Reactive immunization elicits catalytic antibodies for polyester hydrolysis

Da-Wei Chen, Robert J. Kubiak, Jon A. Ashley and Kim D. Janda *

Department of Chemistry, The Scripps Research Institute and the Skaggs Institute for Chemical Biology, 10550 N. Torrey Pines Road, La Jolla, California 92037, USA. E-mail: kdjanda@scripps.edu

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In the search for biocatalysts for degradation of nonnatural polymers, reactive immunization with haptens **7** and **11** was used to prepare catalytic antibodies capable of cleaving short oligomeric esters, as well as the insoluble polyester **25**. These antibodies were found to be highly specific and efficient esterases for oligomers. Triester **24** was preferentially hydrolyzed by an *endo*-cleavage pathway, however, with a higher molecular weight polymer **25** no site specificity could be observed. Catalytic efficiency of the antibodies towards the insoluble polymer **25** was limited due to physical constraints.

Introduction

Accumulation of man-produced waste is a major concern from the standpoint of natural environmental protection. Chemical and engineering communities are actively pursuing inexpensive and energy efficient methods for degradation of synthetic polymers. In this respect, the most promising are biological systems involving microorganisms and/or enzymes.[1] However, it is generally difficult to engineer a biological system capable of handling nonnatural polymers.[2]

Antibodies can be easily designed to bind most organic substances with high affinity and specificity.[3] An antibody capable of high-affinity binding to a transition state of a chemical reaction is by definition a catalyst for this reaction.[4] We and others have reported preparation of catalytic antibodies raised against phosphorus-based transition-state analog haptens which efficiently catalyze hydrolysis of ester, carbonate, carbamate and amide bonds.[5,6] In a previous communication, we described catalytic antibodies which degraded oligomeric esters.[7] These antibodies were raised against a phosphorodithioate transition-state analog originally designed for hydrolysis of aromatic carbonates. Further screening revealed their catalytic properties for oligoester hydrolysis. The most efficient antibody, OB2-48F8, cleaved a diester with $k_{cat} = 2.2 \times 10^{-2}$ min^{-1}, $K_m = 580$ μM and $k_{cat}/k_{uncat} = 1.5 \times 10^3$. This antibody was also found to cleave a sparingly soluble oligoester with release of shorter fragments. Hydrolysis of oligo- and polymeric materials is a new feature of antibody catalysis. Yet, because this capability was discovered partially by accident, we decided to obtain *de novo* catalysts by rational design for hydrolysis of a specific polymeric target. We decided to raise the desired antibodies by reactive immunization[8] which usually yields catalysts superior to those obtained by standard hapten manifolds such as transition-state analogs, "bait-and-switch" or heterologous immunization.[9–11] Based on our previous experience of hapten design and synthesis, we chose 1,4-phenylenesulfonyl-1,4-phenyleneoxycarbonyl-1,3-phenylenecarbonyl polyester **25** as our target. The reactive immunization technique utilizes a reactive hapten, which under physiological conditions may undergo hydrolysis or covalently bind to a B-cell receptor, therefore combining chemical reactivity with transition-state stabilization. In this work we report catalytic antibodies for hydrolysis of ester bonds which can also cleave insoluble polyesters with vastly improved kinetic parameters.

Results and discussion

We prepared two reactive phosphonate diester haptens **7** and **11** (Fig. 1), which differ structurally by the presence of a methylene group between the phosphorus atom and the phenyl benzoate moiety. The main rationale for introduction of the methylene spacer was to improve hydrolytic stability of the reactive hapten. The half-lives of haptens **7** and **11** under physiological conditions were estimated to be about six and twenty four hours, respectively suggesting that the immune response would be directed against the original phosphonate diester, as well as the phosphonate monoester resulting from its hydrolysis. Both haptens were conjugated with the carrier protein keyhole limpet hemocyanin and used to immunize 129Gix$^+$ mice. Monoclonal antibodies (mAbs) were obtained and purified by standard hybridoma techniques.[12–14]

From a single hybridoma fusion, hapten **7** gave rise to 15 mAbs, while a panel of 25 mAbs was obtained with hapten **11**. All antibodies were screened for catalysis by HPLC using triester **24** as a substrate (Fig. 2). A relative activity of each antibody was estimated by comparing the combined signal intensity of all released products. Several active catalysts were found in the first set of antibodies with mAb DC2-5F4 being the most efficient ($V_0 = 4.8 \times 10^{-6}$ M min^{-1} at 1×10^{-6} M antibody and 50 μM **24**) and as such chosen for further evaluation. The antibodies from the second panel were generally much less proficient and the most active mAb, DC3-9B4, showed roughly 10-fold lower activity than DC2-5F4. It should also be noted that all antibodies selected for in depth kinetic analysis demonstrated multiple turnovers and are thus true catalysts. Since substrate **24** has three distinct cleavage sites, there are several hydrolytic pathways by which it can be decomposed to the component building blocks, with one major pathway being preferred (Fig. 3). HPLC analysis of the time course of the reaction consistently showed accumulation of the AB fragment (Compound **21**, Fig. 2. See also Fig. 3 and Table 1, footnote *a* for explanations). At the same time, neither of the trimer-like diester products ABC or BCD could be detected, nor the BC fragment arising from hydrolytic cleavage of the triester **24** on both ends. Therefore, it appears that in the reaction catalyzed by DC2-5F4 an *endo*-cleavage pathway predominates, yet judging from the hapten structure an *exo*-cleavage with release of the phenol A was initially anticipated.

