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Spectrophotometric Substrates for Cytosolic Epoxide Hydrolase[1]

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In this study, we demonstrate the utility of a broad class of spectrophotometric substrates for the assay of cytosolic epoxide hydrolase purified from murine liver. These substrates, epoxy esters or carbonates, cyclize spontaneously upon or during hydrolysis of the epoxide functionality. The alcohol released by cyclization may then be assayed directly or by coupling to a second reaction. The alcohol produced, or its secondary reaction products, can be selected to give an absorption in the visible or near-uv range of the spectrum. This allows the synthesis of a wide variety of useful spectrophotometric substrates. 4-Nitrophenyl (2*S*,3*S*)-2,3-epoxy-3-phenylpropyl carbonate, at pH 6.4 and 25°C, had a V_{max} of 22 μmol min^{-1} mg^{-1} and a K_m of 16 μM when assayed with a conventional spectrophotometer. When assayed under the same conditions with a 96-well plate reader, the measured V_{max} was 15 μmol min^{-1} mg^{-1} and the K_m was 6.6 μM. Some of these compounds were also found to be substrates for glutathione *S*-transferase, microsomal epoxide hydrolase, and porcine liver carboxylesterase. Indeed, 4-nitrophenyl 3,4-epoxy-3-phenylbutanoate was a 3.4-fold better substrate for porcine liver carboxylesterase than 4-nitrophenyl acetate when initial rates of hydrolysis were measured under the same conditions. © 1994 Academic Press, Inc.

Until recently no readily employed, sensitive spectrophotometric assay has been available for the determination of cytosolic epoxide hydrolase (CEH)[5] activity (1). The standard substrate for cytosolic epoxide hydrolase, EC 3.3.2.3, has been *trans*-stilbene oxide (TSO). The structure and kinetic properties of TSO are shown in Table 1. The *cis*-isomer (CSO) is often used as a substrate for MEH, the microsomal epoxide hydrolase (2–5). The TSO and CSO assays, while sensitive and dependable, involve the use of tritium-labeled substrate and a partitioning step against iso-octane and are endpoint assays (2,3). Partition assays are also relatively time consuming and are not easily adaptable to a large variety of different substrates since a radiolabeled substrate must be synthesized and the partitioning step depends on a large difference in solubility between the epoxide substrate and the diol product (2,6). In principle, the use of radiolabeled substrates can be easily avoided and the range of useful substrates greatly enlarged. However, this requires the development of a thin-layer chromatography (TLC), gas chromatography (GC), or high-performance liquid chromatography (HPLC) assay (2). While GC and HPLC assays can be automated to some extent, these assays are also endpoint assays and are very time consuming.

Unlike spectrophotometric assays available for MEH (2,7–9), spectrophotometric assays for CEH have been typified by low sensitivity and/or interference from

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[5] Abbreviations used: CEH, cytosolic epoxide hydrolase; TSO, *trans*-stilbene oxide; MEH, microsomal epoxide hydrolase; CSO, *cis*-stilbene oxide; EQU-5, *trans*-1,2-epoxy-1-(2-quinolyl)pentane; ENP-5, *trans*-1,2-epoxy-1-(4-nitrophenyl)pentane; PLE, porcine liver carboxylesterase; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TMS, trimethylsilyl; S-NEPC, 4-nitrophenyl (2*S*,3*S*)-2,3-epoxy-3-phenylpropyl carbonate; R-NEPC, 4-nitrophenyl (2*R*,3*R*)-2,3-epoxy-3-phenylpropyl carbonate; NEH, 4-nitrophenyl *trans*-3,4-epoxyhexanoate; NEB, 4-nitrophenyl *trans*-3,4-epoxy-4-phenylbutanoate; NΔE, *trans*-4-phenyl-3-butenoic acid; NapEC, 1-naphthyl *trans*-2,3-epoxy-3-phenylpropyl carbonate; NapEE, 1-naphthyl *trans*-3,4-epoxy-4-phenylbutanoate; CEB, 7-coumarinyl *trans*-3,4-epoxy-4-phenylbutanoate; EEB, ethyl *trans*-3,4-epoxy-4-phenylbutanoate; HPB, 3-hydroxy-4-phenyl-4-butanolide.

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TABLE 1

Comparison of Common Cytosolic Epoxide Hydrolase Substrates[a]

Structure	Compound	Assay Temperature	Km (μM)	Vmax (μmol/min/mg)	Type of Assay	Ref.
	TSO	37	5.0	3.3	partition	23
	SO	37	1400	1.92	partition	21
	EQU-5	25	25	8.3	uv-spec	1
	ENP-5	25	1.7	11.7	uv-spec	1
	S-NEPC	25	16	22	vis-spec	---

[a] The initial ratio of 50 μM S-NEPC hydrolysis at 37°C was 19 μmol/min/mg. The initial rate of TSO hydrolysis at the same concentration and temperature has been reported to be 1.2–1.5 μmol/min/mg (12, 23).

aromatic inhibitors of CEH (2,10). Two spectrophotometric substrates for CEH, *trans*-1,2-epoxy-1-(2-quinolyl)pentane (EQU-5) and *trans*-1,2-epoxy-1-(4-nitrophenyl)pentane (ENP-5), which avoid these problems have been developed (1). In addition, EQU-5 and ENP-5 have a higher V_{max} with CEH than TSO (Table 1) and can easily be used under conditions of substrate saturation. However, these substrates depend on differences in the absorbance spectra of the substrate and product. Finding substrate–product pairs with suitable difference spectra is a tedious and often unrewarding process. Thus, the number of useful substrates is limited. Finally, chiral precursors are not available for these substrates.

We have developed a new approach to spectrophotometric substrates for CEH. These substrates employ two functional groups, an epoxide and either a carbonate or an ester. The two functional groups are positioned so that, upon epoxide hydrolysis, cyclization can occur spontaneously (Fig. 1). The amount of alcohol released by cyclization can then be measured either directly or indirectly. For example, if the alcohol released is 4-nitrophenol, the reaction rate is measured by following the increase in absorbance at 405 nm. An advantage of this type of substrate is that, within the bounds of keeping the epoxide and ester/carbonate groups properly positioned, the structure of the substrate can be varied at will. Also, chiral substrates are easily made using either commercially available glycidol enantiomers or glycidol enantiomers prepared via the Sharpless epoxidation (11). One of the epoxy carbonates, 4-nitrophenyl (2*S*,3*S*)-2,3-epoxy-3-phenylpropyl carbonate (S-NEPC), is compared to the recently developed spectrophotometric substrates EQU-5 and ENP-5 and to the standard CEH substrate, TSO. Finally, we show that the S-NEPC assay is adaptable for use in 96-well microtiter plate readers.

