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A High-Throughput, Low-Volume Enzyme Assay on Solid Support

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A high-throughput enzyme assay is described that uses 1 μL or less of enzyme solution for each test. Enzyme solutions are deposited by robotic handling in a throughput of over 1000 tests/h on the surface of silica gel plates that have been preimpregnated with fluorogenic substrates. The reaction is quantitated by fluorescence. The method is compatible with water-insoluble substrates (lipases), water-soluble substrates (glycosidases), whole-protein substrates (proteases), and enzyme inhibition measurements. Hydrolytically labile umbelliferyl esters can be used to assay lipases in this format without background hydrolysis. High throughput and reproducibility were tested by fingerprint analysis of lipases and esterases against 37 different fluorogenic ester substrates. A set of eight fluorogenic unbelliferyl esters was selected for optimal activity screening of lipases and esterases on silica gel plates.

High-throughput enzyme assays are essential tools in enzyme engineering and drug discovery. Such assays are most often spectroscopic assays based on chromogenic or fluorogenic substrates or sensors.[1] One problem encountered in high-throughput screening is to reliably assay many different samples at the lowest possible cost and with very good accuracy. This can be realized by minimizing sample volume and optimizing liquid handling, for example, by using microtiter plates (MTPs) and multichannel pipets. Assays in MTPs have the drawback that two different solutions must be combined and evenly mixed in each well. Pipetting and mixing operations are difficult to carry out in small volumes, and artifacts due to solvent evaporation and substrate precipitation cannot be controlled easily. Herein we report a method for screening enzymes using silica gel plates impregnated with fluorogenic substrates. The method ensures even distrubution of the substrate to each enzyme sample and enables high throughput (>1000 tests/h) while using small reaction volumes (1 μL/assay). The assay is demonstrated for lipases, esterases, glycosidases, and proteases. High throughput and reproducibility were tested by fingerprint analysis of lipases and esterases with fluorogenic ester substrates. Data analysis led to an optimized set of eight substrates for screening lipases and esterases.

RESULTS AND DISCUSSION

Assay Development for Lipases and Esterases (Water-Insoluble Substrates). Lipases and esterases are interfacial enzymes hydrolyzing fatty acid esters, in particular triglycerides. High-throughput assays for these enzymes are mostly based on chromogenic nitrophenyl esters or fluorogenic umbelliferyl esters of fatty acids.[1] However, these substrates are only poorly soluble in water, requiring the addition of cosolvents such as DMF or DMSO to react, and hydrolyze spontaneously or in the presence of noncatalytic proteins.[2] Chemically stable analogues of these chromogenic and fluorogenic esters are known that are compatible with extreme pH and temperature conditions and only react with active enzymes.[3,4] However, these chemically stable substrates also show limited solubility in water and still require a cosolvent to react with the enzymes. We reasoned that an assay format providing a large contact surface area between the substrate and the enzyme might enable an insoluble substrate to react with interfacial enzymes such as lipases and esterases. Because noncatalytic proteins might not be able to solubilize the substrates at the interface, this assay format might show reduced background hydrolysis even with reactive substrates.

Interfacial assay conditions were investigated using substrates adsorbed on porous surfaces such as cellulose-based filter paper, silica gel, alumina, or reversed-phase-C18 TLC plates. We found that lipase substrates **1a/b**−**20a/b** (Chart 1), comprising esters and acyloxymethyl ethers of umbelliferone and 4-methylumbelliferone,[5] could be uniformly distributed on the surface of silica gel TLC glass plates by simply soaking the plates in a dichlo-

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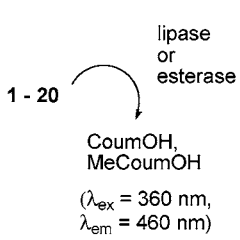
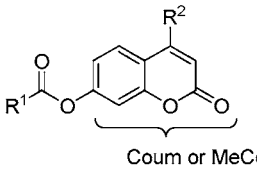
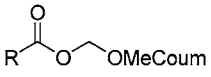
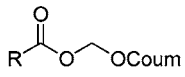
Chart 1. Fluorogenic Ester Substrates Used for Solid-Supported Enzyme Assay







R =
Et **17b**
i-Pr **18b**
c-Pr **19b**
nonyl **20b**



20a (R = nonyl)

