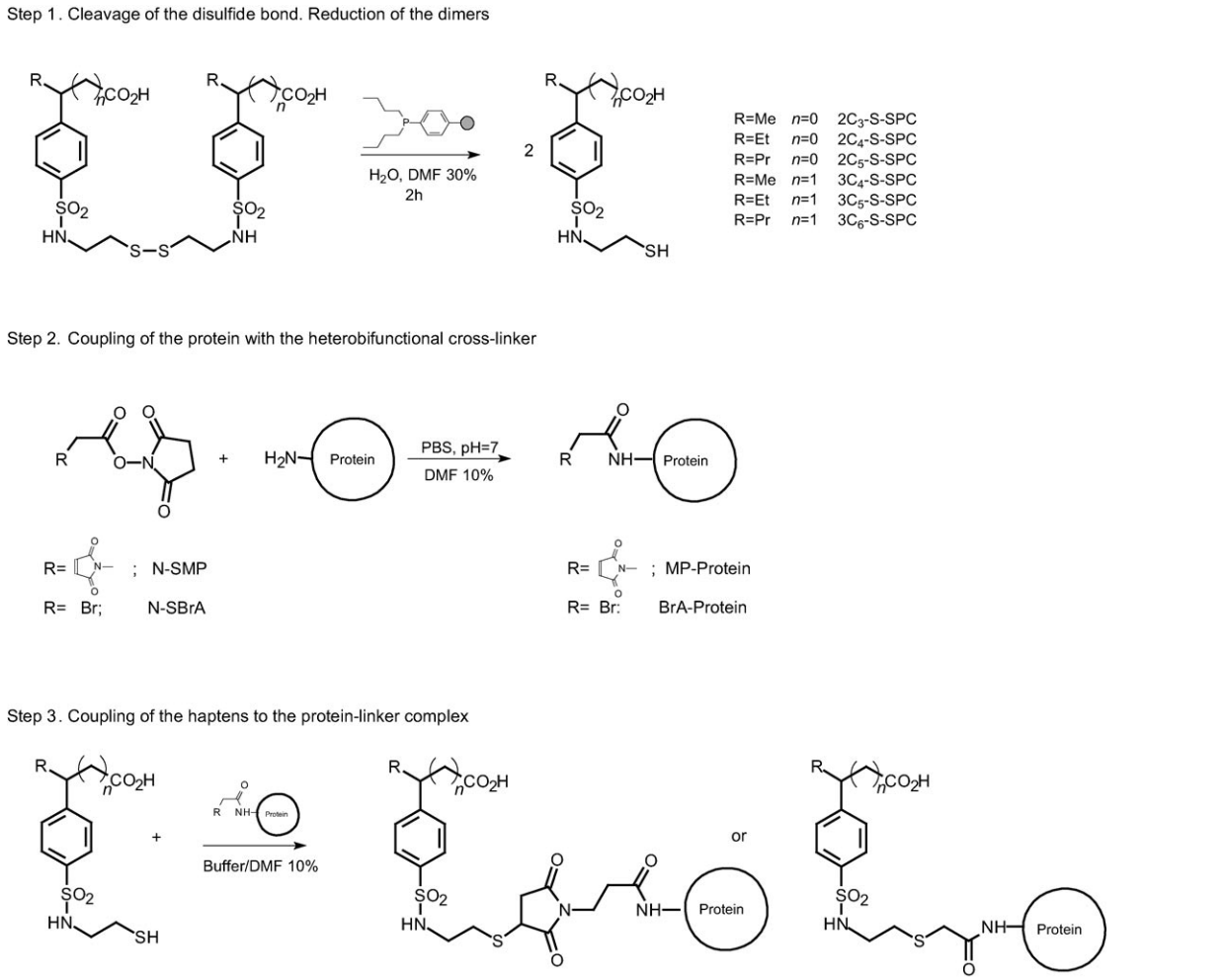
R=Me	*n*=0	$2C_3$-S-SPC
R=Et	*n*=0	$2C_4$-S-SPC
R=Pr	*n*=0	$2C_5$-S-SPC
R=Me	*n*=1	$3C_4$-S-SPC
R=Et	*n*=1	$3C_5$-S-SPC
R=Pr	*n*=1	$3C_6$-S-SPC
R=H	*n*=4	$5C_5$-S-SPC

Scheme 3. Synthesis of SPC–SH.

ensure unique chlorosulfonation at the *para* position, thus preventing reactivity of the *ortho* positions that would lead to polysubstituted derivatives. Protection of the carboxylic acids as methyl esters was sufficient for the case of phenylcarboxylic acids **6** and **7** with longer alkyl chains. The moderate yield of **1a--7a** was attributed to the instability of the chlorosulfonate groups in the aqueous media used to treat the reaction.

Surprisingly, the formation of *meta*-monosubstituted products as by-products in a low yield (6–12%) was also observed for the phenylcarboxylates **1–3** containing the phenyl group at the α position to the carboxylic group, as confirmed by ^{1}H and ^{13}C NMR spectroscopic analysis (see the analytical data for **1b--3b** in the Supporting Information). Finally, the formation of the symmetric dimers was achieved in one step in good yields by directly treating two molecules of chlorosulfonyl derivatives **1a–7a** with one molecule of cystamine dihydrochloride through the formation of two sulfonamide bonds. The hydrolysis of the esters led to the final desired products: the dimers 2-C_3-S-SPC, 2-C_4-S-SPC, 2-C_5-S-SPC, 3-C_4-S-SPC, 3-C_5-S-SPC, 3-C_6-S-SPC, and 5-C_5-S-SPC.

Preparation of the hapten–biomolecule conjugates: For the type-A haptens, *N*-succinimidyl-3-maleimidyl propanoate (*N*-SMP) and *N*-succinimidyl bromoacetate (*N*-SBrA) were used as heterobifunctional cross-linkers to prepare hemocyanin (HC), bovine serum albumin (BSA), ovalbumin (OVA), and conalbumin (CONA) conjugates. Hemocyanin conjugates are selected for use as immunoagents because of the high immunogenic potential of this protein, while the preparation of bioconjugates with albumins was carried out to evaluate them as competitors in the immunoassay. The preparation of the haptenized biomolecules comprised three steps: 1) cleavage of the dimer under reducing conditions, 2) coupling of the linker *N*-SMP (or *N*-SBrA) to the protein, and 3) conjugation of the hapten to the maleidimidylpropanoyl- or bromoacetyl-derivatized proteins (MP–protein and BrA–protein, respectively; Scheme 4). The whole procedure was initially evaluated using 5-C_5-S-SPC as a model hapten, BSA as the protein, and *N*-SMP as the cross-linker. An important aspect of the synthesis was the use of the correct hapten/cross-linker/protein ratio to avoid free epitopes of the cross-linker in the final immunogen conjugate.



Scheme 4. Strategy used to prepare type-A hapten–protein bioconjugates.

Regarding the reduction of the dimer, the efficiency of different reducing agents was evaluated using the Ellman test[34] to follow the formation of the thiol groups. $NaCNBH_3$ was initially chosen with the intention of performing this reaction in aqueous media and in the presence of the protein; however, the degree of conversion was very poor, even with a high excess of $NaCNBH_3$ (the use of 10 equiv of $NaCNBH_3$ for 30 h yielded only a yield of 1.5% of free thiol groups; data not shown). A higher yield was obtained when a stronger, albeit less stable in aqueous media, reducing agent, such as $NaBH_4$, was used, but the results were not satisfactory enough (only a yield of 14% was produced over 5 h by using 5 equiv of $NaBH_4$). In contrast, tributylphosphine (Bu_3P),[35] which requires the presence of water as a coreagent, allowed the quantitative reduction of the dimers by using 1.5 equivalents over only 2 h.

haptens, except for 5-C_5-S-SPC, for which a yield of 70% was reached.