MATERIALS AND METHODS

Reagents

Chemical intermediates, $H_2{}^{18}O$ (97 at.%), *meta*-chloroperbenzoic acid, 3-aminopropyltriethoxysilane, chloro-

FIG. 1. Hydrolysis of 4-nitrophenyl (2*S*,3*S*)-2,3-epoxy-3-phenylpropyl carbonate (S-NEPC) by cytosolic epoxide hydrolase. Subsequent spontaneous cyclization yields 4-nitrophenol, which can be used as a colored reporter for epoxide hydrolysis. From this substrate either a five- or a six-member cyclic carbonate could result. Cyclization could also occur with a direct attack of the oxyanion on the carbonyl group following reaction of the epoxide with either activated water or an anionic amino acid in the enzyme catalytic site.

2,4-dinitrobenzene, and Fast Blue RR were purchased from Aldrich Chemical Co. (Milwaukee, WI). All microtiter plates were obtained from Costar (Cambridge, MA) except for polystyrene plates, which were from Dynatech (Chantilly, VA). BCA reagent and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide were purchased from Pierce Chemical Co. (Rockford, IL). Bradford reagent was obtained from Bio-Rad (Richmond, CA). Alcohol dehydrogenase, NAD, reduced glutathione (GSH), porcine liver carboxylesterase (PLE), bovine serum albumin, fraction V (BSA), and SigmaCote were obtained from Sigma Chemical Co. (St. Louis, MO). Glutathione *S*-transferase (GST), purified from mouse liver, was a gift from Dr. Alan Buckpitt (Department of Veterinary Pharmacology and Toxicology, University of California, Davis, CA). Purified rat liver MEH was a gift from Dr. Thomas Guenthner (Department of Pharmacology, School of Medicine, University of Illinois, Chicago). HPLC solvents were from Fisher (Pittsburgh, PA) or Baker Chemical Co. (Phillipsburg, NJ). HPLC water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Other reagents were of the best quality commercially available.

Cytosolic Epoxide Hydrolase Purification and Assay

CEH was purified from the livers of Swiss Webster mice (Charles River, Cambridge, MA) which had been induced with clofibrate (Ayerst, New York, NY) as previously described (12). The buffer used throughout purification was 76 mM Na/K phosphate (pH 7.4, 0.1 mM EDTA). Protein concentrations were determined using the Pierce BCA assay modified for use in a 96-well plate reader for purified CEH and by the Bradford assay (13), with similar modifications, for all other samples (12). Enzyme activity during purification was followed with the standard [^{3}H]TSO partition assay (2). Purity of the preparations was assessed by SDS–PAGE using 5 μg purified CEH followed by Coomassie blue staining (14). Purified enzyme was stored in glass tubes at 4°C and used within 2 weeks of preparation.

Synthesis of Substrates

All melting points are uncorrected. The ^{1}H nuclear magnetic resonance spectra were determined on a Varian EM-390 spectrometer (Varian, Menlo Park, CA) with tetramethylsilane as the internal standard. All NMR samples were dissolved in $CDCl_3$. The optical rotation at 17°C, $[\alpha]_D^{17}$, was measured in CH_2Cl_2 at $c = 1.0$. The structures and properties are summarized in Table 2. All purified compounds were found to give one uv dense spot after silica gel TLC in each of two different solvent systems.

S-NEPC. To a cooled (ice bath) solution of 1.5 g of (2*S*,3*S*)-3-phenylglycidol and 2.1 g 4-nitrophenyl chloroformate in 30 ml of ether, 0.8 g of pyridine was added dropwise. After stirring for 3 h at room temperature, the ether solution was washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was chromatographed on silica gel with a mobile phase of hexane–ether (3:1). Concentration of the eluate under reduced pressure, followed by recrystallization of the residue from ethanol, gave 0.96 g (31%) of S-NEPC with an $[\alpha]_D^{17} = -60°C$.

4-Nitrophenyl (2R,3R)-2,3-epoxy-3-phenylpropyl carbonate (R-NEPC). R-NEPC was prepared in the same fashion as S-NEPC starting from (2*R*,3*R*)-3-phenylglycidol. The $[\alpha]_D^{17}$, under the same conditions, was +62°C.

4-Nitrophenyl trans-3,4-epoxyhexanoate (NEH). To a cooled (ice bath) solution of 3.3 g of *trans*-3-hexenoic acid and 4.2 g of 4-nitrophenol in 50 ml of tetrahydrofuran, 6.2 g of dicyclohexylcarbodiimide was added. The mixture was stirred for 3 h at room temperature. Next, the precipitate was removed by filtration and the filtrate concentrated under reduced pressure. The residue was purified by chromatography on silica gel with a mobile phase of hexane–ether (5:1) and reconcentrated under reduced pressure. In 30 ml of cooled (ice bath) dichloromethane, 1.6 g of the purified intermediate was mixed with 1.4 g of 85% *meta*-chloroperbenzoic acid. The resulting solution was stirred for 1 h at 0–5°C and then 2 h at room temperature. The solution was then washed with 5% $NaHCO_3$ and brine and dried over Na_2SO_4. After removal of the solvent, the residue was chromatographed on silica gel with a mobile phase of hexane–ethyl acetate (5:1). The eluate was concentrated under reduced pressure and recrystallized from ethanol. The yield was 0.40 g (23%).