R^1	R^2 = H	R^2 = Me
Pr	**1a**	**1b**
i-Pr	**2a**	**2b**
t-Bu	**3a**	**3b**
c-Bu	**4a**	**4b**
pentyl	**5a**	**5b**
c-hexyl	**6a**	**6b**
heptyl	**7a**	**7b**
hept-3-yl	**8a**	**8b**
nonyl	**9a**	**9b**
undecanyl	**10a**	**10b**
tridecanyl	**11a**	**11b**
pentadecanyl	**12a**	**12b**
heptadecanyl	**13a**	**13b**
phenyl	**14a**	**14b**
phenylmethyl	**15a**	**15b**
adamant-1-yl	**16a**	**16b**

romethane solution of the substrate and drying. The substrate-loaded plates were stable for at least two weeks when stored in a dry package at −20 °C. Addition of a small drop (1 μL) of enzyme solution (lipase or esterase, 0.1 mg mL^{-1}) readily induced a reaction, which was detected after a few minutes by the appearance of a bright blue fluorescence indicating the formation of umbelliferone. By contrast, addition of buffer only or a solution of a noncatalytic protein such as bovine serum albumin (BSA, 1 mg mL^{-1}) did not produce a detectable reaction. Addition of glycerol (30% v/v) retarded solvent evaporation, so that even weakly reactive enzymes could be detected by extending incubation times up to 24 h. Under these conditions, there was minimal (<10%) hydrolysis with either BSA or buffer alone. Enzyme activities in the silica gel plate format were comparable to those observed with the same substrates in a classical MTP setup,[6] indicating that the enzymes were not deactivated by the silica gel.

Enzyme solutions were delivered to the substrate-loaded silica gel plates using a liquid-handling HPLC-autosampler robot (Figures 1 and 4). Spotting of 1-μL aliquots formed regular spots of ~3-mm diameter, allowing 144 assays for a 5 × 5 cm plate (Figure 2). Product formation was either judged qualitatively by visual inspection in a TLC−UV chamber or quantitated using a fluorescence MTP reader adapted with a custom-made plate holder and plate-reading routine. When the same enzyme solution was dispensed repetitively onto the entire surface of a substrate-loaded silica gel plate, there was ~±10% fluctuation in the fluorescence reading obtained in each assay across the entire plate. Variations probably reflected slightly uneven substrate distribution and plate surface inhomogeneity.[7]

(6) % v/v DMSO was added as cosolvent in the reference solution assay, which also showed a high level of nonspecific background hydrolysis.

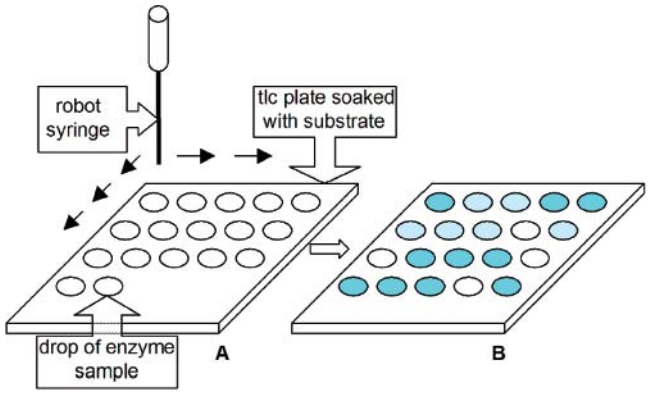


Figure 1. Principle of enzyme assay on silica gel plates.

