Tetrahedron Letters 51 (2010) 1220–1225

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Two-step enzymatic modification of solid-supported bergenin in aqueous and organic media

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ARTICLE INFO

Article history:
Received 12 November 2009
Revised 17 December 2009
Accepted 18 December 2009
Available online 28 December 2009

Keywords:
Solid Phase
Biocatalysis
Non-aqueous
Enzyme cleavage

ABSTRACT

The natural flavonoid bergenin was directly immobilized onto carboxylic acid-functionalized controlled pore glass (carboxy-CPG) at 95% yield. Immobilized bergenin was brominated via chloroperoxidase in aqueous solution and then transesterified with vinyl butyrate in diisopropyl ether by subtilisin carslberg (SC) extracted into the organic solvent via ion-pairing. Enzymatic cleavage of 7-bromo-4-butyrylbergenin from carboxy-CPG (9.6% final yield) was accomplished using lipase B (LipB) in an aqueous/organic mixture (90/10 v/v of water/acetonitrile), demonstrating the feasibility of solid-phase biocatalysis of a natural product in aqueous and non-aqueous media.

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The ability of enzymes to efficiently carry out highly specific reactions on a broad range of substrates has been exploited in many diverse applications, such as pharmaceutical development, protein engineering, and polymer templating and synthesis.[1–5] In the pharmaceutical industry, biocatalysts have been used to generate highly focused lead compound libraries. One approach is termed 'combinatorial biocatalysis,' which has been employed in both aqueous and non-aqueous media as a complement to combinatorial chemistry and rational drug design for the optimization of pharmaceutical compounds.[6–8] Over the past decade, there has been a concerted effort to combine this concept with solid-phase chemistry to merge the selectivity of biocatalysis with the high throughput conferred by solid-phase synthesis.[9] Specific areas of interest include enzymatic modification of immobilized peptides and other small molecules in aqueous[10–14] and non-aqueous[15] media, enzymatic screening of combinatorial libraries of immobilized small molecules,[16–21] substrate removal via enzyme-cleavable linkers,[12,22–25] and preliminary kinetic comparisons of solid versus solution-phase modifications.[15,26–28] To date, enzymatic reactions on insoluble, solid-supported substrates have been carried out primarily in aqueous solution.[29a,b] However, with the recent development of a new protein solubilization method that has broadened the utility of non-aqueous enzymology,[30] we hope to extend the capabilities of solid-phase biocatalysis to include non-aqueous modification of solid-supported pharmaceutical leads and other specialized molecules, taking advantage of the novel synthesis opportunities,[31] tunable specificity,[32] and selectivity[33,34] afforded by such systems.

Successful implementation of this methodology requires two important elements: enzyme-accessible supports, and for the particular case of non-aqueous biocatalysis, organic-soluble enzymes. To satisfy the first requirement, we employed controlled pore glass (CPG), a material commonly used for enzyme immobilization. CPG possesses a large, open pore structure (in contrast to supports that require pre-swelling prior to use), is relatively easy to handle,[24] and, most importantly, provides more favorable access to enzyme than many other supports.[24]

Solid-phase non-aqueous enzymology presents the additional requirement that the enzyme, which is typically insoluble in neat organic solvents, first be made soluble and active in the desired solvent. Although there are a variety of methods for solubilizing enzymes into organic solvents,[35–38] based on previous solubilization studies surfactant ion-pairing via liquid–liquid extraction (EXT)[30,39] and direct solubilization (DS)[30] are particularly promising. Specific advantages of these methods include ease of preparation and high activity in either a particular solvent (extraction) or across a range of solvents exhibiting different polarities (direct solubilization).

The present work helps to establish a foundation for solid-phase combinatorial biocatalysis as a drug development tool. In particular, we describe a means for directly immobilizing potential lead compounds without the use of a linker, provide the first example of non-aqueous solid-phase biocatalysis with a molecule other

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0040-4039/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2009.12.119

than a peptide, and present a method for regioselectively removing potential lead compound derivatives from a solid support in non-aqueous media.

