azide (NaN_3-DMF then H_2/Pd-C/MeOH) to give a Boc-protected primary amine. This was coupled with 24-(penta-*O*-acetylglucuronyl)amino-tetracosanoic acid (prepared by the opening of δ-gluconolactone with 24-amino-tetracosanoic acid (MeOH, 60 °C, DBU, 10 mins), followed by peracetylation (Ac_2O, pyridine)) using EDC/NHS/NMM-Et_3N/CH_2Cl_2. Deacetylation (aqueous ammonia/MeOH) and removal of the Boc groups (CF_3CO_2H 96%, H_2O 4%, 20 °C, 30 mins) yielded the tetra-trifluoroacetic acid salt of **1** as a colorless lyophilisate. Boc = *tert*-butoxycarbonyl; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide; NHS = *N*-hydroxysuccinimide; NMM = *N*-methylmorpholine.

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[15] Transfection complexes were prepared in 10 mM HEPES pH 8.0 buffer by adding a plasmid solution dropwise to an equal volume of bola solution to give a final concentration of 30 $\mu g\,mL^{-1}$ DNA. N:P ratios were calculated assuming the nucleotide residue had a formula weight of 310. The transfection reagents lipofectin and lipofectamine were obtained from Life Technologies Ltd. and superfect from Qiagen Ltd. Cell culture media and additives were obtained from Life Technologies Ltd. Chinese hamster ovarian cells were plated out in 24-well cell culture plates at 10^5 cells per well in 1 mL culture medium (Iscoves complete medium containing 2 mM glutamine and 10% foetal calf serum). After 24 h the cells were washed with 1 mL of Optimem medium per well. Transfection was carried out over 4 h at 37 °C in the presence of 500 µL of Optimem and 167 µL of condensed DNA (5 µg), then the medium was replaced with 1 mL of culture medium and the incubation continued for a total of 48 h. The levels of the activity of the β-galactosidase reporter gene in the cells were assessed using an enzyme activity kit (Promega Ltd) according to the manufacturer's instructions, where the hydrolysis of the substrate *o*-nitrophenyl-β-D-galactopyranoside was measured spectrophotometrically in washed, lysed-cell supernatants. The assay was calibrated against a β-galactosidase standard (Boehringer Mannheim Ltd.; 0.775 mU enzyme activity per ng of protein). The results were normalized for supernatant protein concentration following measurement of the latter using a BCA protein assay kit (Pierce and Warriner Ltd.)

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[18] Jurkat E6.1 cells (ECACC) were cultured in Dulbecco's modified eagles medium (DMEM) (Life Technologies Ltd.) that contained 10% foetal calf serum, 2 nM of glutamine, 50 units mL^{-1} of penicillin, and 50 $\mu g\,mL^{-1}$ of streptomycin. For transfection, cells were washed and re-suspended in serum-free DMEM at 2×10^6 cells mL^{-1} and 0.5 mL per well added to 24-well culture plates. Bola-condensed DNA (200 µL) was added at 4 $\mu g\,well^{-1}$ and the plates incubated for 1 h at 37 °C in 5% CO_2. Incubation was continued for a total of 24 h following addition of 300 µL of 33% foetal calf serum in DMEM. Cells were harvested by centrifugation and washing with phosphate buffered saline. Cells were lysed and supernatants assayed using a β-galactosidase enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Ltd). Reporter gene levels were normalized for cytosolic protein.[15]

A Versatile Periodate-Coupled Fluorogenic Assay for Hydrolytic Enzymes**

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The development of new catalysts is being increasingly followed by using combinatorial and evolutionary methods.[1–3] These approaches require the ability to assay large numbers of samples in parallel. This can be achieved using solid-phase bound assays related to immunoassays[4] and a variety of spectroscopic methods, such as IR thermography[5] and mass spectrometry.[6] One of the most popular methods is the use of chromogenic and fluorogenic substrates as product formation sensors.[1, 7, 8] Herein, we report a new versatile fluorogenic assay for hydrolytic enzymes. The assay couples product formation to the release of a fluorescent signal, achieved via periodate oxidation and albumin-catalyzed β-elimination, and uses nonactivated, chiral substrates.