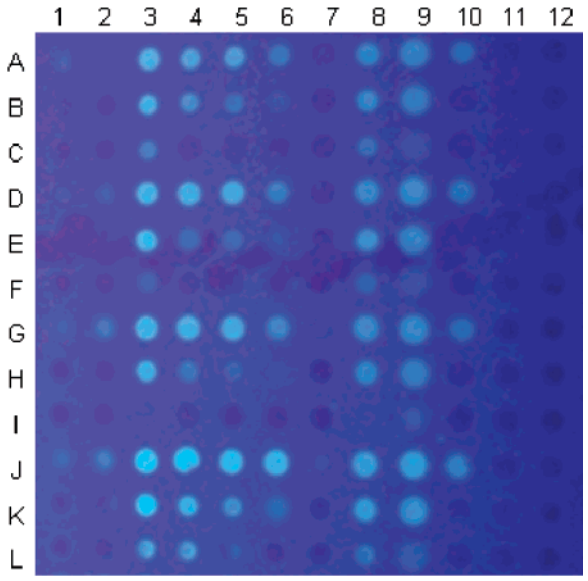


Figure 2. Photo of an enzyme assay on silica gel plate. The plate was soaked with substrate **19b** (2 mM in CH_2Cl_2) and dried. The following enzymes (1 μL/spot) were spotted (100/10/1 μg mL^{-1} in rows A/B/C, D/E/F, G/H/I, J/K/L) Columns: 1, RNL; 2, PPL; 3, E1; 4, E2; 5, PCL2; 6, MML; 7, L7; 8, L8; 9, L9; 10, L10. BSA (1 mg mL^{-1}) was spotted in columns 11 and 12. Rows: ABC, PBS (10 mM phosphate, 160 mM NaCl, pH 7.4); DEF, PBS + 30% v/v MeOH; GHI, PBS + 10% v/v glycerol; JKL, PBS + 30% v/v glycerol. The picture was taken after 120 min at 25 °C.

Glycosidase Assay (Water-Soluble Substrates). Although initially designed for water-insoluble substrates, the assay also proved suitable for water-soluble substrates, as shown for the case of glycosidases. Silica gel plates impregnated with four different 4-methylumbelliferyl glycosides (α-glu, β-glu, α-gal, β-gal) were prepared by soaking the plates in solutions of the substrates in tetrahydrofuran. These plates were remarkably stable and could be kept for several weeks at room temperature without noticeable degradation of substrate. The glycosidase activity assays were carried out by spotting the corresponding glycosidase enzymes in aqueous HEPES buffer. Product formation was visible as a bright fluorescent blue ring under UV after 5 min. The reaction or each of the different glycoside substrates only took place with the corresponding glycosidase, and there was no reaction with

(7) Fluorescence reading of an empty silica gel plate showed a background fluorescence signal or up to 5% of the signal recorded when reading the assays.

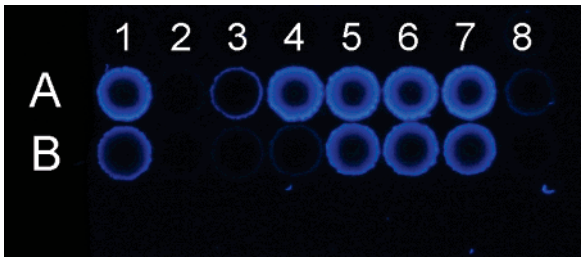


Figure 3. β-Galactosidase activity and inhibition on silica gel plate. The plate was soaked with a 1 mM solution of 4-methyl-umbelliferyl β-D-galactoside in THF and dried. A solution of β-galactosidase from *Aspergillus oryzae* (Sigma G5160, 255 000 units/mg) in 0.1 M HEPES buffer pH 6.8 was spotted (1 μL/spot). The picture was taken after 10 min at 25 °C. Lane A 100 μg/mL enzyme; lane B, 10 μg/mL enzyme. Column 1, no inhibitor, Inhibitor concentrations (μM): column 2, 100; 3, 10; 4, 1; 5, 0.1; 6, 0.01; 7, 0.001; 8A, 1 μg/mL enzyme, no inhibitor; 8B, buffer control.

Figure 4. Autosampler with custom-made MTP/silica gel plate holder. An HPLC needle is mounted on the XY arm at the left and is connected to the washing pump (center). The needle takes 30 μL from one of the MTP wells, distributes 1 μL onto each of the 22 silica gel plates, and goes to a washing cycle before resuming distribution with the next MTP well.

mismatched glycosidase or with BSA. The concentration of enzyme necessary to induce the reaction was the same as for the assay in MTPs.