For step 2, the efficiency of the coupling reaction between the cross-linker and the protein was evaluated with BSA as the model and *N*-SMP at different molar ratios (1:2, 1:5, and 1:10 of lysine residues/*N*-SMP). Table 1 shows the yield

Table 1. Efficiency of the two steps involved in the conjugation strategy used to prepare the immunogens.[a]

Step 2			Step 3			Global conditions	
Lys/*N*-SMP[b]	δ *N*-SMP[c]	Yield [%][d]	MP–protein/hapten[e]	δ hapten[c]	Yield [%][f]	Lys/*N*-SMP/hapten[b]	global yield [%][d]
1:2	14	40–47	–	–	–	–	nt[g]
1:5	18	51–60	1:3	10	55	1:5:2	29–33
–	–	–	1:8	11	61	1:5:5	31–37
–	–	–	1:16	12	66	1:5:10	34–40
1:10	22	63–73	–	–	–	nt	

[a] Conjugation studies related to the ratio of the reagents were carried out with 5-C_5-S-SPC as the model hapten and BSA; the results have been extracted from the analysis of the conjugates by MALDI-TOF mass-spectrometric analysis. [b] Molar ratio used in the conjugation reaction with respect to the lysine residues of the BSA. [c] Number of residues (*N*-SMP or hapten) covalently attached to the BSA. [d] The degree of conjugation calculated on the basis that that BSA has 30–35 free accessible lysine moieties. [e] Molar ratio used in the conjugation reaction with respect to the maleimido residues of the *N*-SMP derivatized protein. [f] The conjugation yield calculated on the basis of the available maleimido residues incorporated in step 2. [g] nt = not tested.

When using *N*-SMP as the cross-linker, the formation of the corresponding hapten-MP-protein conjugates was demonstrated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS); however, when *N*-SBrA was employed, the phosphorous atom of the tributylphosphine, which remained in the mixture after the reduction step, interfered as a result of the formation of the phosphonium salt through the presence of the bromine atom in the BrA-derivatized protein. Complete removal of this reagent, prior to adding the thiol group to the protein, required the introduction of a purification step in which concomitant partial oxidation of the thiol moiety could occur, thus leading again to the dimer. Hence, with the aim of avoiding contact of the bromine atom in the protein with the phosphine group, a polystyrene resin with immobilized di(*n*-butyl)phenylphosphine (0.5 mmol g_{resin}^{-1}) was evaluated as an alternative methodology for reduction, thus using the advantages of solid-phase synthesis. The evolution of the reaction was again followed with the Ellman test. In this case, contrary to the methodology employed before, the reduction had to be performed in the absence of water, otherwise the rate of the reaction proceeded very slowly and several hours were needed for complete reduction to occur, even with an excess of reducing agent (up to 5 equiv). In contrast, an almost complete conversion was accomplished after 2 hours using 1.25 equivalents of the reducing agent only if anhydrous *N*,*N*-dimethylformamide (DMF) was used as the solvent. After this time, water was added and the reaction mixture was stirred for 1 h further. Under these conditions, high yields, which ranged between 95 and 100%, of the reduction products could be accomplished for all the

of these conjugation reactions as measured by MALDI-TOF mass spectrometric analysis. A 1:5 ratio was chosen since 18 maleimido residues (around the 50% of the free lysines derivatized with the linker) were considered enough active points for hapten coupling. Different maleimido residues:hapten molar ratios were evaluated; however, as can be seen in Table 1, increasing the molar ratio only produced a slight augment of the number of hapten residues attached to the protein. The yield of the conjugation products in this step did not ensure free maleimido/Br epitopes, even at a higher hapten/MP/protein molar ratio. In light of these results, we decided to proceed with the conjugation of the *X*-C_z-S-SPC haptens, thus decreasing the lys/*N*-SMP (or *N*-SBrA) molar ratio to 1:2 (around 14 residues; Table 1) and keeping a medium MP (or BrA)–protein/hapten ratio of about 1:8. Thus, the final lys/*N*-SMP (or *N*-SBrA)/hapten molar ratio chosen was 1:2:4. Table 2 shows the degree of conjugation of the different *X*-C_z-S-SPC protein conjugates under these conditions. It can be observed that in spite of the decreased lys/*N*-SMP (or *N*-SBrA) ratio some MP and BrA residues remained. A potential explanation of these results is the possible undesired reaction of the hydroxy groups from the aqueous media with the maleimido or bromide groups, although this supposition has not been proved. Thus, we proceeded to use these conjugates to raise antibodies. Employing a different cross-linker for the coating antigens used in the competitive immunoassay would avoid the interference caused by the polyclonal fraction of antibodies against the cross-linker. Similarly, while HC conjugates were used to raise antibodies, distinct proteins, such as OVA, BSA, and CONA, were used for the preparation of the

Table 2. Hapten density and degree of conjugation of the type-A protein conjugates.[a]

Hapten	*N*-SMP[b]		*N*-SBrA[b]	
	δ Residues[c]	Conjugation [%][e]	δ Residues[c]	Conjugation [%][e]
N-SMP or *N*-SBrA	15[d]	43–50	14[d]	40–47
2-C_3-S-SPC	8	23–27	6	17–20
2-C_4-S-SPC	6	17–20	5	14–17
2-C_5-S-SPC	3	9–10	8	23–27
3-C_4-S-SPC	7	20–23	6	17–20
3-C_5-S-SPC	6	17–20	4	11–13
3-C_6-S-SPC	4	11–13	nc	nc
5-C_5-S-SPC	nc[f]	nc	7	20–23

[a] The molar ratio between lysine residues, the heterobifunctional linker, and free thiol groups was 1:2:4; the data shown correspond to the BSA derivatives of each hapten. [b] *N*-SMP was the cross-linker used to prepare the immunogens, while *N*-SBrA was the cross-linker used to prepare the rest of the antigens. [c] Estimated number of residues (cross-linker or hapten) covalently bound for each molecule of protein, as determined by MALDI-TOF mass-spectrometric analysis. [d] Estimated number of cross-linker residues covalently bound to each molecule of protein determined after step 2. [e] The degree of conjugation calculated on the basis that BSA has 30–35 free accessible lysine residues. [f] nc = not conjugated.

other immunoreagents used in the enzyme-linked immunosorbent assay (ELISA) procedures.

Bioconjugates with type-B haptens could easily be prepared using the water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC). The high polarity of the SPCs allowed the conjugation to be performed in buffer without the need for an organic co-solvent, thus achieving a high degree of conjugation. A hapten density within 16–18 mol of hapten per mol of protein was accomplished for all the cases, except 9-C_9-SPC which had 5 mol of haptens per mol of protein.

Antibodies against SPCs: Previous attempts to use a single SPC hapten congener failed to produce generic antibodies for the short-alkyl-chain SPC family. For this reason, we decided to use equimolar mixtures of the prepared haptens. Hence, white New Zealand rabbits were immunized with type-A haptens ($SPC_{mix\text{-}A}$-MP-HC) and type-B haptens ($SPC_{mix\text{-}B}$–HC)[23] and the evolution of the antibody titer was followed by indirect noncompetitive experiments under homologous conditions by using the analogous mixture conjugated to BSA as the coating antigen ($SPC_{mix\text{-}A}$-MP-BSA and $SPC_{mix\text{-}B}$–HC, respectively). The polyclonal antibodies obtained were named As112, As113, and As114 (type A) and As115, As116, and As117 (type B). Following the plan of heterologous immunization, three more rabbits were inoculated by combining $SPC_{mix\text{-}A}$-MP-HC and $SPC_{mix\text{-}B}$–HC ($SPC_{mix\text{-}AB}$–HC). Although in most of the examples reported heterologous-immunization protocols are performed by alternating two immunogens on each boosting injection (i.e., AABAAB or ABABAB, and so forth), we used an equimolar combination of both mixtures of immunogens on each boosting injection (pseudo-heterologous). Similarly, the evolution of the titer was followed with the homologous equimolar mixture of the BSA conjugates ($SPC_{mix\text{-}AB}$–BSA). The type-AB antisera obtained were named As118, As119, and As120.

Antibody characterization: Antibody characterization was accomplished through the development of competitive enzyme-linked immunosorbent assays for each type of antisera raised. With this aim, the avidity of the antisera raised versus all type-A and -B bioconjugates was tested through noncompetitive indirect ELISAs. As expected, homologous immunoassay combinations (type-A antibody/type-A antigen or type-B antibody/type-B antigen) gave higher antibody titers when the antisera was raised through homologous immunization procedures. In contrast, heterologous type-AB antibodies (As118–120) showed a higher affinity for the type-B antigens than the type-A antigens (see the Supporting Information).

The ability of these antiserums to recognize the SPC_{mix} (a mixture of the six short-chain SPCs 2-C_3-SPC, 2-C_4-SPC, 2-C_5-SPC, 3-C_4-SPC, 3-C_5-SPC, and 3-C_6-SPC) was determined through competitive assays by screening 225 antisera/coating antigen combinations. Heterologous immunoassay combinations (type-A antibody/type-B antigen or type-B antibody/type-A antigen) allowed better detectability to be reached than homologous combinations (see the Supporting Information). Antisera raised against type-A haptens provided better detectability values than type-B and -AB antibodies. Thus, the IC_{50} values recorded were 17.1 ± 7.1 ($N = 11$, type-A antibodies and type-B antigens), 166.7 ± 36.3 ($N = 14$, type-B antibodies and type-A antigens), and 1143 ± 461 ($N = 42$, type-AB antibodies and type-B antigens) $\mu g\,L^{-1}$. One-way analysis of variance (ANOVA) and *t*-tests provided *p* values below 0.0001, thus demonstrating the significance of this result. Table 3 shows the features for the best indirect competitive ELISAs obtained for each family of antibody in terms of detectability and reproducibility.