We recently reported an enantioselective fluorogenic assay for alcohol dehydrogenases based on the detection of carbonyl oxidation products, such as **7** (Scheme 1), by β-elimination of the fluorescent product umbelliferone (**8**) catalysed by bovine serum albumin (BSA).[9, 10] Aldehyde **7** can also be produced from diol **5** or aminoalcohol **6** by oxidation with

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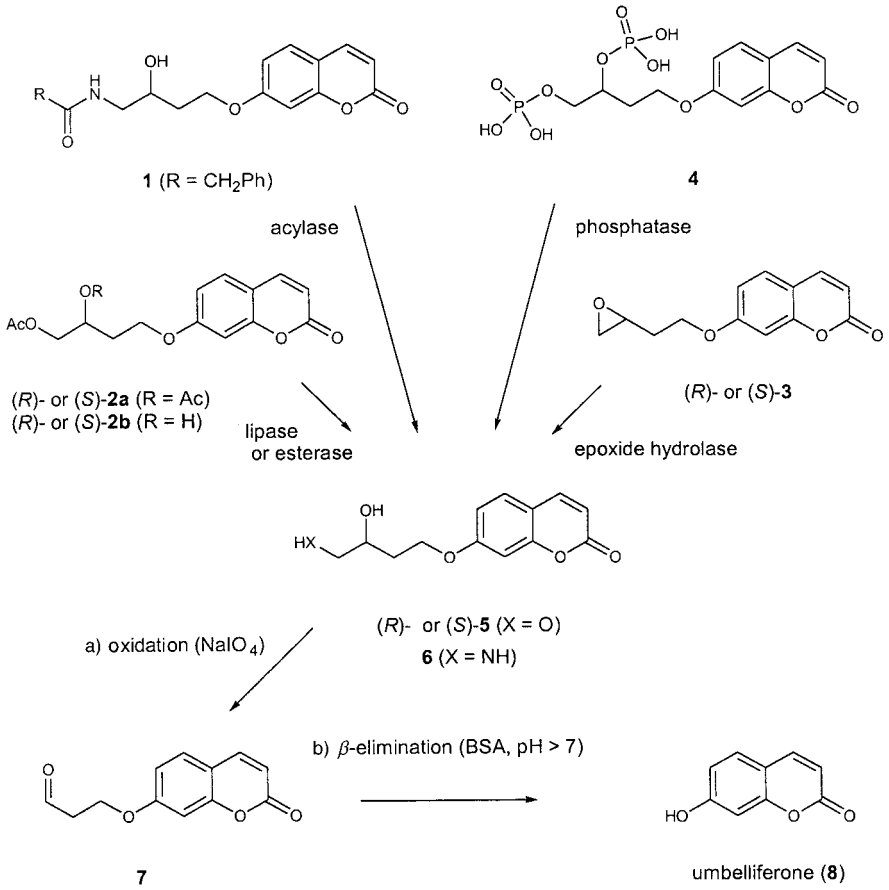

Scheme 1. Periodate-coupled fluorogenic assay for esterases and lipases, epoxide hydrolases, and amidases.

sodium periodate, a reagent known for cleaving diols and aminoalcohols with high chemoselectivity in the presence of other functional groups and used routinely for glycoprotein functionalization.[11] Therefore, we envisioned that hydrolytic enzymes releasing **5** or **6** from precursors resistant to periodate oxidation would become detectable by fluorescence in the presence of periodate and BSA. Amides such as **1** would react with acylases. Diesters (*R*)-**2a** and (*S*)-**2a** or the monoesters (*R*)-**2b** and (*S*)-**2b** should be excellent substrates for lipases and esterases due to their structural resemblance to glycerides.[12] Epoxides (*R*)-**3** and (*S*)-**3** would react with epoxide hydrolases and bis-phosphate **4** would react with phosphatases.

Diol **5** and epoxide **3** was prepared by OsO_4-catalyzed dihydroxylation or *meta*-chloroperbenzoic acid (MCPBA) epoxidation of the parent olefin, respectively. The enantiomers (*S*)-**5** (96.6% enantiomeric excess, *ee*) and (*R*)-**5** (99.2% *ee*) were obtained by dihydroxylation with ADmix α and ADmix β,[13] respectively, and converted into epoxides (*S*)-**3** (87% *ee*) and (*R*)-**3** (96% *ee*) by the one-pot Sharpless procedure[14] or acetylated to (*S*)-**2a** and (*R*)-**2a**. Partial acetylation of either (*S*)-**5** or (*R*)-**5** gave the monoacetates (*S*)-**2b** and (*R*)-**2b**. The racemic aminoalcohol **6** was obtained by aminolysis of the corresponding racemic epoxide **3** with aqueous ammonia[15] and converted to the phenylacetamide **1** by reaction with the acyl chloride. Bis-phosphate **4** was obtained from the racemic diol **5** by phosphorylation with dibenzyl *N*,*N*-diisopropyl phosphoramidite, oxidation with MCPBA, and catalytic hydrogenation (1 atm H_2, Pd/C, aq EtOH, 1 h).