Formation of fluorescent rings showed that the water-soluble substrates underwent latteral diffusion upon spotting. The ring-shaped fluorescence was suitable for qualitative reading but could not be quantified automatically with the MTP reader. Homogeneous spots without lateral diffusion could be obtained by spotting the enzyme solution manually in much smaller volumes (~100 nL) using a glass capillary. Unfortunately, this procedure could not be automated with the available robot. The possibility of tracking enzyme inhibition was tested using an aminocyclopentitol-type inhibitor for β-galactosidases.[8] A buffered solution of β-galactosidase was treated with the inhibitor at various concentrations and spotted on a TLC plate impregnated with 4-methylumbelliferyl β-D-galactoside substrate. Indeed. there was no measurable activity in the presence of the inhibitor, while the control showed full activity (Figure 3).

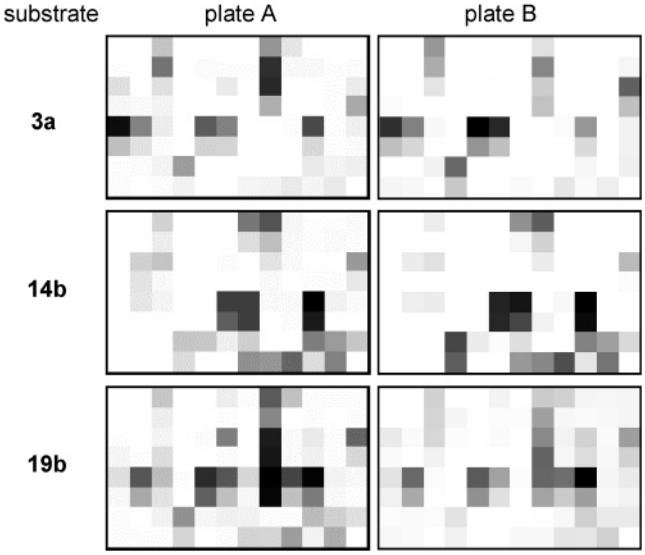


Figure 5. Reproducibility of activity patterns on substrate-impregnated silica gel plates. The net fluorescence is reported as gray scale (white 0, black maximum) after substraction of background (sample B1 with BSA, ~10% of maximum signal). Plate A: 1-μL drops (PBS, 30% v/v glycerol) were spotted. After 24 h at 25 °C, fluorescence intensity was recorded using a fluorescence MTP reader (λ_{ex} = 360 nm, λ_{em} = 460 nm). Plate B: the same experiment was repeated 2 weeks later. The following samples were spotted: A1, fluorescein reference; B1, BSA 1 mg mL^{-1} ; A2−A12, C1−C12, E1−E12, G1−G3, different lipases and esterases, 100 μg mL^{-1} ; B2−B12, D1−D12, F1−F12, H1−H3, the same enzyme series at 10 μg·mL^{-1} ; G5-G12, H5−H12, recombinant lipases.

Protease Assay (Whole-Protein Substrate). The assay was further extended to a protease assay to demonstrate the use of whole-protein substrates. We used a fluorescence assay recently developed in our group based on the indirect detection of free amino acids using the copper−calcein complex.[9] This assay can be used to assay proteases using BSA as substrate. BSA-impregnated silica gel plates were obtained by soaking the plates for a few seconds in a 1 mg mL^{-1} solution of BSA in PBS buffer, followed by drying under vaccuum. The assay was carried out by spotting solutions of three different proteases (trypsin, elastase, and chymotrypsin at 100 μg mL^{-1}) in aqueous buffer with 30% v/v glycerol containing the fluorescence quenched copper−calcein complex, followed by overnight incubation. Fluorescence increase indicative of proteolysis were not visible by the eye under UV but could be detected using the MTP reader at the appropriate wavelength (λ_{ex} = 470 nm, λ_{em} = 509 nm). Proteases reproducibly gave a 100 ± 30% fluorescence increase compared to the fluorescence signal without protease or with a nonproteolytic enzyme. This signal modulation was weaker than for the same assay in solution (>800% increase) but would be sufficient in the context of simple activity detection assays.