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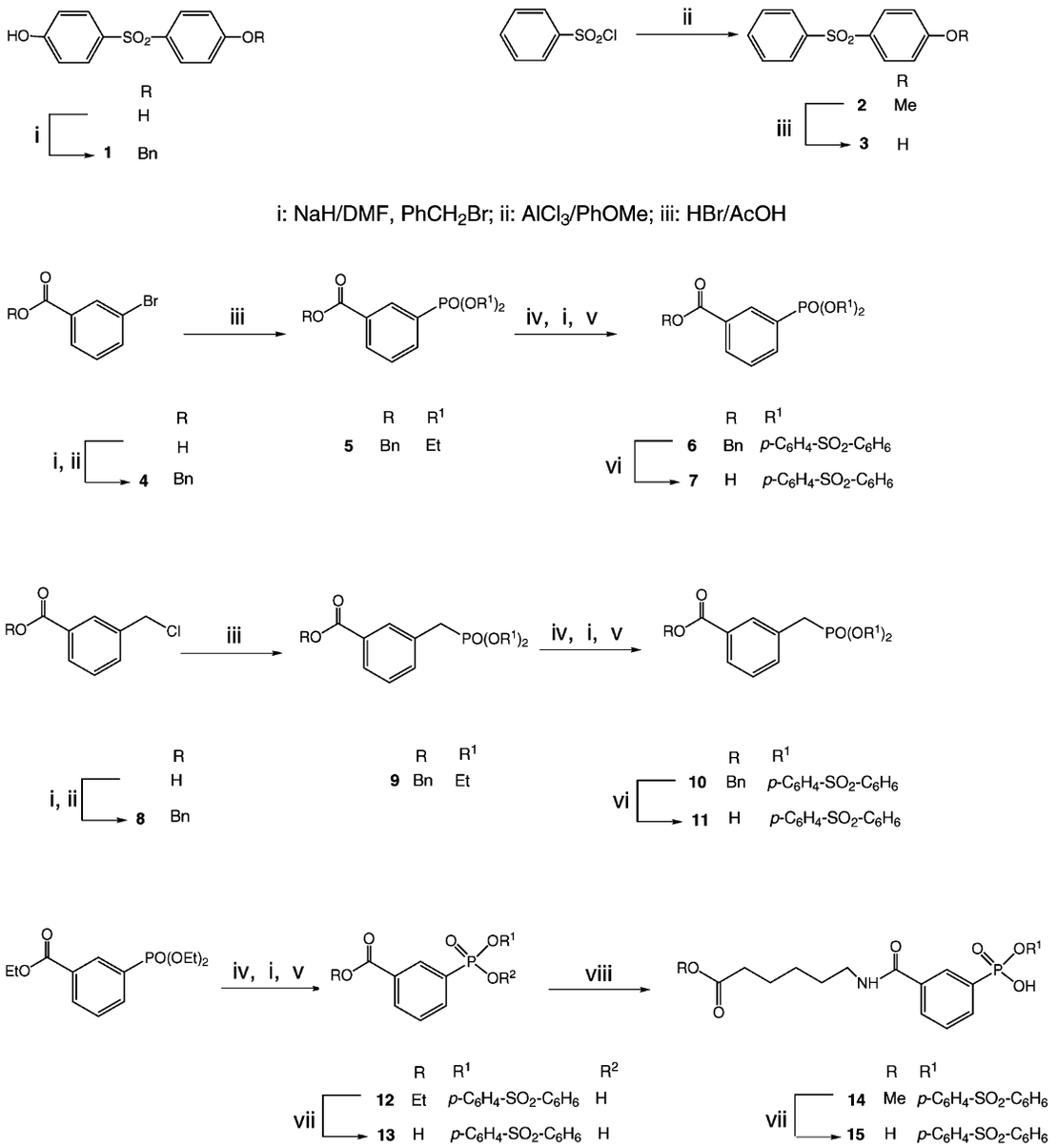

Fig. 1 Synthesis of reactive haptens **7**, **11** and compound **15**.

Due to the limited solubility of **24** under the assay conditions (up to 50 μM in 50 mM MOPS buffer, pH 7.08 with 10% DMSO as a co-solvent) and multiple reaction products, the kinetic evaluation of DC2-5F4 was performed using monoesters as substrates (Table 1). Although DC2-5F4 is the most efficient catalyst for hydrolysis of the CD ester (**23**) providing almost 1.7×10^4-fold rate enhancement, the antibody is most specific for the AB ester, which is indicated by the low K_m value and high specificity coefficient k_{cat}/K_m for this reaction. Since the structure of the AB subunit is most congruent to the hapten **7**, it is reasonable to expect high specificity of the antibody when this particular subunit is used as a substrate. It is less obvious however, why hydrolysis of the triester **24** by mAb DC2-5F4 should result in predominant formation of the AB fragment. One possible explanation is that immunization with the very bulky phosphonate diester causes formation of an extensive active site, which in turn may allow alternative modes for substrate binding. Hence, the triester **24** apparently binds in such a conformation, perhaps somewhat folded, that promotes the observed ester cleavage by the catalytic machinery of the antibody.

The high k_{cat}/k_{uncat} values observed for all substrates studied indicate that the antibody DC2-5F4 is a very efficient esterase. Wolfenden [15] defined the proficiency constant of a biocatalyst as a ratio of the second-order rate specificity constant to the rate constant of the uncatalyzed reaction. Proficiency constants for DC2-5F4 are 1.80, 1.85 and 26×10^9 M^{-1} for the AB, BC and CD substrates, respectively. These values are comparable with those reported for natural lipases and esterases with the proficiency constants in the range of 10^9–10^{12} M^{-1}.[16]

Reactive immunization was designed to induce an amino-acid residue with a nucleophilic side chain (*e.g.* Cys, Ser, His, Thr or Tyr) in the binding site of the resulting antibody.[8] Such an antibody presumably should catalyze hydrolytic reactions by formation of a covalent intermediate with its substrate. Also, antibodies obtained by reactive immunization should be able to efficiently hydrolyze the original hapten with possible irreversible inactivation in the process. Hapten **7** is rapidly decomposed by mAb DC2-5F4 with an observed rate constant $k_{obsr} = 1$ min^{-1} (data not shown). The time course of reaction shows a characteristic initial burst of the phenol product which indicates that indeed the antibody may be covalently modified.