Substrates **1**–**4** were found to be stable in the presence of periodate and BSA in aqueous buffers. By contrast, diol **5** and aminoalcohol **6** released umbelliferone (**8**) in 72% and 85% yield, respectively (100 μM substrate, 1 mM $NaIO_4$, 2 mg mL^{-1} BSA in pH 8.8 borate buffer). The yield of oxidation was independent of the presence of BSA. The reaction was fast ($t_{1/2} = 15$ min (**5**) and 3 min (**6**)) and would thus reveal any hydrolysis of substrates **1**–**4** within minutes.

Phenylacetamide **1** was tested with penicillin G acylase, chymotrypsin, and papain, acetates **2a** and **b** with 25 different esterases and lipases, epoxides **3** with epoxide hydrolase (EH) from *Aspergillus niger*[16] and *Rhodotorula glutinis*,[17] and bis-phosphate **4** with alkaline phosphatase (AP) from calf intestinal mucosa. A time-dependent increase in fluorescence was observed in the cases where a suitable enzymatic activity was present (Figure 1, Tables 1 and 2; similar results were obtained at pH 7.2). HPLC analysis confirmed that fluorescence originated in umbelliferone (**8**). The results were similar with or without preincubation with enzyme before addition of periodate and BSA, to show that these reagents did not affect the enzymes. HPLC analysis of the reactions without periodate and BSA showed that **5** and **6**

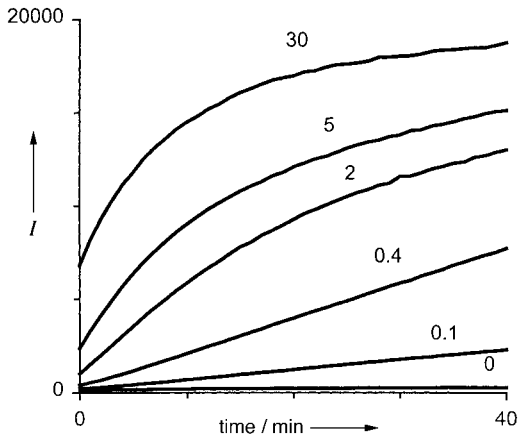

Figure 1. Development of fluorescence intensity (I, at $\lambda = 460$ nm) for the treatment of amide **1** (0.1 mM) at the indicated concentrations of penicillin G acylase (in μg mL^{-1}) in the presence of $NaIO_4$ (1 mM) and BSA (2 mg mL^{-1}) in aq borate buffer (20 mM, pH 8.8, 26 °C). Penicillin G acylase is known to cleave phenylacetamides. There was no reaction in the presence of chymotrypsin or papain (each 0.1 mg mL^{-1}). At high enzyme concentrations, the oxidation/β-elimination of **6** becomes rate limiting, so that the apparent release rate of **8** is not proportional to enzyme concentration.

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Table 1. Apparent reaction rates[a] of acetates **2a** and **2b** with lipases and esterases.