Assays As112/2-C_5–OVA, As115/2-C_5-S-SPC-CH_2-OVA, and As119/2-C_3–BSA were further evaluated to determine the detectability and specificity of the antisera raised through homologous (type A and B) and pseudo-heterologous (type AB) immunization approaches, respectively (see the Supporting Information). Attending to these studies, three ELISA protocols were established. Figure 1 and Table 4 show, respectively, the standard curves and the immunoassay parameters of the three indirect ELISAs obtained by using each group of antibodies (open symbols in the graph). As112 in combination with 2-C_5–OVA as the coating antigen allowed detection of the short-alkyl-chain SPC_{mix} compounds with a limit of detection (LOD) of 0.46 nM (0.11 $\mu g\,L^{-1}$, based on an average of molecular weight of 250) and an IC_{50} value of 3.05 nM (0.76 $\mu g\,L^{-1}$).

Antibody specificity was evaluated to find out the effect of the immunization strategies on the capability of the antibodies to recognize the different SPCs congeners formed during the degradation process. Hence, individual recognition of these SPCs and other related compounds was as-

Table 3. Features of the best competitive immunoassays obtained for the three families of antisera raised against the three types of SPC immunizing haptens.[a]

As	Coating antigen	CA [$\mu g\,mL^{-1}$][b]	As[c]	A_{max}	A_{min}	IC_{50} [$\mu g\,L^{-1}$][d]	Slope	R^2
Type A (As112)[e]	2-C_5-OVA	0.019	1:500	0.865±0.126	0.109±0.046	0.70±0.05	−0.99±0.12	0.997±0.002
	3-C_4–OVA	0.039	1:500	0.755±0.129	0.095±0.027	0.52±0.10	−1.13±0.20	0.995±0.002
	3-C_5–OVA	0.019	1:500	0.779±0.154	0.099±0.024	0.95±0.17	−1.05±0.17	0.965±0.021
	3-C_6–BSA	0.039	1:500	0.688±0.186	0.041±0.003	2.08±0.27	−1.49±0.48	0.966±0.016
Type B (As115)[e]	2-C_5-S-BSA	0.63	1:4000	1.012±0.432	0.032±0.034	42.4±20.4	−1.27±0.21	0.958±0.026
	2-C_5-S-OVA	1.25	1:2000	0.795±0.155	0.035±0.025	35.6±10.9	−1.42±0.26	0.960±0.017
	3-C_5-S-CONA	1.25	1:1000	0.881±0.280	0.044±0.037	31.7±9.9	−1.28±0.25	0.951±0.035
Type AB (As119)[e]	2-C_3–BSA	0.312	1:4000	0.640±0.243	0.028±0.036	29.8±12.5	−0.82±0.19	0.961±0.055
	9-C_9–BSA	0.312	1:1000	0.851±0.137	0.047±0.047	33.1±13.7	−1.05±0.30	0.943±0.068

[a] The parameters were extracted from the four-parameter equation used to fit the standard curves; the data presented correspond to the average of five calibration curves run on 5 different days; each curve was built using two-well replicates. [b] Coating antigen. [c] Dilution factor of the antisera. [d] SPC_{mix} composed of an equimolar mixture of the six short-alkyl-chain SPCs was used as the standard analyte; an average molecular weight of 250 was used to calculate the IC_{50} value. [e] As selected for each type of immunization protocol.

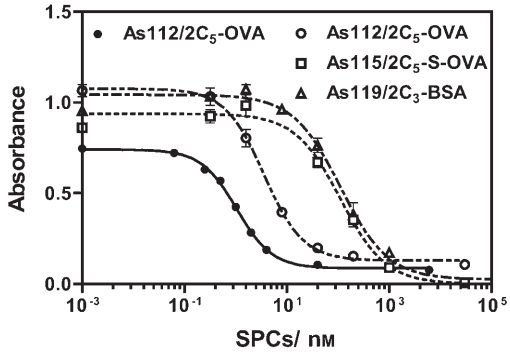


Figure 1. Standard calibration curves obtained with the antibodies produced through homologous (type A and B) and pseudo-heterologous strategies (type AB). Open symbols indicate that an equimolar mixture of the six SPCs was used as the standard analyte. Solid symbols indicate that 3-C_6-SPC was used as the analyte. The curves shown correspond to assays performed over different days. See Table 4 for the immunoassay features.

Table 4. Features of the optimized immunoassays.[a]

Antibody type:	Type A		Type B	Type AB
As/CA combination:	As112/2-C_5–OVA		As115/2-C_5-S-OVA	As119/2-C_3–BSA
Analyte:	mixture of SPCs	3-C_6-SPC	mixture of SPCs	mixture of SPCs
As dilution	1:500	1:500	1:2000	1:4000
CA [$\mu g\,mL^{-1}$]	0.02	0.02	1.25	0.31
A_{max}	0.898±0.221	0.785±0.131	0.914±0.272	0.907±0.358
A_{min}	0.114±0.041	0.079±0.008	0.023±0.020	0.032±0.028
IC_{50} [$\mu g\,L^{-1}$]	0.76±0.11	0.20±0.04	23.3±7.5	29.7±13.6
dynamic	0.23±0.06	0.06±0.02	5.60±3.80	3.61±2.30
range[b] [$\mu g\,L^{-1}$]	2.97±053	0.58±0.04	76.61±22.40	171.72±92.41
LOD [nM; $\mu g\,L^{-1}$]	0.11±0.04	0.03±0.01	1.5± 1.4	0.95±0.92
slope	−1.13±0.13	−1.27±0.24	−1.21±0.39	−0.80±0.16
R^2	0.996± 0.003	0.995± 0.003	0.956±0.030	0.969±0.030
N[c]	16	6	15	12

[a] Three immunoassays have been optimized, one for each family of antibody raised (type-A, -B, and -AB antibodies); the parameters are extracted from the four-parameter equation are used to fit the standard curve. [b] Upper and lower limits of the dynamic range, thus corresponding to the IC_{20} and IC_{80} values. [c] Number of assays used to calculate the data are presented as an average and the standard deviation; the assays were run on different days and each curve was built using two-well replicates.

sessed for the three types of antibody. The antisera raised through homologous or pseudo-heterologous immunization procedures showed a clearly different recognition pattern. Thus, antibodies obtained through pseudo-heterologous procedures showed a wider recognition pattern (see Table 5). Whereas the long-alkyl-chain SPCs tested (9-C_9-SPC and 12-C_{12}-SPC) and LASs were not recognized at all by the As112/2 C_5–OVA (type-A antibodies) and As115/2-C_5-S-SPC-CH_2-OVA (type-B antibodies) immunoassays, these substances cross-reacted significantly (45, 36, and 15%, respectively) in the As119/2-C_3–BSA (type-AB pseudo-heterologous antibodies) assay. Regarding individual recognition of the short-alkyl-chain SPCs, type-A and -B antibodies showed almost the same recognition pattern (3-C_6-SPC $\gg$ 3-C_5-SPC > 2-C_5-SPC > 3-C_4-SPC > 2-C_4-SPC $\approx$ 2-C_3-SPC) with a superior recognition of the congeners with longer alkyl chains, such as 3-C_6-SPC (more than 300%) and 3-C_5-SPC (around 100%), while the shorter-alkyl-chain SPCs 2-C_3-SPC and 2-C_4-SPC were barely recognized. In contrast, in the case of the type-AB antibodies, although 3-C_5-SPC and 3-C_6-SPC were also better recognized (>200 and $\approx$50%, respectively), the rest of the short-chain SPCs showed significant cross-reactivity values (30–35%). Other structurally related environmental contaminants, such as naphthalene and benzene sulfonates, were not recognized by any of the antibodies, thus indicating that the selectivity was only addressed versus the SPC family.