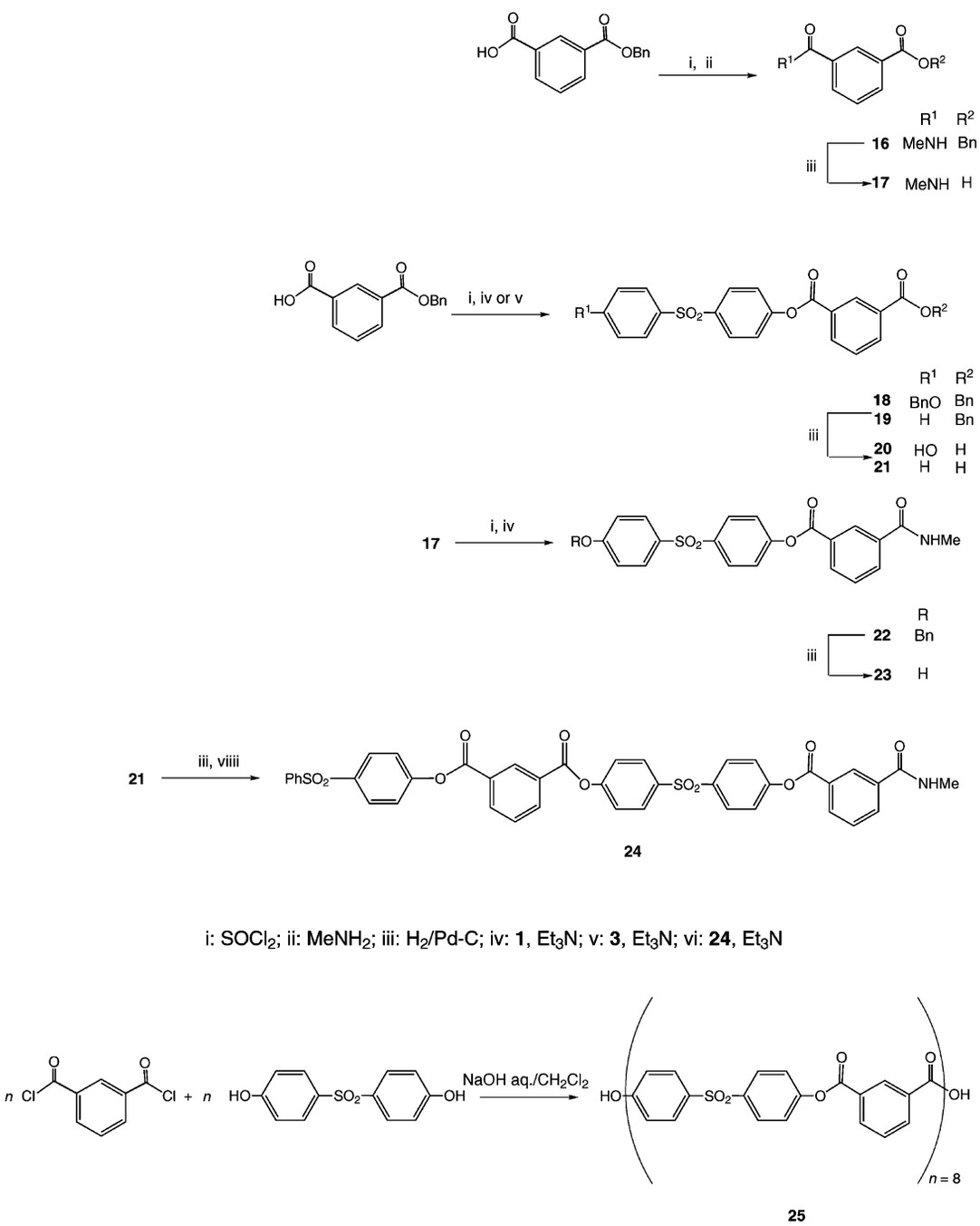

Fig. 2 Synthesis of the assay substrates.

Table 1 Kinetic parameters for mAbs DC2-5F4 and DC2-8E6

Substrate[a]	K_m/μM		k_{cat}/min^{-1}		$k_{cat}/k_{uncat} \times 10^{-4}$	
	DC2-8E6	DC2-5F4	DC2-8E6	DC2-5F4	DC2-8E6	DC2-5F4
AB	25	<2[b]	0.88	~0.72[c]	6.52	~5.3[c]
BC	5.8	23	0.3	0.66	1.88	4.14
CD	27	92	1.3	3.9	5.65	17.0

[a] AB: Compound **21**; BC: Compound **20**; CD: Compound **23**. [b] Below detection limit. [c] Since the K_m value was too low to measure directly, the k_{cat} was estimated from the initial velocity under saturating conditions.

Based on the premise of reactive immunization, one would expect that the phosphonate decomposition product of **7** can be inhibitory for the esterase reactions. Phosphonate monoester **15** was found to be a competitive inhibitor (K_i = 0.33 μM) of DC2-5F4 when tested for hydrolysis of the CD ester. However, fragment A and fragment C were also found to be competitive inhibitors of this reaction with inhibition constants K_i = 6 μM and 1.5 μM, respectively. Neither isophthalic nor isophthalamic acids† were found to have any significant inhibitory properties. These results indicate that reactions catalyzed by DC2-5F4 are strongly product inhibited by the phenolic leaving groups. It

† The IUPAC name for isophthalamic acid is *m*-carbamoylbenzoic acid.

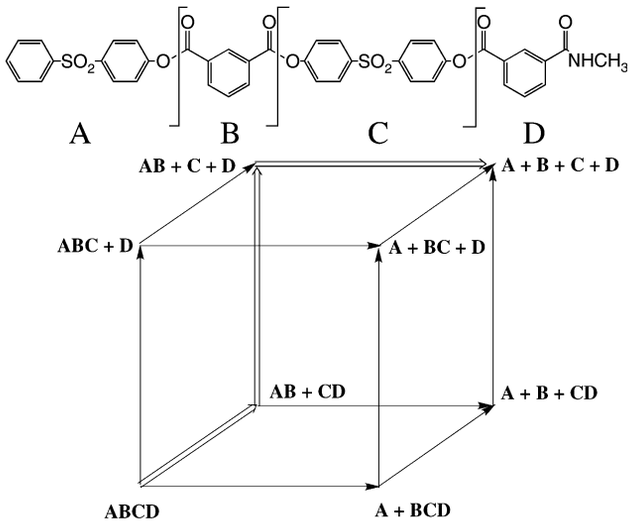

Fig. 3 Possible cleavage pathways for hydrolysis of the triester **24**. The predominant reaction pathway catalyzed by mAb DC2-5F4 is indicated by open arrows. A: (4-phenylsulfonyl)phenol; B: isophthalic acid; C: 4,4′-sulfodiphenol; D: isophthalamic acid.

has been reported recently that, in contrast to transition-state analog immunization, reactive immunization yields antibodies which are not substantially affected by product inhibition.[11] However, all previously described reactive immunization haptens were based on phenylmethylsulfone phosphonate diesters.[8–11] On the other hand, hapten **7** with a diphenylsulfone moiety seems to induce much stronger interactions with the antibody and hence leads to product inhibition.

In order to evaluate catalytic performance of our antibodies under conditions more closely approaching possible applications for polymer degradation, we prepared solid and water insoluble 1,4-phenylenesulfonyl-1,4-phenyleneoxycarbonyl-1,3-phenylenecarbonyl polyester **25** by a procedure reported by Earekson.[17] The average molecular weight of the obtained polymer as determined by MALDI-TOF and multi-angle light scattering (MALS) was $M_w = 3\,233$ which corresponds to $n = 8$. Since it could be expected that an antibody might perform differently with a soluble oligomeric substrate as compared to the high molecular weight and insoluble polymer, all 40 mAbs were re-screened to find the most active catalyst for cleaving the polyester. Somewhat surprisingly, the most active antibody was DC2-8E6, since in the original assay with the triester **24** DC2-8E6 was found to be somewhat inferior to DC2-5F4. Despite a large rate enhancement observed for simple ester substrates (Table 1), DC2-5F4 increased the rate of polymer hydrolysis by only three-fold compared to the background rate (3.6×10^{-3} and 1.1×10^{-3} μM min^{-1}, respectively). On the other hand, DC2-8E6 enhanced the rate of the polymer hydrolysis by factor of five (5.2×10^{-3} μM min^{-1}). In order to explain this apparent discrepancy, we decided to evaluate the kinetic parameters for monoester hydrolysis by mAb DC2-8E6. The comparison between DC2-8E6 and DC2-5F4 (Table 1) indicates that while both antibodies have comparable k_{cat} values, DC2-8E6 has generally lower K_m values. For the BC substrate, the building block of the polymer **25**, the K_m value for DC2-8E6 is about four-fold lower than that for DC2-5F4, which suggests that under conditions of low substrate solubility DC2-8E6 should be superior to DC2-5F4 despite the lower k_{cat} value.

Conclusions

In conclusion, reactive immunization was used to obtain catalytic antibodies that cleave oligomeric esters as well as the congener monoesters. The newly discovered antibodies catalyzed the hydrolysis of an asymmetric triester **24** *via* an *endo*-cleavage pathway, which could be explained by our hapten design. While oligomeric ester cleavage was viable, the catalytic properties of these antibodies towards high molecular weight polymeric esters appeared to be limited by the polymer's solubility. Interestingly, the best catalytic antibody (DC2-5F4) found for hydrolysis of oligoester **24** (Table 1), was not the most proficient catalyst for the insoluble polymer **25**. Thus, while homogeneous assay conditions are required to obtain meaningful kinetic characterization of antibody catalysts, our results did not translate to the most proficient catalyst being the most appropriate for insoluble polymer degradation. Based on these findings caution should be used for substrate selection when screening for antibody depolymerization catalysts. Future research efforts will be directed toward improving the catalytic performance of these types of antibodies against solid polymers through additives to enhance polymer solubility or antibody–polymer surface binding.