Lipase source	$V_{(R)\text{-}\mathbf{2a}}$ [pM s^{-1}]	$V_{(S)\text{-}\mathbf{2a}}$ [pM s^{-1}]	$E_{\mathbf{2a}}$[b]	$V_{(R)\text{-}\mathbf{2b}}$ [pM s^{-1}]	$V_{(S)\text{-}\mathbf{2b}}$ [pM s^{-1}]	$E_{\mathbf{2b}}$[b]
Aspergillus niger	40	265	6.5 (*S*)	3150	3500	1.11 (*S*)
Aspergillus oryzae	–	–	–	1230	1510	1.23 (*S*)
Candida antarctica	4230	3940	1.07 (*R*)	950	1490	1.57 (*S*)
Candida cylindracea	–	–	–	435	545	1.25 (*S*)
Chromobacterium visc.	150	510	3.44 (*S*)	4530	5950	1.31 (*S*)
Pig pancreas	245	400	1.62 (*S*)	1490	3190	2.14 (*S*)
Mucor miehei	–	95	only *S*	670	720	1.08 (*S*)
Pseudomonas cepacia	250	580	2.31 (*S*)	690	1530	2.22 (*S*)
Pseudomonas fluorescens	3820	4290	1.12 (*S*)	13200	16700	1.27 (*S*)
Pseudomonas sp.	2080	1850	1.12 (*R*)	5870	8860	1.51 (*S*)
Pseudomonas sp. Type B	4840	5640	1.17 (*S*)	18100	23600	1.30 (*S*)
Rhizomucor miehei	–	–	–	640	610	1.05 (*R*)
Rhizopus niveus	–	–	–	350	540	1.54 (*S*)
Wheat germ	85	–	only *R*	1330	2170	1.63 (*S*)
Esterase source						
Bacillus sp.	–	–	–	245	–	only *R*
Bac. stearothermophilus	1520	330	4.59 (*R*)	2010	1580	1.27 (*R*)
Bac. thermoglucosidasius	170	55	2.82 (*R*)	640	415	1.54 (*R*)
Horse liver	1570	3790	2.41 (*S*)	4460	7530	1.69 (*S*)
Mucor miehei	–	75	only *S*	265	405	1.54 (*S*)
Thermoanaerobium brockii	–	–	–	215	355	1.66 (*S*)

[a] Apparent rate V as observed in the presence of substrate (0.1 mM), $NaIO_4$ (1 mM), BSA (2 mg mL^{-1}), and lipase or esterase (0.1 mg mL^{-1}), in aq borate (20 mM, pH 8.8, 26 °C). Fluorescence was converted into umbelliferone concentration according to a calibration curve with pure **8** in the same buffer containing sodium periodate and BSA. The error margin on V is ±10 pM s^{-1}. The apparent rate of umbelliferone release from diol **5** is 64300 pM s^{-1}. No data are given when the activity was not distinguishable from the fluorescence curve of substrate without enzyme ($V < 20$ pM s^{-1}). [b] E = enantiomeric ratio between $V_{(R)}$ and $V_{(S)}$. Lipases and esterases *Penicillium roqueforti*, *Mucor javanicus*, *Candida lipolytica*, *Saccharomyces cerevisiae*, and *Rhizopus arrhizus* gave no reaction with either **2a** or **2b**:.

Table 2. Apparent reaction rates with substrates **1** and **3**–**6**.[a]

Substrate	Enzyme	V_{app} [pM s^{-1}]
6	no enzyme	58400
1	Penicillin G acylase (1 μg mL^{-1})	12820
1	α-Chymotrypsin from bovine pancreas (100 μg mL^{-1})	15
1	Papain (100 μg mL^{-1})	−10
1	no enzyme	35
5	no enzyme	46300
3	EH from *Aspergillus niger* (42.0 μg mL^{-1})	20940 (*R*), 23230 (*S*)
3	EH from *Aspergillus niger* (1.3 μg mL^{-1})[b]	1785 (*R*), 2940 (*S*)
3	EH from *Rhodotorula glutinis* (100 μg mL^{-1})	2845 (*R*)[c], 1570 (*S*)[c]
3	no enzyme	720
4	AP from calf intestinal mucosa (100 μg mL^{-1})	565[d]
4	no enzyme	40

[a] Apparent rate of umbelliferone release as observed in the presence of substrate (0.1 mM), $NaIO_4$ (1 mM), and BSA (2 mg mL^{-1}) in aq borate buffer (20 mM, pH 8.8). Fluorescence was converted into umbelliferone concentration according to a calibration curve with pure **8** in the same buffer containing $NaIO_4$ and BSA. The error margin on V is ±20 pM s^{-1}. Similar results were obtained at pH 7.2. [b] Detection limit for the epoxyde hydrolase is 1 μg mL^{-1}. [c] Values at 37 °C, the reported optimal temperature for this enzyme, are $V_{(R)\text{-}\mathbf{3}} = 3500$ and $V_{(S)\text{-}\mathbf{3}} = 2000$ pM s^{-1}. [d] Apparent rate observed in phosphate (20 mM, pH 7.2) is $V_4 = 4380$ pM s^{-1}.

were indeed released by the enzymes marked as active in the fluorescence assay. The assay was specific in that no fluorescence signal was observed when using heat-deactivated enzymes.[18, 19]

The above experiments show that a versatile fluorogenic assay for hydrolytic enzymes is available using the secondary processing of reaction products **5** or **6** to the fluorescent product **8** by sequential periodate oxidation and BSA-catalyzed β-elimination. This assay is also the first system for fluorescence detection of epoxide hydrolase activity.