TABLE 2

Structure and Properties of Synthesized Spectrophotometric Substrates

Compound	Structure	Melting point (o)	Composition (%)	^{1}H -NMR (δ)
S-NEPC		72-73	found: C-60.92, H-4.17, N-4.49 calc.: C-60.95, H-4.13, N-4.44 (C16H13NO6)	3.3-3.5(1H,m); 3.90(1H,d,2Hz); 4.33 (1H,dd,Ja=5Hz,Jb=12Hz); 4.67(1H,dd,Ja=3 Hz,Jb=12Hz); 7.2-7.6 (7H,m); 8.32(2H,d,J=9Hz)
R-NEPC		73-74	found: C-60.92, H-4.17, N-4.49 calc.: C-60.95, H-4.13, N-4.49 (C16H13NO6)	see S-NEPC
NEH		66-67	found: C-57.39, H-5.22, N-5.62 calc.: C-57.37, H-5.18, N-5.58 (C12H13NO5)	1.05(3H,t,J=7Hz); 1.4-1.8(2H,m); 2.6-2.9 (3H,m); 3.0-3.3(1H,m); 7.38(2H,d,J=9Hz); 8.35(2H,d,J=9Hz)
NEB		92-94	found: C-64.03, H-4.41, N-4.60 calc.: C-64.21, H-4.35, N-4.68 (C16H13NO5)	2.8-3.3(2H,m); 3.35-3.6(1H,m); 3.83(1H, d,J=2Hz); 7.2-7.5(7H,m); 8.35(2H,d,J=9 Hz)
NapEC		104-105	found: C-74.78, H-5.01 calc.: C-75.00, H-5.00 (C20H16O4)	3.3-3.5(1H,m); 3.9(1H,d,J=2Hz),4.40 (1H,dd,Ja=5Hz,Jb=12Hz); 4.72(1H,dd, Ja=3Hz,Jb=12Hz); 7.1-8.1(12H,m)
NapEE		64-65	found: C-78.94, H-5. 29 calc.: C-78.95, H-5. 26 (C20H16O3)	3.15(2H,d,J=6Hz); 3.5-3.7(1H,m); 3.92 (1H,d,J=2Hz); 7.3-8.1(12H,m)
CEB		132-134	found: C-70.83, H-4. 38 calc.: C-70.81, H-4. 35 (C19H14O5)	2.9-3.1(2H,m); 3.3-3.5(1H,m); 3.85(1H,d, J=2Hz); 6.42(1H,d,J=10Hz); 7.0-7.8(9H,m)
EEB		n.d.*	found: C-69.69, H-6.73 calc.: C-69.90, H-6. 80 (C12H14O3)	1.26(3H,t,J=7Hz); 2.72(2H,d,J=6Hz); 3.2-3.4(1H,m); 3.73(1H,d,J=2Hz); 4.22(2H,q, J=7Hz); 7.2-7.5(5H,m)

* n.d., Not determined.

4-Nitrophenyl trans-3,4-epoxy-4-phenylbutanoate (NEB). NEB was prepared in the same fashion as NEH. Starting from *trans*-4-phenyl-3-butenoic acid (NΔE) and 4-nitrophenol, the yield was 34%.

1-Naphthyl trans-2,3-epoxy-3-phenylpropyl carbonate (NapEC). To a cooled (ice bath) solution of 3.2 g of cinnamyl alcohol and 5 g of 1-naphthyl chloroformate in 30 ml of ether, 3.2 g of pyridine was added dropwise. After stirring at room temperature for 5 h, the ether solution was washed with brine and dried over Na_2SO_4. After removal of the solvent, the residue was chromatographed on silica gel with a mobile phase of hexane–ethyl acetate (3:1). Concentration under reduced pressure yielded 6.5 g of crude 1-naphthyl cinnamyl carbonate (NapEC).

To a cooled (ice bath) solution of 4 g NapEC in 30 ml of dichloromethane, 3.4 g of 85% *meta*-chloroperbenzoic acid was added. After stirring for 18 h at room temperature, the dichloromethane solution was washed with 5% $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was removed and the residue recrystallized from ethanol. The yield was 3 g (71%).

1-Naphthyl trans-3,4-epoxy-4-phenylbutanoate (NapEE). To a cooled (ice bath) solution of 1.6 g *trans*-4-phenyl-3-butenoic acid, 1.4 g 1-naphthol, and 0.1 g dimethylaminopyridine in 50 ml dichloromethane, 2.1 g dicyclohexylcarbodiimide was added. The resulting solution was stirred overnight at room temperature, the precipitate was removed by filtration, and the filtrate was washed with 5% HCl, 5% $NaHCO_3$, and brine. The

washed filtrate was dried over Na_2SO_4. The solvent was removed and the residue chromatographed on a silica gel column with a hexane–ethyl acetate mobile phase (6:1). The eluate was concentrated under reduced pressure and recrystallized from ethanol. The purified intermediate was epoxidized by the same method used for NEH. The overall yield was 38%.

7-Coumarinyl trans-3,4-epoxy-4-phenylbutanoate (CEB). This compound was synthesized by the same method used for NapEE. The yield was 42% starting from *trans*-4-phenyl-3-butenoic acid and 7-hydroxycoumarin and recrystallized from ethyl acetate.

Ethyl trans-3,4-epoxy-4-phenylbutanoate (EEB). This compound was also prepared with the method used for NapEE. The yield was 72% starting from *trans*-4-phenyl-3-butenoic acid and ethanol.

3-Hydroxy-4-phenyl-4-butanolide (HPB). To a solution of 1.6 g of *trans*-4-phenyl-3-butenoic acid, in 30 ml of dichloromethane at 0–5°C, 2.5 g 85% *meta*-chloroperbenzoic acid was added. The resulting mixture was stirred for 12 h at room temperature, the precipitate removed by filtration, and the filtrate concentrated under reduced pressure. The residue was allowed to stand at room temperature for 2 days. It was then dissolved in ether and the solution was washed with 5% Na_2CO_3 brine and dried over Na_2SO_4. The solution was then concentrated and the residue recrystallized from toluene. The yield was 0.56 g (34%).

Mass Spectrometry

S-NEPC was hydrolyzed by purified CEH in 0.1 ml of $H_2{}^{18}O$ for 1.5 h at 37°C. The product was extracted three times with ethyl ether and dried over Na_2SO_4, and the ether was removed under a stream of nitrogen. The sample was then taken up in 50 μl of acetonitrile, 50 μl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide was added, and the mixture was incubated for 2 h at 60°C. The silylated extract was injected without further treatment onto a Hewlett Packard 5890 GC (Hewlett Packard, Palo Alto, CA) equipped with a DB-5 capillary column (J+W Scientific, Folsom, CA) with a programmed temperature gradient from 125–220°C at 5°C/min. The GC was coupled to a VG TRIO-2 mass spectrometer (VG Industries, Altrincham, England), and the data were collected and analyzed with a VG11-250 data system. Chemical ionization was carried out with isobutane.