Experimental

Synthetic procedures

^{1}H, ^{13}C and ^{31}P NMR spectra were obtained in $CDCl_3$, CD_3OD or Me_2SO-d_6 with chemical shifts relative to internal $CDCl_3$ (^{1}H δ 7.26 ppm and ^{13}C δ_C 77.0 ppm), CD_3OD (^{1}H δ 3.30 ppm and ^{13}C δ_C 49.2 ppm) and DMSO-d_6 (^{1}H δ 2.49 ppm and ^{13}C δ_C 39.0 ppm) standard. Thin-layer chromatography (TLC) was performed using Merck silica gel 60 F_{254} plates. All the solvents were freshly distilled from the appropriate drying agent. All chemicals were obtained from Aldrich and used without further purification. Reactions were carried out in oven- or flame-dried glassware in a dry nitrogen atmosphere.

Benzyl 3-bromobenzoate 4. Into the suspension of 3-bromobenzoic acid (2.0 g, 10.0 mmol) in CH_2Cl_2 (20 mL), DMF (5 μL) and oxalyl chloride (1.05 mL, 12 mmol) were added at room temperature with stirring. The reaction mixture became homogeneous and after additional stirring for 3 h the solvent was removed by evaporation and the residue was dried under vacuum. The residue was redissolved in CH_2Cl_2 (50 mL), and benzyl alcohol (1.03 mL, 10.0 mmol) followed by triethylamine (2.1 mL, 15.0 mmol) were added at 0 °C with stirring. After 2 h, the reaction mixture was washed with water and the organic layer was dried over anhydrous sodium sulfate. The solvent was removed by evaporation and the crude product was purified by column chromatography on silica gel (hexane–EtOAc 10 : 1) to give **4** (2.46 g, 85%) as a colorless oil, ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.20 (1H, t, J 1.5 Hz), 8.01 (1H, dt, J 7.7, 1.5 Hz), 7.69 (1H, ddd, J 8.2, 2.2, 1.1 Hz), 7.46–7.26 (6H, m), 5.36 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 164.99, 135.89, 135.56, 132.56, 131.96, 129.88, 128.59, 128.36, 128.24, 128.21, 122.39, 67.03.

3-(Diethoxyphosphinyl)benzoic acid benzyl ester 5. Into a mixture of **4** (2.46 g, 8.45 mmol) and $NiCl_2$ (55 mg, 0.42 mmol), triethyl phosphite (1.67 mL, 9.73 mmol) was added at 175 °C over 15 min.[18] After the addition was completed, the mixture was stirred for an additional 20 min. After cooling down to room temperature the reaction mixture was subjected to column chromatography on silica gel (hexane–EtOAc 1 : 1) to give **5** as a colorless oil (2.52 g, 86%), ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.52 (1H, dt, J 3.9, 1.1 Hz), 8.27 (1H, ddt, J 8.0, 2.9, 1.1 Hz), 8.03 (1H, ddt, J 20.5, 7.7, 1.5 Hz), 7.58 (1H, ddd, J 12.5, 7.5, 3.7 Hz), 7.50–7.36 (5H, m), 5.41 (2H, s), 4.27–4.03 (4H, m), 1.35 (6H, t, J 7.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 165.40, 136.09, 135.98, 135.59, 133.34, 132.87, 132.76, 130.52, 130.37, 130.14, 128.71, 128.55, 128.37, 128.32, 128.26, 66.99, 62.32, 62.27, 16.26, 16.20; ^{31}P NMR (162 MHz, $CDCl_3$) δ_P 17.63; MALDI-FTMS, Calc. for $C_{18}H_{22}O_5P$: 349.1199 (MH^+). Found: 349.1201.

3-{Bis[4-(phenylsulfonyl)phenoxy]phosphinyl}benzoic acid benzyl ester 6. Into a solution of compound **5** (195 mg, 0.56

mmol) in CH_2Cl_2 (3 mL), was added bromotrimethylsilane (0.22 mL, 1.68 mmol) at room temperature with stirring. The reaction was completed within 3 h as judged by TLC. The solvent was removed by evaporation and the residue was dried thoroughly under vacuum. The residue was dissolved in CH_2Cl_2 (3 mL), and DMF (2 μL) and oxalyl chloride (122 μL, 1.4 mmol) were added with stirring. After 2 h the solvent was evaporated and the residue dried under vacuum. The resulting residue was redissolved in CH_2Cl_2 (5 mL) and (4-phenylsulfonyl)phenol **3**[19] (262 mg, 1.12 mmol) was added followed by triethylamine (195 μL, 1.4 mmol). The reaction mixture was stirred for 12 h. After removal of the solvent, compound **6** was obtained by flash chromatography (CH_2Cl_2–EtOAc 10 : 1) as a white solid (141 mg, 35%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (1H, dt, *J* 14.9, 1.1 Hz), 8.34 (1H, dd, *J* 7.6, 1.4 Hz), 8.10 (1H, ddt, *J* 13.8, 7.6, 1.1 Hz), 7.91–7.88 (8H, m), 7.65–7.56 (3H, m), 7.52–7.46 (4H, m), 7.46–7.38 (5H, m), 7.31–7.28 (4H, m), 5.39 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 164.63, 153.19, 153.13, 140.86, 138.54, 136.18, 136.06, 135.18, 134.86, 133.26, 133.13, 133.02, 131.07, 130.90, 129.78, 129.35, 129.22, 128.97, 128.49, 128.37, 128.24, 127.38, 126.99, 126.65, 124.71, 121.13, 121.09, 115.88, 67.19; ^{31}P NMR (101 MHz, $CDCl_3$) δ_P 11.44; MALDI-FTMS, Calc. for $C_{38}H_{29}O_9NaPS_2$: 747.0883 (MNa^+). Found: 747.0865.

3-{Bis[4-(phenylsulfonyl)phenoxy]phosphinyl}benzoic acid 7. Compound **6** (24 mg, 0.033 mmol) was dissolved in THF (3 mL) and hydrogenolyzed over Pd (10% on carbon, 3.5 mg) under normal pressure. After the reaction was completed, the catalyst was filtered off and the solvent was removed by evaporation to give compound **7** (21 mg, 100%) as a white solid, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (1H, d, *J* 14.6 Hz), 8.36 (1H, d, *J* 7.6 Hz), 8.15 (1H, dd, *J* 13.8, 7.9 Hz), 7.90 (8H, d, *J* 8.5 Hz), 7.70–7.65 (1H, m), 7.60–7.56 (2H, m), 7.53–7.49 (4H, m), 7.31 (4H, d, *J* 8.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 168.77, 153.28, 153.21, 140.95, 138.77, 136.81, 136.69, 135.45, 133.70, 133.60, 133.43, 130.73, 129.96, 129.58, 129.37, 129.12, 127.55, 127.15, 126.77, 124.82, 121.27, 121.22, 116.02; ^{31}P NMR (162 MHz, $CDCl_3$) δ_P 11.19; MALDI-FTMS, Calc. for $C_{31}H_{24}O_9PS_2$: 635.0594 (MH^+). Found: 635.0584.