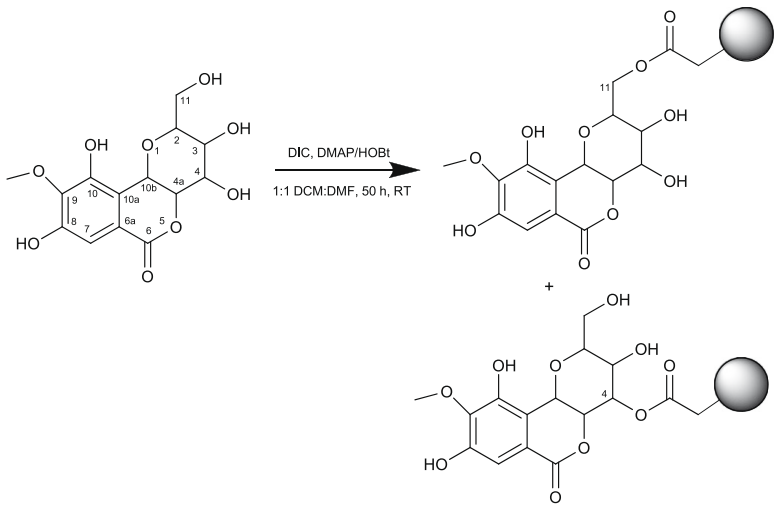
Bergenin was directly immobilized onto carboxylic acid-functionalized controlled pore glass (carboxy-CPG) at 95% yield according to Scheme 1. Immobilization yields were determined by measuring the disappearance of bergenin from solution via LC/MS. To quantify the degree of immobilization at the primary 11-hydroxyl versus secondary hydroxyl groups (most likely the 11-hydroxyl position due to reduced steric hindrance), bergenin was coupled to Boc-L-phenylalanine, a model compound containing a carboxylic acid group mimicking that of carboxy-CPG. Based on LC/MS analysis of the modified sample, the ratio of primary/secondary hydroxyl coupling was ~2:1. In addition, the large pore size (500 Å) of carboxy-CPG, along with the formation of an enzyme-cleavable ester bond between bergenin and the support, suggested that enzymatic cleavage of immobilized bergenin and its potential derivatives should be possible.

As an alternate immobilization method, bergenin was coupled to phenylalanine-CPG with the aim of using α-chymotrypsin (CT) to selectively cleave bergenin from the support. Previous studies suggested that CT can be used in solid-phase synthesis to cleave compounds from a solid support via a phenylalanine linker.[40,25] Bergenin was attached to and cleaved from Phe-CPG according to Scheme 2. For the generation of Phe-CPG, two silanization reagents, triethoxysilylpropanal (C3 silane) and triethoxysilyldecanal (C10 silane), were used to generate aldehyde groups on CPG and provide either a C3 or C10 linker between the surface of the CPG and the aldehyde group. L-Phe was then coupled to the aldehyde CPG. Both 400 Å and 900 Å pore-size CPG were employed in order to ascertain the effect, if any, of pore size on Phe loading.[41] The loading of Phe on both C3 and C10 CPG was determined using elemental analysis by measuring the difference in carbon and nitrogen levels before and after reaction with Phe.