Since some researchers consider *X*-C_6-SPC to be the more

Table 5. SPC pattern of recognition observed using the three types of antibodies.[a]

Antibody type:	Type A		Type B		Type AB	
As/CA combination:	As112/2-C_5-OVA		As115/2-C_5-S-OVA		As119/2-C_3-BSA	
Compound[a]	IC_{50} [nM]	CR [%]	IC_{50} [nM]	CR [%]	IC_{50} [nM]	CR [%]
SPCs	3.05 ± 0.42	100	93.2 ± 30.0	100	118.7 ± 54.2	100
2-C_3-SPC	742	0.4	>30000	<0.01	409	29
2-C_4-SPC	887	0.3	4015	2	330	36
2-C_5-SPC	20	15	849	11	400	30
3-C_4-SPC	215	1	1277	7	409	29
3-C_5-SPC	2.76	110	65.7	142	55	216
3-C_6-SPC	0.84	363	26.8	348	220	54
5-C_5-SPC	17.5	17	>30000	<0.01	–	–
9-C_9-SPC	>30000	<0.01	>30000	<0.01	289	41
12-C_{12}-SPC	>30000	<0.01	>30000	<0.01	330	36
LAS	80	4	>30000	<0.01	766	15
NP	>30000	<0.01	>30000	<0.01	>30000	<0.01
p-TS	>30000	<0.01	>30000	<0.01	>30000	<0.01
EBS	>30000	<0.01	>30000	<0.01	2000	6
p-XS	>30000	<0.01	>30000	<0.01	>30000	<0.01
BDS	>30000	<0.01	>30000	<0.01	>30000	<0.01
SDS	>30000	<0.01	>30000	<0.01	>30000	<0.01
1-NaphSO_3	>30000	<0.01	>30000	<0.01	>30000	<0.01
1,5-NaphdSO_3	>30000	<0.01	>30000	<0.01	>30000	<0.01
1,3,5-NaphtSO_3	>30000	<0.01	>30000	<0.01	>30000	<0.01
2-phepropionic acid	>30000	<0.01	>30000	<0.01	>30000	<0.01
2-phebutyric acid	3613	0.1	>30000	<0.01	>30000	<0.01
2-phepentanoic acid	969	0.3	>30000	<0.01	>30000	<0.01
3-phebutyric acid	>30000	<0.01	>30000	<0.01	>30000	<0.01
3-phepentanoic acid	227	1	>30000	<0.01	>30000	<0.01
3-phehexanoic acid	57	5	>30000	<0.01	>30000	<0.01

[a] The percentage of recognition is expressed as cross-reactivity (CR) according to the expression [IC_{50}(SPC)/IC_{50}(cross-reactant)] × 100; 2-C_3, 2-C_4, 2-C_5, 3-C_4, 3-C_5, and 3-C_6 are the SPCs used as immunogens. LAS = linear alkylbenzenesulfonates, NP = nonylphenol, *p*-TS = *para*-toluensulfonic acid, EBS = *para*-ethyl benzenesulfonic acid, *p*-XS = *para*-xilenesulfonic acid, BDS = 1,3-benzenedisulfonic acid, SDS = sodium docecyl sulfate, 1-naphSO_3 = 1-naphthalenesulfonate, 1,5-naphdSO_3 = 1,5-naphthalenedisulfonate, 1,3,5-naphtSO_3 = 1,3,6-naphthalenetrisulfonate, 2-Phepropionic acid = 2-phenylpropionic acid, 2-Phebutyric acid = 2-phenylbutyric acid, 2-Phepentanoic acid = 2-phenylpentanoic acid, 3-Phebutyric acid = 3-phenylbutyric acid, 3-Phepentanoic acid = 3-phenylpentanoic acid, 3-Phehexanoic acid = 3-phenylhexanoic acid.

abundant short-alkyl-chain SPC found in the environment, assay As112/2 C_5–OVA was characterized for 3-C_6-SPC as a potential indicator of the contamination of water samples with SPCs. 3-C_6-SPC could be detected with a LOD of 0.13 nM (35 ng L^{-1}) and an IC_{50} value of 0.67 nM (181 ng L^{-1}). The calibration curve is shown in Figure 1 (solid symbol).

Discussion

Generic recognition of a chemical family of substances using analytical methods based on biomolecular interactions is an uncertain goal. The possibility of tailor-made bioreceptors has been a matter of investigation mainly by using theoretical chemistry tools or molecular bioengineering methods. Thus, immunochemical methods have often been exploited for their capability to direct antibody specificity through appropriate hapten design by using theoretical methods and models[20,36–39] or recombinant antibody methodologies.[40,41] However, as reported previously, addressing antibody recognition towards the common sulfonic group of the SPCs failed to produce generic antibodies.[23] The great differences in the antibody recognition of the different SPC congeners pointed to a significant contribution of the different sizes and geometries of the alkyl carboxylic moieties to the recognition event. In fact, it has been known since the early 1990s that electrostatic and hydrogen-bonding interactions contribute only a small fraction (ca. 20%) to the free energy of the recognition event (for $K_a = \approx 10^{10}\,M^{-1}$, $\Delta G = \approx 68\,kJ\,mol^{-1}$), while the so-called hydrophobic interactions contribute to the rest of the total energy (see ref. [42–44] and references cited therein), with an energy of about 100 J $Å^{-2}$ of the contact zone.[45] Thus, the main role of the electrostatic and hydrogen-bonding interactions seems to be to keep the two molecules in the correct orientation to allow short-range hydrophobic interactions to be established.

Attending to these precedents, we decided to investigate the potential of the heterologous immunization strategy devised by Masamune and coworkers[27,28,30] to raise antibodies with class-directed specificity against the SPC family. Contrary to homologous immunization procedures, in which only one type of immunizing hapten is used in each boosting injection, this strategy consists of successively immunizing the animal with two different but structurally related haptens. This approach has mainly been used in the catalytic antibody field for zwitterionic transition-state analogues with positive and negative charges, in which the chemical synthesis of the corresponding hapten could be problematic. Instead, heterologous immunization with two individual haptens, containing a different charge, provides the opportunity to simultaneously generate acidic and basic catalytic residues in the antibody-combining site. Thus, Ersoy et al.[46,47] designed three haptens to produce by heterelogous immunization nucleophile-mediated (phenol) amide bond-cleaving catalytic antibodies with a binding pocket with 1) a hydrophobic area, 2) an acidic residue complementary to the oxyanionic transition state, and 3) a basic residue to aid deprotonation of a phenol nucleophile and protonation of the departing amine. These examples point to the possibility of modulating the selectivity and affinity of the immunoresponse by combining immunoconjugates prepared by using two or more haptens without the need to invest time on complex synthetic procedures to preserve all the epitopes on the same hapten.

Hence, from a synthetic point of view, the preparation of SPC type-A and -B haptens was easier than trying to synthesize a single hapten that preserved both epitopes. The introduction of a linker, as in the case of type-A haptens, was easily possible using the corresponding *para*-chlorosulfonic acid derivatives. On the other hand, type-B haptens were conjugated through the carboxylic group of the target molecule. The only challenge was to select a bifunctional linker able to react with the chlorosulfonic group while introducing a chemical functionality that did not interfere with this reaction and was orthogonal to the functionality of the carboxylic group of the SPC. The thiol group complied with these requirements, although it could spontaneously oxidize into the disulfide form. For this reason, we envisaged the synthesis of type-A haptens as disulfide symmetric dimmers to keep this possibility under control. The formation of the symmetric dimers (2-C_3-S-SPC, 2-C_4-S-SPC, 2-C_5-S-SPC, 3-C_4-S-SPC, 3-C_5-S-SPC, 3-C_6-S-SPC, and 5-C_5-S-SPC) was achieved in acceptable yields following the synthetic strategy shown in Scheme 3. The synthesis of haptens as dimers, instead of protecting the thiol group, proved to be a good strategy to prepare hapten bioconjugates. The formation of a dimer allows the oxidation of the thiol group to be kept under control. Furthermore, release of the thiol group can take place under very mild conditions by using a DBPP polystyrene resin immediately before the bioconjugation, without needing to introduce a purification step. Although reduction did also occur in a very high yield using tributylphosphine in aqueous media, the use of the resin avoided the potential interference of the reducing agent in the conjugation step with the derivatized protein (BrA–protein). A lys/*N*-SMP (or *N*-SBrA)/hapten molar ratio of 1:2:4 produced the *X*-C_z-S-SPC protein conjugates with 3–8 hapten residues, although some MP and BrA residues remained. The strategy of synthesizing disulfide symmetric dimers as pre-haptens to further derivatize surfaces or biomolecules, as in described herein, can be of general applicability, especially if highly functionalized small organic molecules need to be coupled. Moreover, thiol groups can frequently be the functionality of choice for a biosensor or in nanobiotechnological applications that involve the use of silver or gold flat or nanostrutured surfaces.