High-Throughput Measurements. High throughput and reproducibility of the silica gel plate assay were tested by activity profiling of fluorogenic ester substrates **1a/b**−**20a/b** against a series of lipases and esterases. A custom-made plate holder allowed robotic pipetting from a 96-well plate into 22 different 5

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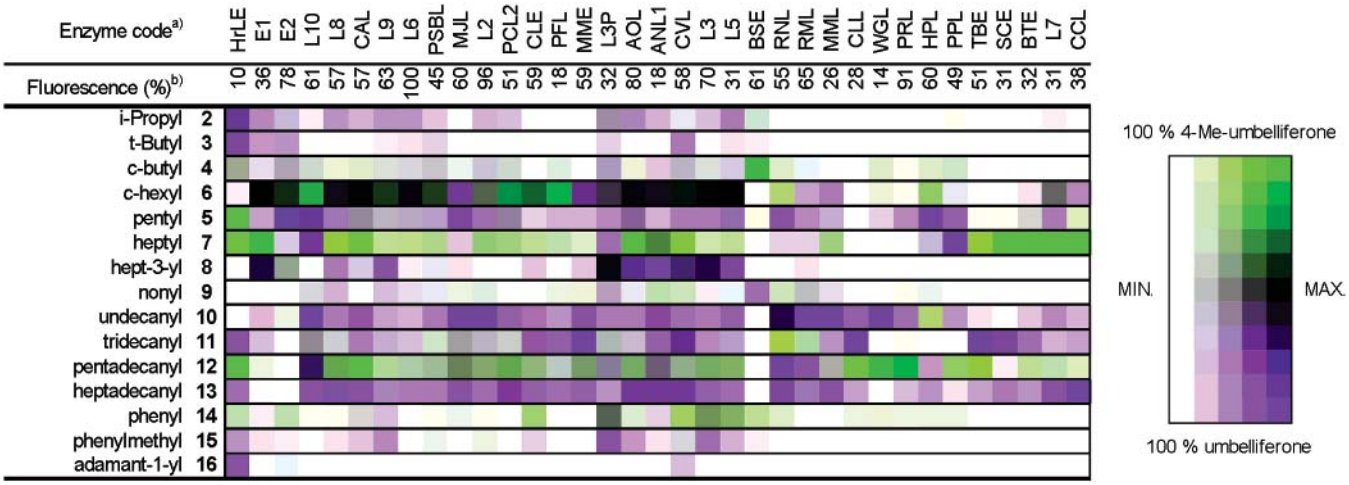

Figure 6. Color-coded activity patterns of fluorogenic esters substrates against lipases and esterases using the silica gel plate assay. Enzyme solutions (0.1 mg mL^{-1} in PBS + 30% v/v glycerol) were spotted (1 μL) on silica gel plates preimpregnated with each substrate (soaking in 2 mM substrate solution in CH_2Cl_2 and drying). The fluorescence signal (λ_{ex} = 360, λ_{em} = 460 nm) was recorded after 24 h at 25 °C. (a) For abbreviation for enzyme use, see Supporting Information. (b) Percentage of maximum observable fluorescence signal observed with the most reactive substrate for each enzyme. The color-coding is calculated relative to this maximum for each column (color code at right). Substrates are ordered by similarity analysis of their reactivity pattern against the different enzymes.

× 5 cm silica gel substrate-loaded silica gel plates (Figure 5). The 96-well plate was filled (200 μL/well) with 96 test solutions with various enzymes and controls (Figure 6). The entire 96-well plate was transferred onto 37 different substrate-loaded silica gel plates in duplicate, amounting to 7104 spotting operations of 1 μL each and 3404 different enzyme reactions. Dispensing of the 96-well plate to 22 silica gel plates was completed in ~3 h, so that the entire double measurement was completed in ~12 h of robot operating time, consuming less than 0.1 mL of each enzyme test solution and less than 0.2 mg of substrate per 5 × 5 cm silica gel plate. Product formation in each spot was quantitated by measuring fluorescence intensity using the fluorescence MTP reader (see above).

Reproducibility was judged by comparing the activity patterns produced by the 96 test samples onto two silica gel plates of the same substrate (Figure 5). Similarity analysis of activity patterns by data clustering showed that two activity measurements with the same substrate were always more similar to one another than to a different substrate. Visual inspection confirmed that the reactivity pattern of each substrate across the 96 test samples was indeed very well conserved between the two measurements. Nevertheless, due to the significant activity fluctuations observed in individual cases, duplicate measurements might be preferable for high-throughput screening with this method.