3-(Chloromethyl)benzoic acid benzyl ester 8. A solution of 2-chloromethylbenzoic acid (1.71 g, 10.0 mmol) in methylene chloride (20 mL) containing a catalytic amount of DMF was treated with oxalyl chloride (1.5 mL, 12.0 mmol). After evolution of gas ceased, the reaction mixture was evaporated to dryness and the residue was dried under vacuum and redissolved in CH_2Cl_2 (20 mL). To the stirred solution benzyl alcohol was added (1.03 mL, 10.0 mmol) followed by triethylamine (2.1 mL, 15.0 mmol). After 6 h at room temperature the reaction mixture was concentrated and the residue was chromatographed on silica gel (hexane–EtOAc 10 : 1) to give **8** as a colorless oil, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (1H, t, *J* 1.8 Hz), 8.04 (1H, dt, *J* 7.9, 1.5 Hz), 7.61 (1H, d, *J* 7.6 Hz), 7.47–7.34 (6H, m), 5.38 (2H, s), 4.62 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 165.80, 137.80, 135.77, 133.08, 130.57, 129.67, 129.61, 128.85, 128.55, 128.26, 128.20, 66.81, 45.41; MALDI-FTMS, Calc. for $C_{15}H_{14}ClO_2$: 261.0677 (MH^+). Found: 261.0685.

3-[(Diethoxyphosphinyl)methyl]benzoic acid benzyl ester 9. A mixture of benzyl 2-chloromethylbenzoate (0.61 g, 2.34 mmol) and triethylphosphite (0.6 mL, 3.51 mmol) was refluxed for 4–6 h and the reaction progress was monitored by TLC. After completion of the reaction, the crude product was purified by column chromatography (hexane–EtOAc 1 : 1) to give **9** (0.56 g, 66%) as a pale yellow oil, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98–7.97 (2H, m), 7.52 (1H, d, *J* 7.9 Hz), 7.45–7.33 (6H, m), 5.36 (2H, s), 4.04–3.98 (4H, m), 3.19 (2H, d, *J* 21.7 Hz), 1.23 (6H, t, *J* 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 166.13, 135.87, 134.40, 134.34, 132.22, 132.13, 130.88, 130.81, 130.32, 128.58, 128.55, 128.50, 128.23, 128.20, 66.73, 62.21, 62.15, 34.17, 32.79, 16.31, 16.25; ^{31}P NMR (162 MHz, $CDCl_3$) δ_P 26.10; MALDI-FTMS, Calc. for $C_{19}H_{23}O_5P$: 363.1356 (MH^+). Found: 363.1343.

3-{Bis[4-(phenylsulfonyl)phenoxy]phosphinylmethyl}benzoic acid benzyl ester 10. Compound **9** (184 mg, 0.51 mmol) was converted into a dichloride and then reacted with **3** as described for compound **6**. Compound **10** (80 mg, 21%) was obtained as a white solid by flash chromatography on silica gel (hexane–EtOAc 1 : 2), ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (1H, s), 7.98 (1H, d, *J* 5.8 Hz), 7.88 (4H, d, *J* 6.2 Hz), 7.82 (4H, d, *J* 7.0 Hz), 7.59–7.56 (2H, m), 7.52–7.49 (5H, m), 7.44–7.36 (6H, m), 7.10 (4H, d, *J* 7.0 Hz), 5.35 (2H, s), 3.56 (2H, d, *J* 17.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 165.64, 153.44, 153.34, 141.08, 138.59, 135.65, 134.40, 134.34, 133.37, 131.07, 130.99, 130.85, 129.84, 129.50, 129.34, 129.11, 128.61, 128.40, 128.29, 127.53, 121.07, 121.03, 66.96, 34.38, 33.00; ^{31}P NMR (101 MHz, $CDCl_3$) δ_P 20.10; MALDI-FTMS, Calc. for $C_{39}H_{31}O_9NaPS_2$: 761.1039 (MH^+). Found: 761.1051.

3-{Bis[4-(phenylsulfonyl)phenoxy]phosphinylmethyl}benzoic acid 11. Compound **10** (36.5 mg, 0.049 mmol) was dissolved in THF (3 mL) and hydrogenolyzed over Pd (10% on carbon, 5 mg). After 1 h, the catalyst was removed by filtration and the solvent was evaporated. Compound **11** (29 mg, 92%) was obtained as a white solid, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.99 (1H, d, *J* 7.9 Hz), 8.05 (1H, s), 7.90–7.85 (8H, m), 7.59–7.56 (3H, m), 7.52–7.48 (4H, m), 7.41 (1H, t, *J* 7.6 Hz), 7.14 (4H, d, *J* 7.9 Hz), 3.61 (2H, d, *J* 21.7 Hz); ^{13}C NMR ($CDCl_3$) δ_C 170.12, 153.45, 153.36, 141.06, 138.69, 135.01, 133.42, 131.54, 130.20, 129.90, 129.58, 129.38, 129.20, 127.56, 121.13, 121.09; ^{31}P NMR (101 MHz, $CDCl_3$) δ_P 20.20; MALDI-FTMS, Calc. for $C_{32}H_{25}O_9PS_2Na$: 671.0575 (MNa^+). Found: 671.0571.

3-{Hydroxy[4-(phenylsulfonyl)phenoxy]phosphinyl}benzoic acid ethyl ester 12. To a stirred solution of diethyl 3-(ethoxycarbonyl)phenylphosphonate[20] (0.286 g, 1.0 mmol) in methylene chloride (10 mL) was added bromotrimethylsilane (0.53 mL, 1.0 mmol). After 2 h at room temperature the reaction mixture was evaporated to dryness and the residue was dried under vacuum and redissolved in methylene chloride (10 mL). The stirred solution was treated with oxalyl chloride (0.25 mL, 2.5 mmol) and a catalytic amount of DMF. After 2 h at room temperature the reaction mixture was evaporated to dryness and dried under vacuum. The residue was dissolved in CH_2Cl_2 (10 mL) and treated with the phenol **1**[21] (0.468 g, 2.0 mmol) and triethylamine (0.35 mL, 2.5 mmol) with stirring. After the reaction was completed as judged by TLC, the reaction mixture was treated with saturated sodium bicarbonate solution (10 mL) and stirred vigorously for 2 h. The pH of the mixture was adjusted to about 7 and the unreacted phenol **1** was extracted with ethyl acetate. The inorganic phase was acidified to pH 1 and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and evaporated to give crude phosphonate monoacid **12** (0.21 g, 47%) as a colorless oil, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (1H, dt, *J* 15.0, 1.5 Hz), 8.24 (1H, dd, *J* 7.9, 1.5 Hz), 7.95–7.88 (3H, m), 7.82 (2H, d, *J* 8.8 Hz), 7.57–7.47 (4H, m), 7.20 (2H, dd, *J* 8.8, 1.2 Hz), 4.39 (2H, q, *J* 7.0 Hz), 1.39 (3H, t, *J* 7.0 Hz); MALDI-FTMS, Calc. for $C_{21}H_{18}O_7PSNa$: 469.0487 (MNa^+). Found: 469.0474.