Antibodies raised against $SPC_{mix\text{-}A}$-MP-HC provided the best immunochemical detectability, which indicated the great importance of the alkanoic moieties as antigenic determinants. From this point of view, pseudo-heterologous immunization procedures did not enhance the avidity of the antisera for the target analytes. In contrast, this strategy produced antibodies with a much broader recognition pattern. Thus, although there was better recognition of short-alkyl-chain SPCs of five and six carbon atoms, those with three and four carbon atoms were also recognized with 30–35% cross-reactivity. Similarly, long-alkyl-chain SPCs were recognized to a significant extent (45–40%). In contrast, antibodies raised against only type-A or -B haptens recognized 3-C_5- and 3-C_6-SPCs almost exclusively, while other short-alkyl-chain SPCs were only barely recognized. Although with some limitations, pseudo-heterologous immunization procedures may offer some advantages, thus overcoming the synthetic difficulties derived from preparing a complex hapten and preserving all the most important antigenic epitopes of a molecule. Moreover, it could be the right choice to produce generic (class-selective) antibodies, although the general applicability of this hypothesis should be proved with other antibodies against different target analytes.

Finally, the excellent features of the immunochemical method developed using As112/2-C_5-OVA, with a LOD of 35 ng L^{-1} and an IC_{50} value of 181 ng L^{-1} for 3-C_6-SPC, suggest the potential applicability of these antibodies as screening tools to detect the contamination of water samples by anionic surfactants using SPCs as indicators.

Experimental Section

Organic chemistry

Chemicals and instruments: Thin layer chromatography (TLC) was performed on 0.25-mm precoated silica gel. Unless otherwise indicated purification of the reaction mixtures was accomplished by flash chromatography using silica gel as the stationary phase. ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz or at 500 and 125 MHz, respectively, using trimethylsilane (TMS) as the internal reference. IR spectra were measured on a Bomen MB 120 FTIR spectrophotometer (Hartmann & Braun, Québec, Canada). High-resolution mass spectrometry (HRMS) by electronic impact (EI) was performed on a Micromass Autospec spectrometer (Unity of Mass Spectrometry, Universidad de Santiago de Compostela, Spain). The chemical reagents used for the synthesis of the haptens were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The six short-chain SPCs used as standards were prepared as previously described.[23]

Synthesis of haptens

General: The six short-chain SPCs were directly used as type-B haptens. The synthesis of type-A haptens is reported herein (see Scheme 3). The precursors **1**, **2**, **4**, and **7** were obtained from commercial sources, whereas **3**, **5**, and **6** were synthesized as previously described.[23] The spectroscopic characterization of the intermediates **1′–7′** and **1a–7a** can be found in the Supporting Information section.

Phenyl carboxylate esters (1′–7′): The phenyl carboxylic acids **1–5** were protected as butyl esters and phenyl carboxylic acids **6** and **7** as methyl esters. Briefly, the acid was dissolved in BuOH (10 equiv) or MeOH (10 equiv) in a round-bottom flask and few drops of H_2SO_4 were subsequently added. The reaction mixtures were left to stir at room temperature until the total disappearance of the starting material was observed by TLC. The reaction mixtures were treated with a solution of saturated $NaHCO_3$, and the products were extracted with Et_2O. The organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The obtained products were purified by flash chromatography on silica gel eluting with a hexane/Et_2O gradient to obtain them in a pure form.

General procedure for the preparation of the chlorosulfonyl derivatives (1a–7a): Chlorosulfonic acid (3 equiv) was added dropwise to a round-bottom flask fitted with a trap containing a 1 M NaOH solution and kept under argon. The ester **1′–7′** (1 equiv) was then slowly added to change the color of the solution from yellow to brown. The reaction mixture was left to stir at room temperature until the total disappearance of the starting material was observed by TLC. The crude product was poured onto ice/water and a yellow precipitate formed, which was extracted with hexane. The organic phase was washed with a saturated solution of $NaHCO_3$, dried over $MgSO_4$, filtered, and evaporated to dryness. The obtained yellow oil was then purified by flash chromatography on silica gel eluting with a hexane/Et_2O gradient to yield the corresponding 4-chlorosulfonylphenyl derivatives.

General procedure for the synthesis of the dimers 2-C_3-S-SPC, 2-C_4-S-SPC, 2-C_5-S-SPC, 3-C_4-S-SPC, 3-C_5-S-SPC, 3-C_6-S-SPC, and 5-C_5-S-SPC: Cystamine hydrochloride (0.5 equiv) was added to a solution of the corresponding chlorosulfonyl phenylalkanoic esters (**1a**–**7a**; 1 equiv) in anhydrous CH_2Cl_2 (final concentration: 0.05–0.08 M) in a round-bottom flask and stirred for few minutes. Triethylamine (2.1 equiv) was added, which initially produced an off-white suspension that rapidly became clear. As the reaction progressed, a white precipitate corresponding to $HNEt_3Cl$ appeared. When complete disappearance of the starting material was observed by TLC, the reaction mixture was dissolved on pouring into water and extracted with CH_2Cl_2, dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. The dimers obtained were purified by flash chromatography on silica gel eluting with a hexane/EtOAc gradient. Subsequently, the ester compounds were hydrolyzed in MeOH with 1 M KOH at room temperature until the total disappearance of the starting materials was observed by TLC. The MeOH was evaporated and the crude product was washed with Et_2O. The aqueous phase was acidified with concentrated HCl to pH 5.5, and a white precipitate was formed that was extracted with EtOAc. The organic phase was dried over $MgSO_4$, filtered, and evaporated to isolate the desired acids.

2,2′-Dithiobis[5-{4-(*N*-ethylsulfamoyl)}-2-phenyl propanoic acid] (2-C_3-S-SPC): Obtained in 40 mg, 70% yield. ^{1}H NMR (300 MHz, CD_3OD): $\delta=$ 1.49 (d, $J=7$ Hz, 6H), 2.65 (t, $J=7$ Hz, 4H), 3.13 (t, $J=6.5$ Hz, 4H), 3.84 (q, $J=7$ Hz, 2H), 7.53 (d, $J=8$ Hz, 4H), 7.81 (d, $J=8$ Hz, 4H) ppm; ^{13}C NMR (75 MHz, CD_3OD): $\delta=18.5$, 38.7, 43.1, 46.5, 128.3, 129.6, 140.5, 147.7, 165.4 ppm; IR (KBr): $\tilde{\nu}=3273$ (NH and OH st), 2981–2937 (C–H st), 1714 (C=O st), 1598 (ArC–C), 1319 ($-SO_2N$ st as), 1157 ($-SO_2N$ st si) cm^{-1}; HR-MS (ESI-TOF-MS): m/z calcd for $C_{22}H_{28}N_2NaO_8S_4$ [$M+Na^+$]: 599.0626; found: 599.0634.

2,2′-Dithiobis[5-{4-(*N*-ethylsulfamoyl)}-2-phenyl butanoic acid] (2-C_4-S-SPC): Obtained in 337 mg, 62% yield. ^{1}H NMR (300 MHz, CD_3OD): $\delta=$ 0.91 (t, $J=7$ Hz, 6H), 1.80 (ddq, $J=17.5$, 7.5, 7 Hz, 2H), 2.11 (ddq, $J=$ 15, 7.5, 7 Hz, 2H), 2.65 (t, $J=6$ Hz, 4H), 3.14 (t, $J=6.5$ Hz, 4H), 3.58 (t, $J=8$ Hz, 2H), 7.53 (d, $J=8.5$ Hz, 4H), 7.82 (d, $J=8$ Hz, 4H) ppm; ^{13}C NMR (75 MHz, CD_3OD): $\delta=12.5$, 27.6, 38.7, 43.1, 54.4, 128.3, 130.0, 140.6, 146.1, 176.7 ppm; IR (KBr): $\tilde{\nu}=3272$ (NH and OH st), 2968–2877 (C–H st), 1706 (C=O st), 1597 (ArC–C), 1322 ($-SO_2N$ st as), 1157 ($-SO_2N$ st si) cm^{-1}; HR-MS (ESI-TOF-MS): m/z calcd for $C_{24}H_{32}N_2NaO_8S_4$ [$M+Na^+$]: 627.0939; found: 627.0935.