Optimal Substrate Set for Lipases and Esterases. Clustering of enzyme activity patterns showed that several substrates behaved identically across all enzymes tested. A subset of 15 functionally different acyl groups was selected, and the enzyme reactivity data for the corresponding umbelliferyl ester and 4-methyl umbelliferyl ester was measured for 35 lipases and esterases and computed as enzyme activity fingerprints (Figure 6).[10] The fingerprint data showed that six substrates were sufficient to cover most of the observed reactivity, namely, the C6, C12, and C18,ester of umbelliferone (**5a**, **10a**, **13a**) and the cyclohexanecarboxylic, C8, and C16,ester of 4-methylumbelliferone (**6b**, **7b**, **12b**). In addition the isobutyryl ester of umbelliferone **2a** and cyclobutylcarboxylic ester of 4-methylumbelliferone **4b** showed singular reactivites with esterases. This subset of eight different substrates (**2a**, **4b**, **5a**, **6b**, **7b**, **10a**, **12b**, **13a**) might be sufficient for a broad-based detection of interfacial lipase and esterase activities using the silica gel plate assay.

CONCLUSION

The experiments above demonstrate a high-throughput enzyme assay based on fluorogenic substrates adsorbed on silica gel plates. The reactivity of the enzymes at the silica gel surface appears to be similar to that in solution. Evaporation of water from the silica gel plate limits the reaction time to ~15 min without cosolvent and several hours in the presence of glycerol. The assay is compatible with water-insoluble, water-soluble, whole-protein substrates, and enzyme inhibition measurements. Water-insoluble lipase substrates were found to react at the surface of silica gel plates without addition of any cosolvent. Under these conditions, the background hydrolysis of these rather labile umbelliferyl esters was negligible. It should be noted that nitrophenyl esters were not stable at the silica gel surface and also did not yield a sufficiently strong color for product detection.

The silica gel plate assay presented here offers a practical alternative to microtiter plate-based high-throughput screening and allows miniaturization by ~100-fold (100 to 1 μL assay) at essentially no cost. The substrate conditioning method is broadly applicable with fluorogenic substrates and sensors. This simple method might be preferable to more sophisticated assays in microarray format, which require complex substrate attachement or dispensing procedures,[11] and are based on microarrayers and microarray scanners that are still relatively expensive instruments.

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EXPERIMENTAL SECTION

Preparation of Substrate-Impregnated Silica Gel Plates and Dispensing of Enzyme Samples. Lipases and Esterase Assay. Silica gel TLC glass plates G-25 (Macherey-Nagel (silica gel 40−63 μm, surface 550 m^2/g, layer 0.25 mm) were soaked in a solution of substrate (**1a/b**−**20a/b**, 2 mM in CH_2Cl_2) for 2 min and dried. The 1-μL aliquots of enzyme samples (in PBS: 10 mM phosphate, 160 mM NaCl, pH 7.4) with 30% v/v glycerol) were spotted at 25 °C. Product formation was recorded after 2−24 h.

Glycosidase Assay. Silica gel TLC glass plates were soaked in a solution of 4-methylumbelliferyl glycoside (2 mM in THF) for ~2 min and dried. Enzyme samples (~100 nL) in 0.1 M HEPES buffer pH 6.8 were spotted manually using a thin glass capillary. Product formation was measured by digital photos under UV after 15 min.

Protease Assay. Silica gel TLC glass plates were soaked in a solution of BSA (0.1 mg mL^{-1} in PBS) for 10 s and dried under vaccuum. The proteases (trypsin, chymotrypsin, elastase, thermolysin, each 100 μg mL^{-1}) were conditioned in PBS + 30% v/v glycerol containing 10 μM $CuCl_2$, 1 μM calcein (F 21039) and spotted as a 1-μL drop using the autosampler. Fluorescence was recorded after 24 h at 25 °C using a fluorescence MTP reader (λ_{ex} = 470 nm, λ_{em} = 509 nm). All plates prepared for assay were used immediately or were stored in a drybox in a refrigerator at −20 °C for later use. The maximum storage time tested was 2 weeks, after which there was no noticeable degradation.

Autosampler. Automatic sample delivery was carried out with a Gilson 233XL on-line column switching and Gilson 402 syringe pump with Gilson 735 sampler software v. 5.20.

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SUPPORTING INFORMATION AVAILABLE

Synthesis of **1**−**20a/b**; List of commercially available lipases and esterases; spectral data, ^1H NMR and ^{13}C NMR, for **1**−**20a/b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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