3-{Hydroxy[4-(phenylsulfonyl)phenoxy]phosphinyl}benzoic acid, 13. Compound **12** obtained in the previous step without any further purification was treated with lithium hydroxide monohydrate (30 mg, 0.70 mmol) in acetonitrile–water (4 : 1, 10 mL). After stirring for 5 h the reaction mixture was concentrated and the crude product was purified by chromatography on silica gel (CH_2Cl_2–MeOH 2 : 1) to afford **13** as a white

powder (69 mg, 70%), ^{1}H NMR (400 MHz, CD_3OD) δ 8.46 (1H, d, *J* 13.5 Hz), 7.99 (1H, dd, *J* 7.6, 1.4 Hz), 7.90–7.78 (5H, m), 7.62–7.51 (3H, m), 7.38–7.34 (1H, m), 7.27 (2H, d, *J* 8.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ_C 158.86, 143.44, 136.56, 134.46, 134.33, 134.20, 133.64, 133.54, 132.44, 130.50, 130.37, 128.44, 128.31, 122.63, 122.59; ^{31}P NMR (162 MHz, CD_3OD) δ_P 11.77; MALDI-FTMS, Calc. for $C_{19}H_{15}O_7PSNa$: 441.0168 (MNa^+). Found: 441.0176.

6-(3-{Hydroxy[4-(phenylsulfonyl)phenoxy]phosphinyl}benzoylamino)hexanoic acid methyl ester 14. A mixture of compound **13** (32 mg, 0.076 mmol) and ε-aminocaproic‡ acid methyl ester hydrochloride (15 mg, 0.084 mmol) was treated with EDC–HCl (16.5 mg, 0.084 mmol) in the presence of triethylamine (24 μL, 0.17 mmol) in CH_2Cl_2–DMF (2 : 1, 3 mL). After 12 h the reaction mixture was quenched with 1 M HCl and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and concentrated. Chromatography (CH_2Cl_2–MeOH 4 : 1) afforded **14** as a pale yellow oil (16 mg, 38%), ^{1}H NMR (400 MHz, CD_3OD) δ 8.1 (1H, dt, *J* 15.0, 1.5 Hz), 7.82–7.69 (6H, m), 7.57–7.34 (4H, m), 7.15 (2H, d, *J* 8.0 Hz), 3.52 (3H, s), 3.40 (2H, m), 2.24 (2H, t, *J* 8.0 Hz), 1.57–1.48 (4H, m), 1.31–1.27 (2H, m); ^{13}C NMR (100 MHz, CD_3OD) δ_C 175.87, 169.79, 143.39, 136.89, 135.70, 135.49, 134.38, 131.13, 131.02, 130.62, 130.52, 130.43, 129.36, 129.23, 128.45, 122.60, 51.98, 40.80, 34.66, 30.08, 27.48, 35.69; ^{31}P NMR (162 MHz, CD_3OD) δ_P 10.24; MALDI-FTMS Calc. for $C_{26}H_{28}NO_8PSNa$: 568.1165 (MNa^+). Found: 568.1160.

6-(3-{Hydroxy[4-(phenylsulfonyl)phenoxy]phosphinyl}benzoylamino)hexanoic acid, 15. Compound **14** (16 mg, 0.029 mmol) was treated with lithium hydroxide monohydrate (3.7 mg, 0.088 mmol) in acetonitrile–water (4 : 1, 2.5 mL). After 2 h the solvent was evaporated and the residue was purified by preparative TLC (CH_2Cl_2–MeOH 3 : 1) to give **15** (9.3 mg, 60%), ^{1}H NMR (400 MHz, CD_3OD) δ 8.21 (1H, dt, *J* 12.0, 1.4 Hz), 7.92–7.84 (4H, m), 7.81 (2H, d, *J* 8.8 Hz), 7.62–7.58 (1H, m), 7.56–7.51 (2H, m), 7.48–7.43 (1H, m), 7.25 (2H, dd, *J* 9.1, 1.2 Hz), 3.37 (2H, m), 2.17 (2H, t, *J* 7.3 Hz), 1.69–1.59 (4H, m), 1.44–1.37 (2H, m); ^{13}C NMR (100 MHz, CD_3OD) δ_C 182.91, 143.36, 137.35, 135.92, 135.42, 134.39, 131.30, 130.95, 130.52, 130.43, 130.19, 129.15, 128.44, 127.66, 122.60, 120.08, 41.09, 39.16, 30.32, 28.22, 27.46; ^{31}P NMR (101 MHz, $CDCl_3$) δ_P 10.3; MALDI-FTMS Calc. for $C_{25}H_{26}NO_8PSNa$: 554.1014 (MNa^+). Found: 554.1029.

Isophthalamic acid methyl benzyl diester 16. Monobenzyl isophthalate[22] (1.55 g, 6.05 mmol) was treated with $SOCl_2$ (2.21 mL, 30.3 mmol) at 50 °C for 2 h. After the excess of $SOCl_2$ was evaporated, the residue was dried under vacuum and then redissolved in CH_2Cl_2 (10 mL). Methylamine (2.6 mL, 30.3 mmol, 40% aqueous solution) was added at 0 °C with stirring. After 1 h the organic layer was separated and washed with water and brine. The solvent was removed by evaporation and the residue chromatographed on silica gel (hexane–EtOAc 1 : 1). Recrystallization from hexane–EtOAc afforded **16** (0.81 g, 50%) as white needles, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (1H, t, *J* 1.8 Hz), 8.20 (1H, dt, *J* 7.7, 1.5 Hz), 8.04 (1H, dt, *J* 8.4, 1.1 Hz), 7.56–7.36 (6H, m), 6.22 (1H, br s), 5.39 (2H, s), 3.04, 3.02 (3H, d); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 167.16, 165.69, 135.65, 134.94, 132.37, 131.85, 130.39, 128.85, 128.62, 128.39, 128.31, 127.50, 67.06, 26.86; MALDI-FTMS Calc. for $C_{16}H_{16}NO_3$: 270.1125 (MH^+). Found: 270.1126.

Isophthalamic acid 17. Compound **16** (0.81 g, 3 mmol) was dissolved in MeOH (15 mL) and hydrogenolyzed at 40 psi over Pd (10% on carbon, 0.3 g). The reaction was completed in 2 h. The catalyst was filtered off and the solvent was removed to give compound **17** (0.5 g, 92%) as a white powder, ^{1}H NMR (400 MHz, CD_3OD) δ 8.62 (1H, br s), 8.46 (1H, t, *J* 1.4 Hz), 8.16 (1H, d, *J* 7.6 Hz), 8.01 (1H, d, *J* 7.6 Hz), 7.57 (1H, t, *J* 7.6 Hz), 2.93, 2.92 (3H, d); ^{13}C NMR (100 MHz, CD_3OD–$CDCl_3$ 10 : 1) δ_C 169.96, 169.20, 136.16, 133.76, 132.75, 132.60, 129.99, 129.72, 27.34; MALDI-FTMS, Calc. for $C_9H_{10}NO_3$: 180.0655 (MH^+). Found: 180.0655.