2,2′-Dithiobis[5-{4-(*N*-ethylsulfamoyl}-]2-phenyl pentanoic acid] (2-C_5-S-SPC): Obtained as 600 mg, 68% yield. ^{1}H NMR (300 MHz, CD_3OD): $\delta=$ 0.92 (t, $J=7$ Hz, 6H), 1.29 (ddq, $J=14.5$, 8.5, 8 Hz, 4H), 1.75 (ddt, $J=$ 15.5, 7.5, 6 Hz, 2H), 2.04 (ddt, $J=15.5$, 7.5, 6 Hz, 2H), 2.65 (t, $J=6$ Hz, 4H), 3.14 (t, $J=6.5$ Hz, 4H), 3.68 (t, $J=8$ Hz, 2H), 7.53 (d, $J=8.5$ Hz, 4H), 7.82 (d, $J=8$ Hz, 4H) ppm; ^{13}C NMR (75 MHz, CD_3OD): $\delta=14.1$, 21.7, 36.6, 38.6, 43.0, 52.4, 128.2, 130.0, 140.4, 146.2, 176.7 ppm; IR (KBr): $\tilde{\nu}=3274$ (NH and OH st), 2960–2873 (C–H st), 1708 (C=O st), 1596 (ArC–C), 1322 ($-SO_2N$ st as), 1158 ($-SO_2N$ st si) cm^{-1}; HR-MS (ESI-TOF-MS): m/z calcd for $C_{26}H_{37}N_2O_8S_4$ [$M+H^+$]: 633.1433; found: 633.1444.

2,2′-Dithiobis[5-[4-(*N*-ethylsulfamoyl)]-3-phenyl butanoic acid] (3-C_4-S-SPC): (680 mg, 76% yield). ^{1}H NMR (300 MHz, CD_3OD): $\delta=1.32$ (d, $J=7$ Hz, 6H), 2.63 (d, $J=7.5$ Hz, 4H), 2.66 (t, $J=6$ Hz, 4H), 3.13 (t, $J=$ 6.5 Hz, 4H), 3.31 (tq, $J=7$ Hz, $J=7$ Hz, 2H), 7.48 (d, $J=8$ Hz, 4H), 7.78 (d, $J=8$ Hz, 4H) ppm; ^{13}C NMR (75 MHz, CD_3OD): $\delta=22.4$, 37.6, 38.7, 43.0, 43.5, 128.3, 128.9, 139.7, 152.7, 175.7 ppm; IR (KBr): $\tilde{\nu}=3276$ (NH and OH st), 2968–2873 (C–H st), 1708 (C=O st), 1603 (ArC–C), 1322 ($-SO_2N$ st as), 1157 ($-SO_2N$ st si) cm^{-1}; HR-MS (ESI-TOF-MS): m/z calcd for $C_{24}H_{32}N_2NaO_8S_4$ [$M+Na^+$]: 627.0939; found: 627.0933.

2,2′-Dithiobis[5-{4-(*N*-ethylsulfamoyl)}-3-phenyl pentanoic acid] (3-C_5-S-SPC): Obtained as 630 mg, 67% yield. ^{1}H NMR (300 MHz, CD_3OD): $\delta=$ 0.78 (t, $J=7$ Hz, 6H), 1.63 (ddq, $J=13.5$, 7.5, 6.5 Hz, 2H), 1.77 (ddq, $J=$ 13.5, 7.5, 6.5 Hz, 2H), 2.60 (dd, $J=15.5$, 9 Hz, 2H), 2.66 (t, $J=6$ Hz, 4H), 2.72 (dd, $J=15.5$, 6.5 Hz, 2H), 3.09 (tt, $J=9$, 6 Hz, 2H), 3.15 (t, $J=$ 6.5 Hz, 4H), 7.44 (d, $J=8.5$ Hz, 4H), 7.80 (d, $J=8.5$ Hz, 4H) ppm; ^{13}C NMR (75 MHz, CD_3OD): $\delta=12.2$, 30.0, 38.7, 41.5, 42.9, 44.9, 128.1, 129.6, 139.5, 150.9, 175.8 ppm; IR (KBr): $\tilde{\nu}=3525$ (OH st), 3270 (NH st), 2963–2875 (C–H st), 1710 (C=O st), 1598 (ArC–C), 1324 ($-SO_2N$ st as), 1159 ($-SO_2N$ st si) cm^{-1}; HR-MS (ESI-TOF-MS): m/z calcd for $C_{26}H_{36}N_2NaO_8S_4$ [$M+Na^+$]: 655.1252; found: 655.1242.

2,2′-Dithiobis[5-{4-(*N*-ethylsulfamoyl)}-3-phenyl hexanoic acid] (3-C_6-S-SPC): Obtained as 64 mg, 25% yield. ^{1}H NMR (300 MHz, CD_3OD): $\delta=$ 0.87 (t, $J=7$ Hz, 6H), 1.17 (tq, $J=7$, 7 Hz, 4H), 1.66 (m, 4H), 2.58 (dd, $J=16$, 8.5 Hz, 2H), 2.65 (t, $J=6$ Hz, 4H), 2.70 (dd, $J=16$, 6.5 Hz, 2H), 3.14 (t, $J=6.5$ Hz, 4H), 3.19 (tt, $J=8.5$, 6 Hz, 2H), 7.44 (d, $J=8.5$ Hz, 4H), 7.79 (d, $J=8.5$ Hz, 4H) ppm; ^{13}C NMR (75 MHz, CD_3OD): $\delta=$ 14.3, 21.5, 38.6, 39.3, 42.0, 43.0, 43.1, 128.2, 129.6, 139.7, 151.2, 175.7 ppm; IR (KBr): $\tilde{\nu}=3526$ (OH st), 3271 (NH st), 2963–2873 (C–H st), 1710 (C=O st), 1598 (ArC–C), 1324 ($-SO_2N$ st as), 1160 ($-SO_2N$ st si) cm^{-1}; HR-MS (ESI-TOF-MS): m/z calcd for $C_{28}H_{40}N_2NaO_8S_4$ [$M+Na^+$]: 683.1565; found: 683.1585.

2,2′-Dithiobis[5-{4-(*N*-ethylsulfamoyl)}phenyl pentanoic acid] (5-C_5-S-SPC): Obtained as 102 mg, 37% yield. ^{1}H NMR (300 MHz, CD_3OD): $\delta=$ 1.67 (m, 8H), 2.33 (t, $J=7$ Hz, 4H), 2.64 (t, $J=7$ Hz, 4H), 2.73 (t, $J=$ 7 Hz, 2H), 3.12 (t, $J=6.5$ Hz, 4H), 7.40 (d, $J=8.5$ Hz, 4H), 7.76 (d, $J=$ 8.5 Hz, 4H) ppm; ^{13}C NMR (75 MHz, CD_3OD): $\delta=25.3$, 31.7, 34.7, 36.3, 38.7, 43.1, 128.2, 130.3, 139.2, 149.2, 179.7 ppm; IR (methyl ester) (KBr): $\tilde{\nu}=3280$ (-NH- st), 2948–2864 (C–H st), 1731 (C=O st), 1598 (ArC–C), 1328 ($-SO_2N$ st as), 1159 ($-SO_2N$ st si) cm^{-1}; HR-MS (ESI-TOF-MS): m/z calcd for $C_{26}H_{37}N_2O_8S_4$ [$M+H^+$]: 633.1433; found: 633.1415.

Immunochemistry

Chemicals and immunochemicals: Di(*n*-butyl)phenylphosphine polystyrene resin (DBPP; with a derivatization of 0.5 mmol g_{resin}^{-1}) was purchased from NovaBiochem (Läufelfingen, Switzerland). Specific immunoreagents (antibodies and protein and enzyme conjugates) were prepared as described below. The SPC standards and the phenyl carboxylic acids used in the cross-reactivity studies were synthesized as described previously.[23] LAS standards were a gift from PETRESA (Cádiz, Spain). The heterobifunctional cross-linkers *N*-succinimidyl-3-maleimidyl propanoate (*N*-SMP) and *N*-succinimidyl bromoacetate (*N*-SBrA) used to prepare the protein conjugates were synthesized following the procedures described by Nielsen and Buchardt[48] and Bernatowicz and Matsueda,[49] respectively. Short-alkyl-chain SPCs and the corresponding immunoreagents were used as mixtures in some experiments. Thus, screening experiments to find out the best immunoassay conditions were carried out using SPC_{mix} as an equimolar mixture of 2-C_3-SPC, 2-C_4-SPC, 2-C_5-SPC, 3-C_4-SPC, 3-C_5-SPC, and 3-C_6-SPC. Immunization protocols were performed with mixtures of hapten–protein conjugates. The term $SPC_{mix\text{-}A}$-MP (or -CH_2)-protein designates an equimolar mixture of six immunogens prepared by covalent attachment of the type-A haptens (2-C_3-S-SPC, 2-C_4-S-SPC, 2-C_5-S-SPC, 3-C_4-S-SPC, 3-C_5-S-SPC, and 3-C_6-S-SPC) to the protein, through the corresponding *N*-SMP or *N*-SBrA cross linkers, respectively. Similarly, the term $SPC_{mix\text{-}B}$–protein defines an equimolar mixture of six immunogens prepared by covalent attachment of type-B haptens (2-C_3-SPC, 2-C_4-SPC, 2-C_5-SPC, 3-C_4-SPC, 3-C_5-SPC, and 3-C_6-SPC) to the protein. Finally, an equimolar mixture of the twelve immunogens prepared with both types of haptens have been designated as $SPC_{mix\text{-}AB}$–protein. The buffers used can be found in the Supporting Information.