Most commercially available fluorogenic enzyme substrates are reactive derivatives of phenols and anilines. Their chemical reactivity makes them completely unstable under certain screening conditions, such as elevated temperature or impure extracts,[1a] and furthermore risks any high-throughput screening based on these products of isolating catalysts that are not capable of cleaving less-reactive substrates of synthetic interest. Our periodate-coupled assay overcomes these limitations by using completely nonactivated amides, esters, epoxides, and phosphates of aliphatic alcohols and amines.[20] In addition, and in contrast to our previous assay based on alcohol dehydrogenase,[21] the periodate oxidation scheme can be readily extended to include a broad variety of substrates related to **1**–**4** but bearing different substituents and includes further reaction types, that produce diol **5**, aminoalcohol **6**, or related compounds, as products. These advantages, together with the possibility of using optically pure substrates for assaying enantioselectivity, make the present assay very attractive for screening catalysis.

Experimental Section

The *ee* of (*R*)- and (*S*)- diols **5** and epoxides **3** were determined by analysis on a chiral HPLC column OD-H (Daicel; 25 cm × 0.46 cm i.d.) using an hexane/*i*PrOH (8/2) eluent; the *R* isomers eluted first in each case. The absolute configuration of diols (*R*)- and (*S*)-**5** were assigned on the basis of the Sharpless mnemonic device applied to the parent olefin. Monoacetates (*R*)- and (*S*)-**2b** and diacetates (*R*)- and (*S*)-**2a** were prepared from the corresponding (*R*)-**5** and (*S*)-**5** diols. The configuration of the diols (*R*)-**5** and (*S*)-**5** is maintained in the corresponding epoxides (*R*)-**3** and (*S*)-**3**. (*R*)-**3**: m.p. 60–63 °C; $[\alpha]_D^{20} = +21.8$ (*c* 0.45, $CHCl_3$); elemental analysis for $C_{13}H_{12}O_4$: calcd: C 67.23, H 5.21; found: C 67.39, H 5.52. (*S*)-**3**: m.p. 61–64 °C; $[\alpha]_D^{20} = -23.0$ (*c* 0.3, $CHCl_3$); elemental analysis: found: C 66.97, H 5.08; ^{1}H NMR ($CDCl_3$): $\delta = 7.58$ (d, 1H, $J = 9.3$ Hz), 7.31 (d, 1H, $J = 8.3$ Hz), 6.75–6.81 (m, 2H), 6.18 (d, 1H, $J = 9.8$ Hz), 4.03–4.19 (m, 2H), 3.05–3.14 (m, 1H), 2.78 (t, 1H, $J = 4.9$ Hz), 2.53 (dd, 1H, $J = 4.9$, 2.4 Hz), 2.04–2.20 (m, 1H), 1.79–1.97 (m, 1H). (*R*)-**5**: m.p. 94–96 °C; $[\alpha]_D^{20} = +17.9$ (*c* 0.55, CH_3OH); elemental analysis for $C_{13}H_{14}O_5$: calcd: C 62.39, H 5.64; found: C 62.12, H 5.41. (*S*)-**5**: m.p. 92–93 °C; $[\alpha]_D^{20} = -22.4$ (*c* 0.46, CH_3OH); elemental analysis: found: C 62.11, H 5.87; ^{1}H NMR (CD_3OD): $\delta = 7.83$ (d, 1H, $J = 9.8$ Hz), 7.47 (d, 1H, $J = 8.3$ Hz), 6.85–6.92 (m, 2H), 6.21 (d, 1H, $J = 9.3$ Hz), 4.16–4.23 (m, 2H), 3.81–3.93 (m, 1H), 3.54 (d, 2H, $J = 6.3$ Hz), 1.97–2.13 (m, 1H), 1.74–1.91 (m, 1H). (*R*)-**2b**: semisolid; $[\alpha]_D^{20} = +9.3$ (*c* 0.3, $CHCl_3$); elemental analysis for $C_{15}H_{16}O_6$: calcd: C 61.64, H 5.52; found: C 61.91, H 5.79. (*S*)-**2b**: semisolid; $[\alpha]_D^{20} = -9.6$ (*c* 0.31, $CHCl_3$); elemental analysis: found: C 61.86, H 5.77; ^{1}H NMR ($CDCl_3$): $\delta = 7.61$ (d, 1H, $J = 9.8$ Hz), 7.33 (d, 1H, $J = 7.8$ Hz), 6.78–6.83 (m, 2H), 6.21 (d, 1H, $J = 8.8$ Hz), 4.01-4.29 (m, 5H), 2.09 (s, 3H), 1.91–2.00 (m, 2H). (*R*)-**2a**: m.p. 76–78 °C; $[\alpha]_D^{20} = +9.6$ (*c* 0.65, $CHCl_3$); elemental analysis for $C_{17}H_{18}O_7$: calcd: C 61.07, H 5.43; found: C 60.84, H 5.21. (*S*)-**2a**: m.p. 74–76 °C; $[\alpha]_D^{20} = -9.8$ (*c* 0.5, $CHCl_3$); elemental analysis: found: C 61.15, H 5.69; ^{1}H NMR ($CDCl_3$): $\delta = 7.61$ (d, 1H, $J = 9.8$ Hz), 7.34 (d, 1H,