Benzene-1,3-dicarboxylic acid 4-{[4-(benzyloxy)phenyl]sulfonyl}phenyl benzyl diester 18. Monobenzyl isophthalate (0.419 g, 1.6 mmol) was treated with neat thionyl chloride (1.1 mL, 15 mmol) for 2 h at 50 °C. The reaction mixture was evaporated and dried under vacuum. The residue was dissolved in CH_2Cl_2 (20 mL) and treated with phenol **1** (0.51 g, 1.5 mL) and triethylamine (0.33 mL, 2.4 mmol). After 3 h at room temperature the solution was washed with water and dried over anhydrous sodium sulfate. The organic phase was concentrated to afford **18** as a white powder (0.86 g, 96%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (1H, t, *J* 1.4 Hz), 8.35 (2H, dd, *J* 7.9, 1.8 Hz), 8.00 (2H, d, *J* 8.8 Hz), 7.99 (2H, d, *J* 8.8 Hz), 7.62 (1H, t, *J* 7.9 Hz), 7.48–7.45 (2H, m), 7.42–7.35 (10H, m), 7.05 (2H, d, *J* 9.1 Hz), 5.41 (2H, s), 5.12 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 165.19, 163.51, 162.53, 153.92, 139.79, 135.60, 135.48, 134.87, 134.39, 132.92, 131.31, 130.85, 129.84, 129.08, 128.96, 128.65, 128.58, 128.38, 128.28, 127.38, 122.51, 115.34, 70.27, 67.13; MALDI-FTMS, Calc. for $C_{34}H_{26}O_7NaS$: 601.1291 (MNa^+). Found: 601.1287.

Benzene-1,3-dicarboxylic acid benzyl 4-(phenylsulfonyl)phenyl diester 19. Monobenzyl isophthalate (0.768 g, 3.0 mmol) was activated with thionyl chloride (1.1 mL, 15 mmol) for 2 h. After $SOCl_2$ was removed by evaporation the residue was redissolved in chloroform (20 mL) and treated with phenol **3** (0.702 g, 3.0 mmol) and triethylamine (3.3 mL, 24 mmol) in a procedure analogous to that described for **18**. Chromatography (hexane–EtOAc 2 : 1) afforded **19** (1.24 g, 88%) as orange crystals, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (1H, t, *J* 1.4 Hz), 8.35 (2H, dd, *J* 8.0, 1.8 Hz), 8.03 (2H, d, *J* 8.8 Hz), 7.97 (2H, d, *J* 7.4 Hz), 7.63–7.58 (2H, m), 7.53 (2H, t, *J* 8.1 Hz), 7.46 (2H, d, *J* 6.6 Hz), 7.42–7.36 (5H, m), 5.42 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 165.26, 163.57, 154.27, 141.27, 139.15, 135.54, 134.99, 134.46, 133.36, 131.40, 130.96, 129.49, 129.37, 129.15, 129.02, 128.64, 128.46, 128.35, 127.67, 122.65, 67.20; MALDI-FTMS, Calc. for $C_{27}H_{20}O_6NaS$: 495.0873 (MNa^+). Found: 495.0893.

Benzene-1,3-dicarboxylic acid {4-[(4-hydroxyphenyl)sulfonyl]phenyl} ester 20. Compound **18** (0.86 g, 1.45 mmol) was dissolved in chloroform and hydrogenolyzed at 40 psi over Pd (10% on carbon, 0.3 g). After the reaction was completed methanol was added and the catalyst was filtered off. The solvent was removed to give **20** as a white powder (0.55 g, 95%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (1H, t, *J* 1.8 Hz), 8.42–8.36 (2H, m), 8.00 (2H, d, *J* 8.9 Hz), 7.85 (2H, d, *J* 8.8 Hz), 7.66 (1H, t, *J* 7.9 Hz), 7.38 (2H, d, *J* 8.8 Hz), 6.93 (2H, d, *J* 8.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ_C 168.35, 165.02, 163.78, 155.61, 141.50, 135.92, 135.18, 132.79, 132.41, 132.18, 131.15, 130.55, 130.24, 130.01, 123.99, 117.09; MALDI-FTMS, Calc. for $C_{20}H_{14}O_7NaS$: 421.0352 (MNa^+). Found: 421.0335.

Benzene-1,3-dicarboxylic acid mono[4-(phenylsulfonyl)phenyl] ester 21. Compound **20** (1.24 g, 2.6 mmol) was dissolved in chloroform (20 mL) and hydrogenolyzed at 40 psi over Pd (10% on carbon, 0.3 g). After 6 h, methanol (20 mL) was added and the catalyst was filtered off. The solvent was evaporated to give **21** (0.952 g, 96%) as a white crystalline solid, ^{1}H NMR (400 MHz, CD_3OD) δ 8.77 (1H, t, *J* 1.5 Hz), 8.37 (1H, dt, *J* 7.9, 1.2 Hz), 8.33 (1H, dt, *J* 7.9, 1.2 Hz), 8.07 (2H, d, *J* 8.8 Hz), 7.99 (2H, d, *J* 7.0 Hz), 7.71–7.64 (2H, m), 7.62–7.58 (2H, m), 7.51 (2H, d, *J* 8.8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 166.33, 163.43, 154.29, 140.96, 138.79, 134.69, 134.01,

‡ The IUPAC name for caproic acid is hexanoic acid.

133.89, 131.57, 130.48, 129.87, 129.67, 129.36, 128.96, 127.43, 123.55; MALDI-FTMS, Calc. for $C_{20}H_{14}O_6NaS$: 405.0403 (MNa^+). Found: 405.0422.

3-[(Methylamino)carbonyl]benzoic acid 4-{[4-(benzyloxy)-phenyl]sulfonyl}phenyl diester 22. Compound **17** (0.179 g, 1.0 mmol) was treated with $SOCl_2$ (20.73 mL, 10 mmol) at 50 °C for 2 h. The excess of thionyl chloride was evaporated and the residue was dried under vacuum and then dissolved in CH_2Cl_2 (20 mL). Compound **5** (0.34 g, 1.0 mmol) and triethylamine (1.4 mL, 10 mmol) were added at room temperature with stirring. After 20 h, the organic layer was washed with water and brine, and then was dried over anhydrous sodium sulfate. The solvent was removed and the crude product was recrystallized from CH_2Cl_2–hexanes to give **22** (0.225 g, 45%) as a white powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (1H, t, J 1.5 Hz), 8.29 (1H, dt, J 8.2, 1.2 Hz), 8.11 (1H, dt, J 8.5, 1.2 Hz), 7.99 (2H, d, J 9.1 Hz), 7.89 (2H, d, J 9.1 Hz), 7.61 (1H, t, J 7.3 Hz), 7.41–7.34 (7H, m), 7.06 (2H, d, J 9.1 Hz), 5.12 (2H, s), 3.06, 3.05 (3H, d); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 166.81, 163.72, 162.63, 153.98, 139.70, 135.59, 135.22, 132.81, 132.69, 129.84, 129.08, 128.96, 128.68, 128.34, 127.41, 122.53, 115.41, 70.34, 26.88; MALDI-FTMS, Calc. for $C_{28}H_{23}NO_6NaS$: 524.1138 (MNa^+). Found: 524.1125.