Preparation of the immunoreagents using type-A haptens: Haptens 2-C_3-S-SPC, 2-C_4-S-SPC, 2-C_5-S-SPC, 3-C_4-S-SPC, 3-C_5-S-SPC, and 3-C_6-S-SPC were conjugated to HC, BSA, CONA, and OVA with *N*-SMP or *N*-SBrA through a three-step procedure (see Scheme 4). All the bioconjugates (hapten-MP-protein or hapten-CH_2-protein and MP–protein or BrA–protein) were purified by dialysis against 0.5 mM phosphate-buffered saline (PBS; 4×5 L) and MilliQ water (1×5 L) and stored freeze dried at −40 °C. Stock solutions of 1 mg mL^{-1} were prepared in PBS buffer and stored in aliquots at −40 °C. Working aliquots were stored at 4 °C in 10 mM PBS at 1 mg mL^{-1}.

Step 1: Reduction of the dimer to generate the thiol group: The DBPP resin (25 mg, 12.5 μmol of reducing agent, 1.25 equiv) was added to a solution of the dimers (10 μmol) in anhydrous DMF (160 μL) and kept under argon. The reaction mixture was gently stirred at room temperature for 2 h. MilliQ water (40μL) was then added to the suspension, which was stirred for 1 h more until complete reduction of the dimer was observed using the Ellman test.[34] The suspension was then filtered through a syringe provided with a filter (0.45-μm porous size). The re-

tained resin was washed with water (2×120 μL). Finally, the collected fractions were combined and used immediately for conjugation (step 3).

Step 2: Activation of the protein: Simultaneously to step 1, the cross-linkers *N*-SMP (or *N*-SBrA, 20 μmol) in anhydrous DMF (100 μL) were added dropwise to a solution of the protein (HC, BSA, OVA, or CONA; 20 mg) in PBS (1.5 mL). The reaction mixtures were stirred at room temperature for 2 h and then purified by passing the solution through a Sephadex G-25 desalting column and eluting with PBS buffer. The fractions containing the protein conjugate (MP–protein or BrA–protein, depending on the cross-linker used) were collected, combined, and then split in two equal volumes. One of them was used for conjugation with the hapten and the other one was stored separately for further inspection of this reaction step by MALDI-TOF-MS.

Step 3: Coupling of the hapten to the protein: The solutions of the reduced haptens (ca. 20 μmol) obtained in step 1 were added to the derivatized proteins (10 mg) prepared in step 2. The reaction mixtures were stirred for 2 h at room temperature to obtain the corresponding hapten-MP-protein or hapten-CH_2-protein conjugates depending on the cross-linker used.

Preparation of the immunoreagents using type-B haptens: Haptens 2-C_3-SPC, 2-C_4-SPC, 2-C_5-SPC, 3-C_4-SPC, 3-C_5-SPC, 3-C_6-SPC, 5-C_5-SPC, and 9-C_9-SPC were coupled to OVA through the carboxylic group by using the active ester method following a standard methodology[50] by activating the haptens (10 μmol) with *N*,*N*′-dicyclohexylcarbodiimide (DCC; 50 μmol) and *N*-hydroxysuccinimide ester (NHS; 25 μmol) in anhydrous DMF (200 μL) and adding the protein (10 mg) in a borate buffer (1.8 mL). A second batch of type-B BSA conjugates was prepared using EDC[51] (see the Supporting Information).

Hapten density analysis: The hapten densities were calculated by MALDI-TOF mass spectrometric analysis by determining the molecular weight (see the Supporting Information).

Polyclonal antisera: As112, As113, and As114 (type-A antibodies) were obtained by immunizing female white New Zealand rabbits weighting 1–2 kg with $SPC_{mix\text{-}A}$-MP-HC following an already described protocol.[52] The preparation of As115, As 116, and As117 (type-B antibodies) using $SPC_{mix\text{-}B}$–HC (conjugated by the mixed anhydride (MA) method) has been described previously.[23] As118, As119, and As120 (type-AB antibodies) were obtained in the same way but combining type-A and -B haptens in an equimolar mixture of the twelve immunogens ($SPC_{mix\text{-}AB}$–HC): six of type A ($SPC_{mix\text{-}A}$-MP-HC) and six of type B ($SPC_{mix\text{-}B}$–HC; MA method). The evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the antisera to microtiter plates coated with an equimolar mixture of the corresponding BSA conjugates ($SPC_{mix\text{-}A}$-MP-BSA and $SPC_{mix\text{-}AB}$–BSA). After an acceptable antibody titer was observed, the animals were exsanguinated and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at −40 °C in the presence of 0.02 % NaN_3.

Evaluation of the antibody features: The detectability and specificity of the antibodies raised by homologous and heterologous immunization procedures were assessed by determining the IC_{50} values, the LOD values, and the selectivities of the competitive ELISAs developed for each antibody type.

ELISA general protocol: The plates were coated with the antigens (100 μL well^{-1} in coating buffer) overnight at 4 °C and covered with adhesive plate sealers. The next day, the plates were washed four times with phosphate-buffered saline containing Tween-20 (PBST; 300 μL well^{-1}), and solutions of the analyte (50 μL well^{-1} in PBST; only PBST for zero analyte) and the antisera (50 μL well^{-1} in PBST) were added and incubated for 30 min at room temperature. The plates were washed as before, and a solution of antiIgG–HRP (1:6000 in PBST) was added to the wells (100 μL well^{-1}) and incubated for a further 30 min at room temperature. The plates were washed again, and the substrate solution was added (100 μL well^{-1}). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL well^{-1}), and the absorbances were read at 450 nm. The standard curve obtained was fitted to a four-parameter logistic equation according to the following formula: $Y=\{(A-B)/1-(x/C)^D\}+B$, 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.

Optimized indirect ELISAs: Microtiter plate assays were established for each type of antibody using the best As/coating antigen (CA) combinations. Thus, for the antibodies raised against $SPC_{mix\text{-}A}$-MP-HC, As112 (1:500) was used on plates coated with 2-C_5-OVA (0.02 μg mL^{-1}). For the case of the antibodies prepared using $SPC_{mix\text{-}B}$–HC, As115 (1:2000) was used in combination with 2-C_5-S-OVA (1.25 μg mL^{-1}). Finally, for the antibodies raised using the whole mixture $SPC_{mix\text{-}AB}$–HC, the As119 (1:4000) combined with 2-C_3–BSA (0.31 μg mL^{-1}) gave the best results. The microplates were processed as described in the general protocol. For the combination As112/2-C_5-OVA, PBST used at the competitive step contained 1 % BSA. The SPC_{mix} was used as the standard analyte. In some experiments performed using ELISA with As112/2-C_5-OVA, only 3-C_6-SPC was used as the standard analyte.

Specificity studies: Stock solutions of each SPC and several structurally related compounds were prepared in H_2O or dimethyl sulfoxide (DMSO)/MeOH (6 mM) and stored at 4 °C. Standard curves were prepared in PBST by serial dilution (30 μM–64 pM). Each IC_{50} value was determined in the competitive experiments following the optimized protocol. The cross-reactivity values were calculated according to the equation $[IC_{50}(SPC)/IC_{50}(\text{cross-reactant})]\times 100$.[53]

Acknowledgements

This study was supported by the Ministry of Science and Technology (contract numbers: AGL2004–20341-E and NAN2004–09415-C05–02) and by the European Community (NMP2-CT2003–505485). We thank PETRESA for providing samples of the technical LASs. The AMR group is a Grup de Recerca de la Generalitat de Catalunya and receives support from the Departament d′Universitats, Recerca i Societat de la Informació la Generalitat de Catalunya (expedient 2005SGR 00207).