$J = 8.8$ Hz), 6.74 – 6.82 (m, 2H), 6.22 (d, 1H, $J = 9.3$ Hz), 5.24 – 5.35 (m, 1H), 4.32 (dd, 1H, $J = 11.7$, 3.4 Hz), 3.97 – 4.15 (m, 2H), 4.10 (dd, 1H, $J =$ 11.7, 5.9 Hz), 2.05 – 2.16 (m, 2H), 2.05 (s, 6H). **4**: ^{1}H NMR (D_2O, 300 MHz): $\delta = 7.88$ (d, 1H, $J = 9.2$ Hz), 7.50 (d, 1H, $J = 8.5$ Hz), 6.96 – 6.91 (m, 2H), 6.23 (d, 1H, $J = 9.2$ Hz), 4.53 (m, 1H), 4.20 (m, 2H), 4.00 (m, 2H), 2.12 (m, 2H); ^{13}C NMR (D_2O/CD_3COCD_3, 75 MHz): $\delta = 164.6$, 163.2, 156.3, 146.8, 130.8, 114.6, 113.9, 112.9, 102.5, 66.0, 60.7, 58.2, 32.2; ^{31}P NMR (D_2O, 81 MHz): $\delta = 4.1$ (d, $J = 16.2$ Hz), 3.3 (d, $J = 16.2$ Hz); FAB-MS: 410 [M^+], 433 [$M + Na^+$]. All other new compounds gave satifsfactory spectral data.

All substrates and products prepared as 1 mM stock solutions in 50 % aq. acetonitrile. All reagents and buffers were prepared in deionized milliQ water. Enzymes were diluted from stock solutions (1 mg mL^{-1}) in phosphate buffer saline (PBS; 160 mM NaCl, 10 mM phosphate, pH 7.4), BSA from a stock solution (40 mg mL^{-1}) in buffer (20 mM borate, pH 8.8) or PBS. $NaIO_4$ was diluted from a freshly prepared stock solution in water (10 mM). Assays (0.1 – 0.2 mL) were followed in individual wells of round-bottom polypropylene 96-well plates (Costar) using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems, filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm). Commercial enzyme preparations were purchased from Fluka or Sigma.

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Formation of a Giant Supramolecular Porphyrin Array by Self-Coordination**

Kazuya Ogawa and Yoshiaki Kobuke*

Methodologies for the preparation of multi-porphyrin arrays[1–5] have been exploited to successfully assemble arrays of more than ten porphyrins.[6] The formation of extremely long porphyrin arrays by supramolecular coordination[7] and by covalently linking[8] porphyrins have recently been reported. We have been trying to extend our previous methodology of self-coordination[9] and we report here a giant multi-porphyrin array formed by the accumulation of dimeric imidazolyl-substituted Zn porphyrins. These systems should have the excellent photophysical properties of a chlorophyll substitute and enable the transfer of excitation energy to be studied.

Meso-(*N*-methyl)imidazolylporphyrinatozinc compounds such as **1b** afforded quantitatively a dimer of a slipped cofacial orientation **2** by the complementary coordination of an imidazolyl group to the Zn^{II} center (Scheme 1).[9] The coordination was very strong, but the pentacoordinating Zn^{II} ion prohibited further extension of the coordination structure.

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