High-Performance Liquid Chromatography

HPLC was carried out with a Perkin–Elmer Bio 410 HPLC (Perkin–Elmer, Norwalk, CT) equipped with a Perkin–Elmer LC 235 diode array detector, a Supelcosil LC–(R)-urea column (Supleco, Bellefonte, PA), and a Hewlett Packard 4490 recording integrator.

S-NEPC (50 nmol) was incubated at 25°C with either 1.2 μg of CEH in 1 ml of buffer or 1 ml of buffer alone for both 60 and 105 s or at 37°C with either 1.1 μg of CEH in 1.0 ml of buffer or in 1.0 ml of buffer alone for 60 s. Then 200 μl was injected onto the HPLC and the presence of the product, 4-nitrophenol (retention time, 2.96 min), was monitored at 355 nm and the presence of the substrate, S-NEPC (retention time, 9.55 min), was monitored at 270 nm. A water/acetonitrile solvent system was employed to separate S-NEPC and 4-nitrophenol and prepare the column for repeat injections. The solvent during injection and for the first 2 min was water. Then the following gradient was used: (a) 2–8 min, to 100% acetonitrile; (b) 8–11 min, hold 100% acetonitrile; (c) 11–14 min, to 100% H_2O; and (d) 14–22 min, hold 100% H_2O. The flow rate was 2 ml/min. Incubations and subsequent injections were repeated 3 times for the 60-s incubations at 25°C, 4 times for the 105-s incubations at 25°C, and 10 times for the incubations at 37°C. The CEH used for the 25 and 37°C incubations was from different purifications. Standard curves for both product and substrate were constructed by injecting 200 μl of from 50 to 1.56 μM solutions of both substrate and product in buffer. Injections were repeated at least 6 times for each concentration.

The cyclic product, HPB (retention time, 13.78 min), was chromatographed at a flow rate of 1 ml/min with a solvent system of aqueous 25% acetonitrile, 15% methanol, and 2.5% tetrahydrofuran. Synthetic HPB was compared to the products of CEH hydrolysis of NEB (retention time, 11.68 min) by both retention time and comparison of the uv spectra of the peaks. NEB was injected onto the column for assay after CEH hydrolysis without prior cleanup.

Assay with Spectrophotometric Substrates

Substrates producing 4-nitrophenol were assayed by following the appearance of 4-nitrophenolate at 405 nm. The effective extinction coefficient of 4-nitrophenol was determined by measuring the absorbance of 50 and 20 μM solutions of 4-nitrophenol in assay buffer. Assays were carried out in a Cary 219 spectrophotometer (Varian, Palo Alto, CA) unless otherwise noted. The dual-beam mode was used to allow the use of a reagent blank and the slit was 1 nm. Readings were taken for at least three independent samples. Assays for kinetics were performed at least four separate times in triplicate each time. The triplicate samples were assayed at the same time using a rotating turret cuvette holder with a 1-s dwell and a 20-s cycle time. Assays were carried out at 25°C. The temperature was maintained in the spectrophotometer cell with a Polytemp circulating bath (Polyscience, Niles, IL). Stock solutions of substrates (100×) in absolute ethanol, or other solvent as described below, were either prepared in advance and stored at −80°C or

prepared fresh. The solution of stock substrate was kept on ice during use.

Substrates producing 1-naphthol were followed by the appearance of color at 450 nm. This color results from the reaction of 1-naphthol with Fast Blue RR (15). The molar extinction coefficient of the Fast Blue RR adduct of 1-naphthol was determined in assay buffer and found to be 10,600 M^{-1} cm^{-1}. Buffer for the reaction was prepared by adding 3 mg/ml Fast Blue RR to the assay buffer, mixing, and filtering to remove any remaining clumps of dye. The dye solution was prepared immediately before use to avoid high background due to spontaneous color formation. All assays were carried out under the conditions outlined above. Substrate was dissolved in absolute ethanol on the day of use and diluted 100-fold into the assay buffer. Stock substrate was stored on ice during use.

CEB was assayed with a Shimadzu RF5000U fluorimeter (Shimadzu, Kyoto, Japan). Umbelliferone production was monitored by irradiating at 364 nm and observing fluorescence at 448 nm. The extinction coefficient of umbelliferone was determined by measuring the fluorescence of 5 μM umbelliferone in buffer plus 2.5 mg/ml BSA. Assays were carried out in a 1.5-ml cell, without stirring, at room temperature and repeated three times. A separate reagent blank was measured to determine the background rate of hydrolysis. A stock solution of CEB was prepared on the day of use by dissolving the substrate in absolute ethanol. The stock substrate was stored on ice and diluted 100-fold into assay buffer for use.

EEB was assayed by coupling the production of ethanol to the production of acetaldehyde and accompanying reduction of NAD, with alcohol dehydrogenase (240 units/cuvette). The production of NADH was followed at 340 nm under the conditions outlined above. The rate of NADH production was also measured in the presence of either 0.5% ethanol or 0.5 unit of porcine liver carboxylesterase. The stock substrate solution, in methyl sulfoxide, was prepared fresh, kept at room temperature, and diluted 100-fold for use. At least three separate assays were carried out for each sample.

S-NEPC was also assayed using a V_{max} 96-well plate reader (Molecular Devices, Palo Alto, CA). The screening assays were carried out in 200 μl at 25°C. The initial rate of CEH-catalyzed S-NEPC hydrolysis was measured in polystyrene, polyvinyl chloride, polyethylene terephthalate glycol, and polypropylene microtiter plates. All plates were tested untreated or pretreated with 25 mg/ml BSA, 2% Triton-100, and 2% Tween 20. All plates were pretreated by filling wells with the indicated solution for 2 h, rinsing with glass distilled water, and drying. The plates were used immediately after preparation. Polypropylene plates were also pretreated with SigmaCote according to the manufacturer's recommendations and 3-aminopropyltriethoxysilane in 95% aqueous ethanol as described (16). The kinetics of CEH-catalyzed S-NEPC hydrolysis were measured in untreated polypropylene plates with an assay volume of 250 μl. All assays using 96-well plates were performed at least twice in quadruplicate. The effective extinction coefficient of 4-nitrophenol was determined in assay buffer for both 200- and 250-μl well volumes.

Unless otherwise indicated all assay buffers were Na^+/K^+ phosphate with an ionic strength of 0.2 and of the pH indicated. EDTA (0.1 mM) and BSA (2.5 mg/ml) were added to help stabilize enzymatic activity.