[1] A. Oubiña, B. Ballesteros, P. Bou, R. Galve, J. Gascón, F. Iglesias, N. Sanvicens and M.-P. Marco in *Immunoassays for Environmental Analysis, Vol. 21* (Ed.: D. Barceló), Elsevier, Amsterdam, The Netherlands, **2000**, pp. 289–340.
[2] Y. Xu, N. Yamamoto, K. D. Janda, *Bioorg. Med. Chem.* **2004**, *12*, 5247–5268.
[3] J. Emneus in *Integrated Immuno Extraction Sampling and Portable Biosensor Prototype for In-field Monitoring,* EC, Environmental and Climate Program ENV4-CT97–0476, Lund **1997–2000**.
[4] P. Schöberl, *Tenside Surfactants Deterg.* **1989**, *26*, 86–94.
[5] T. P. Knepper, M. Kruse, *Tenside Surfactants Deterg.* **2000**, *37*, 41–47.
[6] A. Marcomini, G. Pojana, A. Sfriso, J. M. Quiroga, *Environ. Toxicol. Chem.* **2003**, *19*, 2000–2007.
[7] V. Andreu, Y. Picó, *Anal. Chem.* **2004**, *76*, 2878–2885.
[8] V. M. Leon, A. Gomez-Parra, E. Gonzalez-Mazo, *Environ. Sci. Technol.* **2004**, *38*, 2359–2367.
[9] J. A. Perales, M. A. Manzano, D. Sales, J. M. Quiroga, *Int. Biodeterior. Biodegrad.* **2003**, *51*, 187–194.
[10] M. Mosche, U. Meyer, *Water Res.* **2002**, *36*, 3253–3260.
[11] J. Vives-Vego, R. López-Amorós, T. Guindulain, M. T. García, J. Comas, J. Sánchez-Leal, *J. Surfactants Deterg.* **2000**, *3*, 303–308.
[12] P. Eichhorn, S. V. Rodrigues, W. Baumann, T. P. Knepper, *Sci. Total Environ.* **2002**, *284*, 123–134.
[13] P. Eichhorn, M. E. Flavier, M. L. Paje, T. P. Knepper, *Sci. Total Environ.* **2001**, *269*, 75–85.
[14] E. Gonzalez-Mazo, V. M. Leon, M. Saez, A. Gomez-Parra, *Sci. Total Environ.* **2002**, *288*, 215–226.
[15] W.-H. Ding, J.-H. Lo, S.-H. Tzing, *J. Chromatogr. A* **1998**, *818*, 270–279.

[16] P. Eichhorn, T. P. Knepper, F. Ventura, A. Diaz, *Water Res.* **2002**, *36*, 2179–2186.
[17] E. Gonzalez-Mazo, M. Honing, D. Barcelo, A. Gomez-Parra, *Environ. Sci. Technol.* **1997**, *31*, 504–510.
[18] C. A. Spinks, G. M. Wyatt, H. A. Lee, M. R. A. Morgan, *Bioconjugate Chem.* **1999**, *10*, 583–588.
[19] P. Cliquet, E. Cox, W. Haasnoot, E. Schacht, B. M. Goddeeris, *J. Agric. Food Chem.* **2003**, *51*, 5835–5842.
[20] C. A. Spinks, G. M. Wyatt, S. Everest, R. Jackman, M. R. A. Morgan, *J. Sci. Food Agric.* **2002**, *82*, 428–434.
[21] P. Degelmann, J. Wenger, R. Niessner, D. Knopp, *Environ. Sci. Technol.* **2004**, *38*, 6795–6802.
[22] V. Kolar, A. P. Deng, M. Franek, *Food Agric. Immunol.* **2002**, *14*, 91–105.
[23] M.-C. Estevez, R. Galve, F. Sanchez-Baeza, M.-P. Marco, *Anal. Chem.* **2005**, *77*, 5283–5293.
[24] T. Korpimaki, V. Hagren, E. C. Brockmann, M. Tuomola, *Anal. Chem.* **2004**, *76*, 3091–3098.
[25] T. Korpimaki, J. Rosenberg, P. Virtanen, T. Karskela, U. Lamminmaki, M. Tuomola, M. Vehniainen, P. Saviranta, *J. Agric. Food Chem.* **2002**, *50*, 4194–4201.
[26] T. Korpimaki, J. Rosenberg, P. Virtanen, U. Lamminmaki, M. Tuomola, P. Saviranta, *Protein Eng.* **2003**, *16*, 37–46.
[27] T. Tsumuraya, H. Suga, S. Meguro, A. Tsunakawa, S. Masamune, *J. Am. Chem. Soc.* **1995**, *117*, 11390–11396.
[28] H. Suga, O. Ersoy, S. F. Williams, T. Tsumuraya, M. N. Margolies, A. J. Sinskey, S. Masamune, *J. Am. Chem. Soc.* **1994**, *116*, 6025–6026.
[29] O. Ersoy, R. Fleck, A. Sinskey, S. Masamune, *J. Am. Chem. Soc.* **1996**, *118*, 13077–13078.
[30] T. Tsumuraya, N. Takazawa, A. Tsunakawa, R. Fleck, S. Masamune, *Chem. Eur. J.* **2001**, *7*, 3748–3755.
[31] T. W. Green, P. G. M. Wuts, *Protective Groups in Organic Synthesis*, Wiley, Canada, **1999**.
[32] S. S. Wong, *Chemistry of Protein Conjugation and Cross-linking*, CRC Press, Boca Raton, Fl, **1993**.
[33] H. T. Clarke, G. S. Babcock, T. F. Murray, *Organic Synthesis, Vol. I*, **1932**.
[34] P. W. Riddles, R. L. Blakeley, B. Zerner, *Anal. Biochem.* **1979**, *94*, 75–81.
[35] J. T. Ayers, S. R. Anderson, *Synth. Commun.* **1999**, *29*, 351–358.
[36] B. Ballesteros, D. Barcelo, F. Sanchez-Baeza, F. Camps, M. P. Marco, *Anal. Chem.* **1998**, *70*, 4004–4014.
[37] R. Galve, F. Camps, F. Sanchez-Baeza, M. P. Marco, *Anal. Chem.* **2000**, *72*, 2237–2246.
[38] N. Sanvicens, F. Sanchez-Baeza, M. P. Marco, *J. Agric. Food Chem.* **2003**, *51*, 3924–3931.
[39] M. C. Estevez, M. Kreuzer, F. Sanchez-Baeza, M. P. Marco, *Environ. Sci. Technol.* **2006**, *40*, 559–568.
[40] J. Brichta, M. Hnilova, T. Viskovic, *Vet. Med.* **2005**, *50*, 231–252.
[41] W. Kusharyoto, J. Pleiss, T. T. Bachmann, R. D. Schmid, *Environ. Prot. Eng.* **2002**, *15*, 233–241.
[42] V. G. Omelyaneko, W. Jiskoot, J. N. Herron, *Biochemistry* **1993**, *32*, 10423–10429.
[43] C. J. van Oss, *Mol. Immunol.* **1995**, *32*, 199–211.
[44] J. N. Herron, D. M. Kranz, D. M. Jameson, E. W. J. Voss, *Biochemistry* **1986**, *25*, 4602–4609.
[45] G. Zeder-Lutz, N. Rauffer, D. Altschuh, M. H. V. Van Regenmortel, *J. Immunol. Methods* **1995**, *189*, 131–140.
[46] O. Ersoy, R. Fleck, M. J. Blanco, S. Masamune, *Bioorg. Med. Chem.* **1999**, *7*, 279–286.
[47] O. Ersoy, R. Fleck, A. Sinskey, S. Masamune, *J. Am. Chem. Soc.* **1998**, *120*, 817–818.
[48] O. Nielsen, O. Buchardt, *Synthesis* **1991**, 819–821.
[49] M. S. Bernatowicz, G. R. Matsueda, *Anal. Biochem.* **1986**, *155*, 95–102.
[50] J. Gascón, A. Oubiña, B. Ballesteros, D. Barceló, F. Camps, M.-P. Marco, M.-A. González-Martinez, S. Morais, R. Puchades, A. Maquiera, *Anal. Chim. Acta* **1997**, *347*, 149–162.
[51] C. Wittmann, B. Hock, *J. Agric. Food Chem.* **1991**, *39*, 1194–1200.
[52] B. Ballesteros, D. Barceló, F. Camps, M.-P. Marco, *Anal. Chim. Acta* **1997**, *347*, 139–147.
[53] Spectroscopic data (^{1}H and ^{13}C NMR, IR, and high-resolution mass spectra) of most of the intermediates obtained during the SPCs synthesis, results from the screening of the different antisera raised regarding the recognition of SPC_{mix}, and the immunochemical performance of the antibodies under different physicochemical conditions are given in the Supporting Information.

Received: August 7, 2007
Published online: December 10, 2007