3-[(Methylamino)carbonyl]benzoic acid 4-[(4-hydroxyphenyl)-sulfonyl]phenyl ester 23. Compound **22** (0.225 g, 0.45 mmol) was suspended in methanol (20 mL) and hydrogenolyzed at 40 psi over Pd (10% on carbon, 48 mg). After 2 h the catalyst was filtered off and the solvent was removed to give **23** (0.184 g, 99%) as a white powder, ^{1}H NMR (400 MHz, CD_3OD) δ 8.6 (1H, s), 8.28 (1H, d, J 7.6 Hz), 8.11 (1H, d, J 7.9 Hz), 7.99 (2H, d, J 8.8 Hz), 7.79 (2H, d, J 8.8 Hz), 7.64 (1H, t, J 7.6 Hz), 7.45 (2H, d, J 8.5 Hz), 6.91 (2H, d, J 8.8 Hz), 2.92 (3H, s); ^{13}C NMR (100 MHz, CD_3OD) δ_C 165.17, 163.85, 155.69, 141.58, 136.34, 133.88, 133.65, 132.45, 131.18, 130.62, 130.29, 130.06, 129.84, 124.01, 117.10, 116.84, 116.84, 27.112; MALDI-FTMS, Calc. for $C_{21}H_{17}NO_6NaS$: 434.0669 (MNa^+). Found: 434.0661.

Benzene-1,3-dicarboxylic acid 4-(4-{3-[(methylamino)-carbonyl]benzoyloxy}phenylsulfonyl)phenyl 4-(phenylsulfonyl)-phenyl diester 24. Compound **21** (65.5 mg, 0.17 mmol) was treated with thionyl chloride (0.25 mL, 3.4 mmol) and then reacted with compound **23** (70 mg, 0.17 mmol) and triethylamine (0.24 mL, 1.7 mmol) in CH_2Cl_2 (10 mL) according to the procedure described above. After 20 h, the organic layer was washed with water and dried over anhydrous sodium sulfate. The solvent was removed and the residue was decanted with methanol to give compound **24** (81 mg, 61%) as a white powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (1H, s), 8.52 (1H, s), 8.45 (2H, dt, J 6.2, 2.0 Hz), 8.29 (1H, d, J 7.9 Hz), 8.10 (1H, d, J 7.9 Hz), 8.06–8.03 (6H, m), 7.96 (2H, d, J 7.0 Hz), 7.71 (1H, t, J 7.9 Hz), 7.62–7.58 (2H, m), 7.55–7.51 (2H, m), 7.44–7.39 (6H, m), 6.42 (1H, br s), 3.04, 3.03 (3H, d); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 166.78, 163.64, 163.29, 163.23, 154.45, 154.32, 154.15, 141.20, 139.27, 138.95, 138.77, 135.45, 135.30, 133.40, 132.89, 132.70, 131.92, 129.56, 129.52, 129.43, 129.38, 129.20, 128.99, 128.32, 127.65, 122.74, 122.62, 122.54, 26.94; MALDI-FTMS Calc. for $C_{41}H_{30}NO_{11}S_2$: 776.1255 (MH^+). Found: 776.1250.

Poly(1,4-phenylenesulfonyl-1,4-phenyleneoxycarbonyl-1,3-phenylenecarbonyl) 25. Compound **25** was prepared by procedure reported by Earekson.[17] In order to remove low molecular weight fragments potentially trapped within the solid matrix, the obtained material was thoroughly extracted with 1,4-dioxane, washed with methanol and dried under vacuum, MALS (10.0 mg mL^{-1} in PhOH–$(CHCl_2)_2$ 3 : 2 wt) M_w = 3 233 g mol^{-1}; MALDI-TOF (MNa^+) 1570.59, 1953.73, 2336.46, 2718.66, 3100.85, 3482.45, 3863.89, 4624.87, 5767.32, 7290.22.

Antibody production and purification

Haptens **7** and **11** were conjugated with the carrier protein keyhole lymphet hemocyanin (KLH) by the sulfo-NHS method.[8] Monoclonal antibodies were prepared by standard techniques.[12,13] Purification of the antibodies was performed accordingly to procedures described previously.[14]

Screening for catalytic antibodies

The screening assays were performed in MOPS buffer (50 mM, pH 7.08) with 10% DMSO as a co-solvent and substrate **24** concentration of 100 μM (partially insoluble). Reactions were initiated by adding an appropriate amount of antibody stock solution to achieve the final antibody concentration of 20 μM and each reaction was agitated overnight using an orbitary shaker. Reaction mixtures were analyzed by HPLC using a C-18 VYDAC column with a gradient mobile phase (t = 0, 10% acetonitrile, 90% water [0.1% TFA]; t = 15 min, 50% acetonitrile, 50% water [0.1% TFA]; t = 20 min, 80% acetonitrile, 20% water [0.1% TFA]; t = 25 min, 10% acetonitrile, 90% water [0.1% TFA]; t = 30 min, 10% acetonitrile, 90% water [0.1% TFA]; flow rate, 1 mL min^{-1}) with UV detection at λ = 240 nm. A relative activity of each antibody was estimated by comparing the combined signal intensity of all released products. Screening of antibodies for catalytic activity against the solid polymer was performed on a rotary shaker as a 0.8% suspension of **25** in buffer (50 mM MOPS, pH 7.08) at 20 °C and in the presence of 20 μM antibody. The rate of the reaction was determined by following the release of isophthalic acid. The uncatalyzed reactions were carried out under the same conditions without the antibody.

Kinetic measurements

Kinetic parameters for hydrolysis of individual esters were obtained by varying substrate concentrations up to at least 3 × K_m (2–300 μM) whenever possible in the buffer system described above. Each reaction was initiated by addition of antibody to the substrate solution (final concentration of mAb 1–2 μM, 200 μL). At appropriate time intervals, an aliquot (30 μL) of the reaction mixture was removed and quenched in methanol (30 μL). Reaction progress was monitored by following the release of the phenolic product by reversed-phase HPLC with isocratic mobile phase (70% water [0.1% TFA], 30% acetonitrile for phenol A and 80% water [0.1% TFA], 20% acetonitrile for phenol C) with UV detection at 256 nm or 240 nm, respectively. Concentration of the product was determined by comparison with product standards. The reaction was followed for no more than 10% of conversion, during which the progress curves were linear. Kinetic parameters were calculated by using Lineweaver–Burk analyses of the raw data with Cricket Graph III 1.5.3 computer program, Computer Associates Int. (copyright 1992–1995).

Inhibition constants K_i, product inhibition determination

The K_i values of inhibitor **15** and fragments A through D were determined from their ability to inhibit the hydrolysis of the CD ester by mAb DC2-5F4. Due to the problems associated with tight binding inhibitors ([I] ≈ [mAb]) the assays were performed as to measure the inhibitor concentration required to reduce the catalytic activity by 50% of the original value (IC_{50}) over a broad range of inhibitor concentrations.[23] The assay was started by addition of the antibody (final mAb concentration 1.29 μM, 200 μL) to the CD substrate solution (100 μM) and the appropriate inhibitor. Reaction progress was monitored by HPLC as described above.

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