GST was assayed at pH 6.4 without BSA, in the presence of 1 mM GSH. MEH was assayed at pH 8.75 in 100 mM Tris buffer without BSA or EDTA. PLE (0.25 unit/assay; 1 unit = 1 μmol ethyl butyrate hydrolyzed per minute at pH 8.0 and 25°C) was assayed at pH 6.4 without BSA. GST, MEH, and PLE were assayed under the same conditions as those used for CEH, and rates were also measured by following the production of 4-nitrophenol at 405 nm. Assays were carried out three separate times in duplicate.

RESULTS

Assay Validation

The hydrolysis of both S-NEPC and NEB should produce a cyclic product which is formed during the release of 4-nitrophenol. The lactone, HPB, was found in the CEH hydrolysate of NEB and was shown to be identical to the synthetic lactone by retention time on HPLC and comparison of their uv spectra (data not shown). The ether-extracted, TMS-derivatized CEH hydrolysate of S-NEPC was shown to contain the expected cyclic product by GC–MS (data not shown).

If these compounds are to be used for investigating CEH kinetics, the rate of cyclization must not be rate limiting. The rate of disappearance of S-NEPC was compared with the appearance of 4-nitrophenol by separation on HPLC and quantitation of the two peaks. At 25°C, the rates of S-NEPC disappearance and 4-nitrophenol appearance were not significantly different ($P < 0.01$, $n = 7$). The rate was 13 ± 0.99 μmol min^{-1} mg^{-1}. The rates at 37°C were also not significantly different ($P < 0.01$, $n = 13$). The rate of S-NEPC disappearance was 20.5 ± 4.16 nmol min^{-1} and the rate of 4-nitrophenol appearance was 20.7 ± 2.33 nmol min^{-1}. This assay was run at 37°C, in addition to 25°C, to show that the rate of substrate disappearance was equal to the rate of product appearance even at the higher temperatures and rates. The higher temperature also allows comparison to the standard TSO assay. The rate of CEH-catalyzed hydrolysis of 50 μM S-NEPC was 19 ± 3.9 μmol min^{-1} mg^{-1} at 37°C.

The pH maximum with S-NEPC was 6.4 (Fig. 2). The linear range, with respect to enzyme, was from 0.0 to 0.90 μg/ml CEH and, with respect to time, was from less

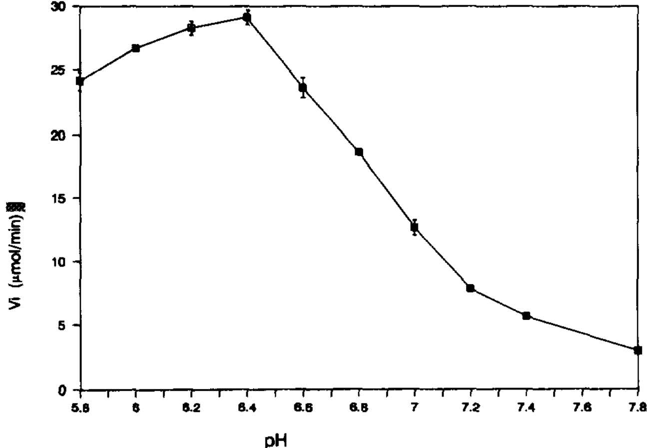

FIG. 2. Rate of S-NEPC hydrolysis vs pH. The rate of reaction was followed and the hydrolysis conditions were [S-NEPC] = 50 μM and [CEH] = 1.8 μg/ml in phosphate buffer (I = 0.2, [EDTA] = 0.1 mM, [BSA] = 2.5 mg/ml, and pH as indicated), T = 25°C, and n = 4. Standard deviation is indicated by error bars when it is larger than the point shown. The data were corrected for the changing extinction coefficient of 4-nitrophenol.

than 1 min to more than 4 min, depending on the concentration of CEH (Fig. 3). The rate of CEH-catalyzed hydrolysis was also found to depend on the presence of BSA. The rate was maximal at 2.5 mg BSA/ml (Table 3). Kinetics, carried out with S-NEPC, were shown to follow Michaelis-Menten kinetics and gave a V_{max} of 22 $\pm$ 0.79 μmol min^{-1} mg^{-1} and a K_m of 16 $\pm$ 0.74 μM [n = 4] (Fig. 4). Since a significant percentage of S-NEPC was hydrolyzed during the linear phase of hydrolysis, kinetic constants were calculated using the average substrate concentration during the time period measured (17).

Rate vs Substrate

The rates of hydrolysis of various substrates by CEH were also compared (Table 4). The most active substrate in terms of specific activity was found to be CEB, the umbelliferone ester, which gave an activity of 994,000 $\pm$ 217,000 mOD min^{-1} mg^{-1} or 16.5 $\pm$ 3.60 μmol min^{-1} mg^{-1}. However, CEB had a spontaneous rate of hydrolysis of 11% of the CEH catalyzed rate, while the spontaneous rate of S- and R-NEPC hydrolysis was only 3% that of the CEH-catalyzed rate under the same conditions. Carbonates were more active as substrates than the respective esters (compare S- and R-NEPC with NEB and NapEC with NapEE), although in these series the carbonyl is further from the epoxide than the carbonyl of the ester. An ethyl group *trans* to the ester group had lower activity than the homologous compound with a phenyl group (compare NEH and NEB). The *S*-enantiomer was slightly less active than the

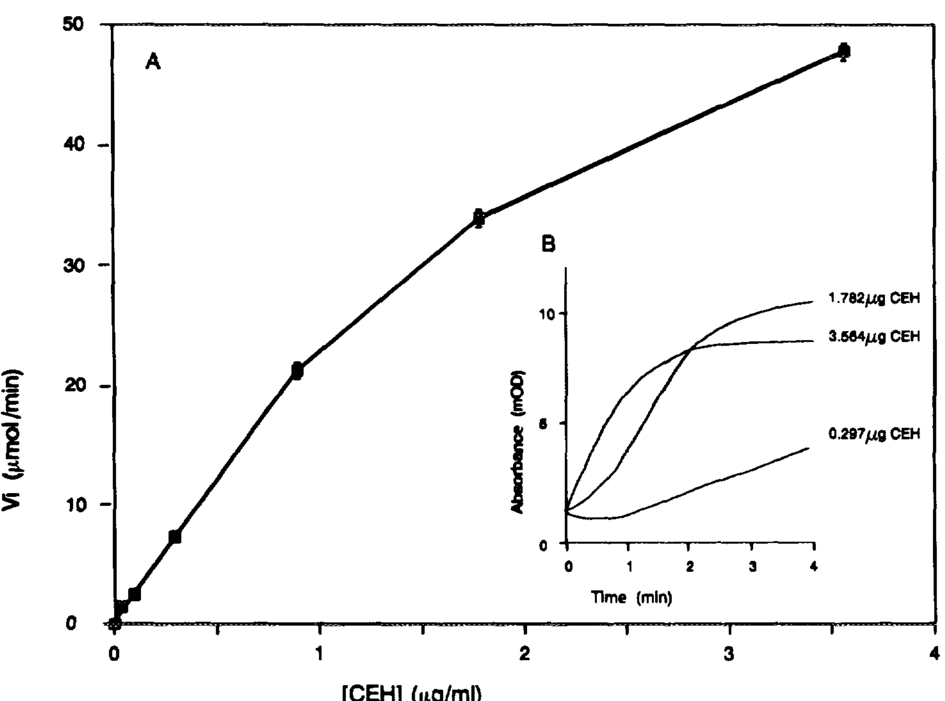

FIG. 3. Rate of S-NEPC hydrolysis vs cytosolic epoxide hydrolase concentration (A) and time (B). The rate of reaction was followed at 405 nm and the hydrolysis conditions were [S-NEPC] = 50 μM, [CEH] = as noted, pH 6.4 (phosphate buffer, I = 0.2, [EDTA] = 0.1 mM, [BSA] = 2.5 mg/ml), T = 25°C, and n = 4. Standard deviation is indicated by error bars when it is larger than the point shown.

TABLE 3

Rate of S-NEPC Hydrolysis vs BSA Concentration[a]

BSA (mg/ml)	0.0	0.05	0.10	0.50	1.0	2.5	10
V_i (mOD/min)	44.0 ± 1.50	50.2 ± 1.20	60.6 ± 1.95	75.0 ± 0.774	78.9 ± 4.08	83.4 ± 2.08	79.8 ± 4.53

[a] All assays performed at 25°C in pH 6.4 phosphate buffer (I = 0.2, 0.1 mM EDTA) with 50 μM S-NEPC and 1.78 μg/ml CEH. All assays were repeated four times.

corresponding *R*-enantiomer (compare S-NEPC and R-NEPC). EEB, the ethyl ester, showed no ethanol production when incubated with CEH. However, EEB was a substrate of CEH as judged by detection of the corresponding diol by HPLC (data not shown).

NΔE, which has no epoxide functionality, gave no color production. Unexpectedly, NapEP, which would need to form a four-member ring to cyclize, did give some color production. However, the rate of color production was the same after CEH was boiled and the color production was found to be dependent on the presence of BSA. Color production was ultimately determined to be due to an interaction between CEH and Fast Blue RR which was enhanced by the presence of BSA.

Activity of the Substrates with Different Enzymes

The activity of S-NEPC and NEB was tested against GST, MEH, and PLE (Table 5). GST showed approximately equal activity with S-NEPC (0.0394 μmol min^{-1} mg^{-1}) and NEB (0.0345 μmol min^{-1} mg^{-1}). Under the same conditions the rate of GST with 50 μM CDNB was 0.246 μmol min^{-1} mg^{-1}. MEH showed low activity with both S-NEPC and NEB. The activity with S-NEPC was 0.00155 μmol min^{-1} mg^{-1} and 0.0104 μmol min^{-1} mg^{-1} with NEB. When tested with 50 μM CSO, MEH had a specific activity of 0.0464 μmol min^{-1} mg^{-1}. PLE rapidly hydrolyzed both S-NEPC and NEB. S-NEPC gave a specific activity of 4.61 μmol min^{-1} mg^{-1} and NEB, the more active substrate, had a specific activity of 13.8 μmol min^{-1} mg^{-1}. 4-Nitrophenyl acetate had a specific activity of 4.11 μmol min^{-1} mg^{-1} with PLE under the same assay conditions. In light of these observations it is not surprising that the activity ratios of purified and crude fractions of murine liver CEH are different when TSO (176, 78.4) and S-NEPC (65.2, 55.2) are used as substrates.

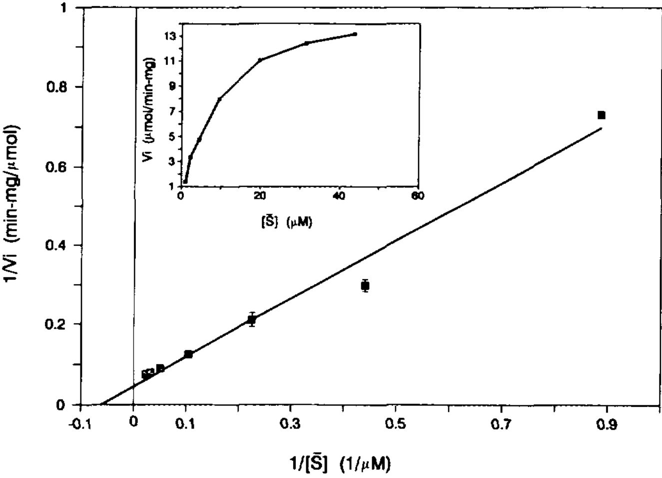

FIG. 4. Kinetics of S-NEPC hydrolysis with cytosolic epoxide hydrolase. The data are shown in both the double reciprocal (A) and the V_i vs [S] (B) forms. The hydrolysis conditions were [S-NEPC] = as indicated, [CEH] = 0.756 μg/ml, pH 6.4 (phosphate buffer, I = 0.2, [EDTA] = 0.1 mM, [BSA] = 2.5 mg/ml), T = 25°C, and n = 4. Each assay was done in triplicate. The indicated substrate concentrations are the mean concentration present during the linear portion of the assay. Standard deviation is indicated by error bars when larger than the point shown and R, the correlation coefficient for the regression line, = 0.985.

Rates with Different Instruments

The rate in all 96-well microtiter plates was somewhat less than that observed with the spectrophotometer (Table 6). All pretreatments tested, soaking with 25 mg/ml BSA, 2% Triton X-100, 2% Tween 20, Sigma-Cote, or 3-aminopropyltriethoxysilane, either had no effect on or decreased the observed rate. The activity was lowest when polystyrene plates were employed. The activity measured in untreated polypropylene plates was closest to that observed with the Cary and these plates were selected for the kinetic determination. The values for kinetic parameters obtained with polypropylene plates were V_{max} = 15 ± 3.3 μmol min^{-1} mg^{-1} and K_m = 6.6 ± 1.3 μM.

DISCUSSION

Epoxy esters and epoxy carbonates have been found to be very useful substrates for the assay of purified or partially purified CEH (Table 4). They were not useful for crude CEH preparations due to interference from esterases and GSTs. The mechanism of color production appears to be cyclization of the oxyanion produced following an attack on the substrate epoxide by either active water or an anionic amino acid or, as shown in Fig. 1, cyclization of the intermediate diol carbonate (or ester) which results in the release of 4-nitrophenol (Fig.

TABLE 4

Cytosolic Epoxide Hydrolase Activity vs Substrate Structure[a]

Substrate	Conc. (μM)	Activity OD/min/mg	Activity μmol/min/mg
S-NEPC	20	35.2±5.79	9.26±1.52
R-NEPC	20	44.8±1.43	11.8±0.377
NEH	20	0.180±0.0138	0.474±0.0363
NEB	20	7.12±1.125	1.87±0.297
NΔE	20	0±0	0±0
NapEC	20	12.0±3.47	1.13±0.327
NapEE	20	5.79±0.0957	0.546±0.00903
NapEP	20	0±0	0±0
CEB	50	994±217	16.5±3.60
EEB	50	0±0	0±0

[a] Assayed at 25°C in pH 6.4 phosphate buffer (I = 0.2, 0.1 mM EDTA, 2.5 mg/ml BSA) with 0.76 μg/ml CEH. Assays were carried out at least three times in duplicate.

1) or some other easily measured alcohol. A large variety of epoxy esters and carbonates can be synthesized, and they allowed CEH to be assayed using a microtiter plate reader.

The pH maximum of CEH for S-NEPC catalysis was 6.4 (Fig. 2). S-NEPC and other substrates producing 4-nitrophenol were not useful below pH 5.8. This results from the increasing loss of sensitivity as the percentage of 4-nitrophenolate anion decreases as the pH decreases. The rate of color production is linear with increasing protein to a CEH concentration of 1 μg/ml. By monitoring both the disappearance of S-NEPC and the appearance of 4-nitrophenol, it was shown that the hydrolysis of S-NEPC and not the production of 4-nitrophenol was the rate-limiting step in color production at both 25 and 37°C. It will be necessary to make sure that

TABLE 5

Activity of Glutathione S-transferase, Microsomal Epoxide Hydrolase, and Porcine Liver Carboxylesterase with Selected Substrates[a]

Enzyme	Substrate	Initial velocity	
		mOD/min/mg	μmol/min/mg
GST	S-NEPC	234 ± 10.5	0.0394
	NEB	205 ± 7.86	0.0345
MEH	S-NEPC	29.3 ± 3.01	0.00155
	NEB	176 ± 31.3	0.0104
PLE	S-NEPC	13,500 ± 1,410	4.61
	NEB	40,500 ± 2,890	13.8

[a] Assayed at 25°C in pH 6.4 phosphate buffer (I = 0.2), $[S]$ = 50 μM, and 0.075 mg/ml GST (1 mM GSH), or 86 μg/ml PLE. MEH assayed in 100 mM Tris (pH 8.75) with 40 μg/ml MEH, T = 25°C, and $[S]$ = 50 μM. Specific activity of GST with CDNB was 0.246 μmol/min/mg, of MEH with *cis*-stilbene oxide was 0.0464 μmol/min/mg, and of PLE with 4-nitrophenyl acetate was 4.11 μmol/ml/mg. Assays were carried out three times in duplicate.

the rate of cyclization is not rate limiting if a substrate with a different structure is employed for kinetics.

The substrates with the best sensitivity and lowest background were S- and R-NEPC. CEH had a higher specific activity with CEB but the background was also much higher. It was found that, at the concentrations tested, carbonates were better substrates than esters, and a *trans*-phenyl group was preferred to a *trans*-ethyl group although substrates with longer hydrocarbon chains are likely to have improved rates. When the chiral substrates were compared, the R-enantiomer, R-NEPC, was a slightly better substrate. The leaving ability of the alcohol produced also influenced the observed rate, with CEB-releasing 7-hydroxycoumarin showing a high rate and EEB-releasing ethanol showing a low rate. However, with the limited number of substrates examined we cannot rule out the possibility that cyclization of EEB fails to occur in the enzyme catalyic site because of the orientation of the substrate. The substrate with the worst leaving group, EEB, did not cyclize to produce ethanol under the conditions of the assay even though it was a substrate of CEH.

NΔE and NapEP were found not to produce a colored product as a result of CEH-mediated hydrolysis, as was predicted from their structures. NΔE has no epoxide functionality and, therefore, should not be a substrate. NapEP must form a three- or four-member ring on cyclization. Since this is such an energetically unfavorable process, there should be no color production. It is interesting to note that there was some color produced during the assay of NapEP. The color production was traced to the direct interaction of Fast Blue RR with CEH.

As mentioned, one drawback of this class of substrates was found to be the lack of specificity. Epoxy carbonates/esters were also found to be substrates for

TABLE 6

Comparison of Rates of Hydrolysis in Cuvets vs Microtiter Plates[a]

Instrument	Sample cell	Run	N	Rate (μM/min)
Cary 219	1.5 ml quartz cuvet	1	6	9.21 ± 0.568
		2	40	8.72 ± 0.292
V_{max} plate reader	PVC microtiter plate	1	8	6.68 ± 1.04
	Polypropylene microtiter plate	1	8	8.15 ± 0.674
		2	40	8.59 ± 1.11
	PETG microtiter plate	1	8	7.14 ± 0.611
	Polystyrene microtiter plate	1	8	3.54 ± 0.0179

[a] Assays carried out in phosphate buffer (pH 6.4, I = 0.2, [BSA] = 2.5 mg/ml, 2% ethanol), 50 μM S-NEPC, T = 25°C, and 1.3 μg/ml CEH for both run 1 and run 2. Assay volumes were 1 ml for the Cary and 0.2 ml for the V_{max} plate reader. All plates were tested after pretreatments with 25 mg/ml BSA, 2% Triton X-100, or 2% Tween 20. Polypropylene plates were also tested after treatment with Sigma-Cote and 3-aminopropyltriethoxysilane. All combinations showed either no change or decreased CEH activity relative to untreated plates.

GST, MEH, and carboxylesterase (Table 5). S-NEPC was found to be more selective for CEH than NEB. Interference from GSTs is easily avoided by preincubation with diethyl maleate, which reacts with the available GSH, or by dialysis to remove any GSH present (18). Increased selectivity with respect to esterases might be achieved with the use of secondary alcohols. A similar strategy resulted in a large decrease in ester-mediated pyrethroid metabolism (19,20). Given the activity seen with these other enzymes, these substrates should not be used to quantitate the level of CEH activity in crude preparations unless it is known that interfering enzymes have been completely inhibited. Either EQU-5 or ENP-5 is a better substrate in this respect. Since they contain only the epoxide functionality, they are not esterase substrates and GST activity can be eliminated.

The use of TSO and CSO offers the advantage of a selective assay of CEH and MEH (2,21). Since monosubstituted epoxides are better substrates for MEH than for CEH (2,4,22), we attempted the synthesis of 4-nitrophenyl 2,3-epoxypropyl carbonate as a substrate for MEH. However, this compound proved to be too unstable for easy purification and use.

When compared to other common substrates for CEH, S-NEPC has the highest V_{max} and a similar K_m (Table 1). The initial rate of CEH with 50 μM S-NEPC at 37°C was 19 ± 3.9 μmol min^{-1} mg^{-1} which is considerably higher than the 1.2–1.5 μmol min^{-1} mg^{-1} measured with 50 μM TSO (12,23). The pH maximum, 6.4, with S-NEPC is also considerably lower than that for TSO, 6.5–7.4 (23), or EQU-5, 6.8 (1). Unfortunately, the low pH maximum of S-NEPC substantially decreases the sensitivity of the assay and makes use of the epoxy carbonate/ester substrates for full characterizations of CEH's response to pH impossible. One should be able to obtain more sensitive assays by using chromophores with a more acidic maximum absorbance. The linear range of the S-NEPC assay is similar to those employing EQU-5 and ENP-5 with respect to enzyme protein, 0.9 μg vs 1 and 0.5 μg, respectively (1). CEH has been shown to be linear with respect to time with TSO as the substrate to 25 μg of cytosol (3), or approximately 0.125 μg of purified CEH. Both the EQU-5 and ENP-5 assays are linear with respect to time for significantly longer than S-NEPC, 12 and 8 min, respectively, vs 3 min for S-NEPC. In general, the loss of linearity over time is a function of consumption of substrate and/or product inhibition and is therefore dependent on the concentration of enzyme present in the assay. This can be seen in Fig. 3. With increasing enzyme concentration and reaction rate, the linear portion of the rate curve becomes shorter.

It was also observed that with decreasing concentrations of CEH there was an increasing lag time. The most likely explanation is that there was a CEH-dependent buildup of an intermediate in the production of 4-nitrophenol. Since it was shown that S-NEPC disappearance and 4-nitrophenol appearance are identical, a possible explanation is that the cyclization of the diol intermediate is mediated by CEH, and buildup of the diol concentration is required before the rate becomes linear. It also is possible that epoxide hydrolases act by the enzyme-mediated attack of an ionic group such as an aspartic acid on the epoxide substrate, resulting in a covalent hydroxy ester bond. Slow breakdown of the resulting product also could result in the lag phase seen. In either case, at very early times in the hydrolysis of S-NEPC or at very low CEH concentrations, the rates of S-NEPC disappearance and 4-nitrophenol appearance should not be identical. The discovery of CEH-mediated cyclization of diepoxides is consistent with this possibility (24,25).

Finally, it has been shown that these substrates can be used in conjunction with a 96-well plate reader. The kinetic parameters were V_{max} = 15 μmol min^{-1} mg^{-1} and K_m = 6.6 μM using polypropylene plates. These values are somewhat lower than those obtained using a standard spectrophotometer (V_{max} = 22 μmol min^{-1} mg^{-1} and K_m = 16 μM). The coefficients of variation for V_{max} (22 and 3.6%, respectively) and K_m (8.1 and 4.6%, respectively) were higher for the plate reader than the spectrophotometer. Other enzyme assays where direct comparisons were made between a 96-well plate reader and a spectrophotometer also showed differences in the measured rates (26). In addition, this difference is within the normal range of variability seen between different preparations of purified CEH (data not shown). As has been shown with other enzymes (27,28), the use of S-NEPC

together with a 96-well plate reader can greatly increase the speed with which CEH assays can be carried out, allowing facile kinetic characterization of CEH and CEH inhibitors. However, it must be kept in mind that the type of plate employed may be crucial to the success of the assay. Microtiter plates are designed to adsorb protein and this may severely affect the outcome of the assay. Also, some plate readers may have errors in rereading the same well and reading the same sample in different wells, which would introduce large errors into the data (29).

In summary, a new class of spectrophotometric substrates for CEH has been demonstrated, allowing measurement of CEH activity in the visible range of the spectrum and avoiding any interference from common, uv-dense inhibitors of CEH. These substrates are not specific for CEH and may prove to be useful for GST, MEH, and carboxylesterases as well. NEB was more active with GST, MEH, and PLE than S-NEPC and was a better substrate for PLE than 4-nitrophenol acetate under the assay conditions employed. The more active members of this class of substrate, S-NEPC, R-NEPC, and CEB, are the most sensitive spectrophotometric substrates for CEH found to date and the V_{max} with S-NEPC is higher than that of any commonly used CEH substrate. Other advantages of this class of substrates are the great structural diversity possible, limited only by the need for a sufficiently good leaving group, and the ease with which chiral substrates can be synthesized. Indeed, it should be possible to increase the V_{max} of this type of substrate with the use of a long alkyl chain in place of the 3-phenyl ring (30–32). Use of these substrates in kinetic plate readers will allow a large increase in the speed with which CEH can be assayed. However, it must be remembered that the rate of epoxide hydrolysis must be known to be rate limiting if these substrates are to be used for quantitative measurements. In short, epoxy carbonates and esters will be very useful in the study of purified CEH. With further refinement they should be even more sensitive and selective than they are at present.

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