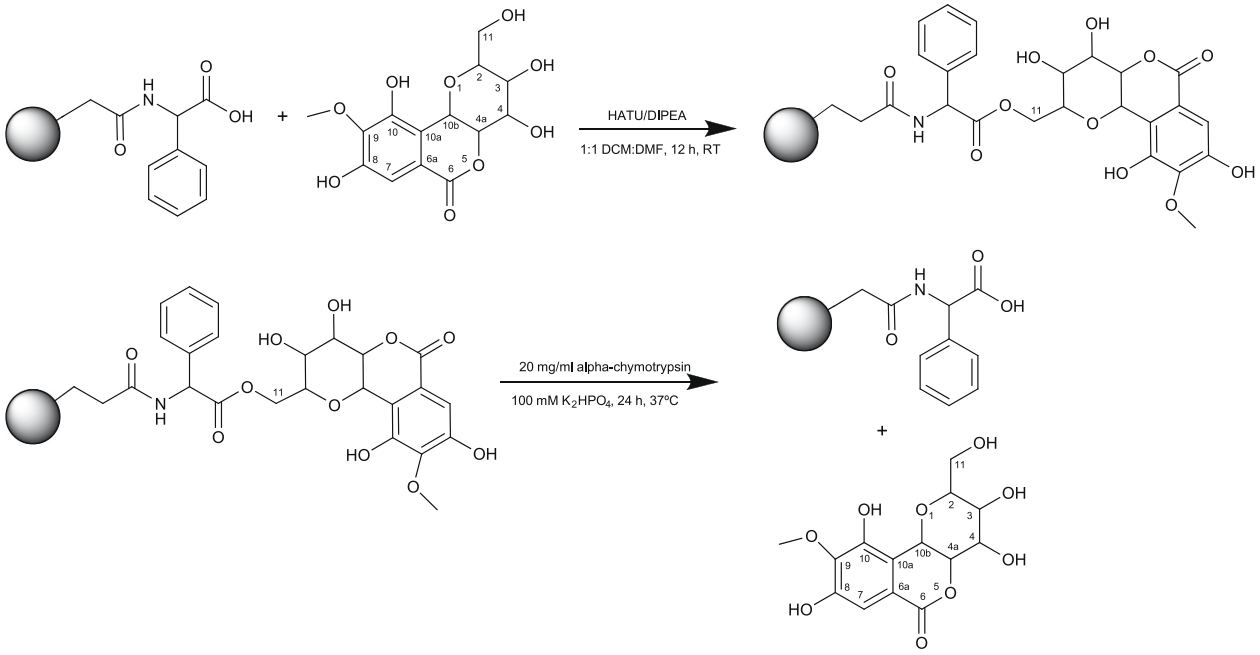
In the case of the C10 linker, no Phe attachment was detected for either pore-size CPG, possibly due to crowding by the comparatively long linker within the smaller (400 Å) pores, and the smaller overall surface area for the larger pore (900 Å) CPG. To preclude both possible problems, subsequent reactions employed the 400 Å CPG functionalized by the C3 silane. Attachment of Phe to the modified CPG resulted in an amino acid loading of 63.9 μmol/g according to elemental analysis. Bergenin coupling onto the Phe-CPG was carried out using conditions analogous to those used for the solution phase coupling reaction between bergenin and Boc-L-Phe (Table 1). The HATU/DIPEA combination yielded the highest solution coupling efficiency for the esterification reaction (71%) and therefore was used to attach bergenin to Phe-CPG. The maximum loading of bergenin to the Phe-CPG was 34.2 μmol/g (53% coupling yield), as determined by monitoring the reaction progress with LC/MS. Removal of attached bergenin was accomplished using H_2SO_4, SC, and CT (Table 2). Cleavage with CT gave a 9.3% cleavage yield, which was two times higher than SC (4.7%). Although this result shows the feasibility of using a Phe linker for selective cleavage of bergenin using CT, this yield was lower than that obtained using solubilized lipase B (lipB) or SC to remove bergenin directly from carboxy-CPG in organic solvents. Thus, Phe-CPG was not employed in subsequent solid-phase biocatalytic reactions.

Solid-supported bergenin was halogenated by chloroperoxidase (CPO) as the first step of a two-step enzymatic modification (Scheme 3). When performed for ~20 h, halogenation conversions of nearly 70% were obtained with solution phase bergenin. Although it was not expected that conversions for solid-phase bergenin halogenation would be as high, successful aqueous phase modifications of immobilized natural products could prove to be particularly useful for substrates that are poorly soluble in water, as is typically the case with many potential pharmaceutical lead compounds.

In the case of solid-supported bergenin, halogenation was carried out under similar conditions to the solution phase reaction, with the following modifications: the CPO/bergenin and KBr/bergenin ratios were increased by approximately 70- and 25-fold, respectively. Under these conditions, a solid-phase halogenation conversion of 5.9% was obtained, thereby demonstrating the ability of the CPO to access at least a portion of the solid-supported bergenin. Efforts to improve this conversion included the following: (1) increasing the H_2O_2/CPO ratio (4–8-fold), (2) running consecutive halogenation reactions with complete removal and replenishment of enzyme solution between reactions (up to six times), and (3) enzyme pre-incubation (at 4, 25 °C) in the absence of H_2O_2 (18–24 or 96 h). It was thought that allowing the enzyme to diffuse through the porous CPG network prior to H_2O_2 addition may enable better access of CPO to the solid-supported bergenin before enzyme inactivation by H_2O_2. Of these measures, only the enzyme pre-incubation (at either temperature for ~20 h) afforded any significant increase in solid-phase halogenation conversion—nearly a three-fold increase from 5.9% to 17.3% indicating that slow diffusion of the enzyme into the CPG pores is limiting.



Scheme 1. Bergenin immobilization onto carboxy-terminated controlled pore glass (carboxy-CPG).



Scheme 2. Immobilization of bergenin on L-phenylalanine-functionalized controlled pore glass (Phe-CPG) and cleavage by α-chymotrypsin.

Table 1
Reaction yields for coupling of bergenin to Boc-L-Phe in solution using various coupling reagents

Reagent	Yield (%)
DIC, HOBt, DMAP	21
DIC, DMAP	46
HATU, DIPEA	71
BrOP, DIPEA	1.5
PyBrOP, DIPEA	11

Table 2
Cleavage yields for bergenin immobilized on Phe-CPG using different reaction conditions

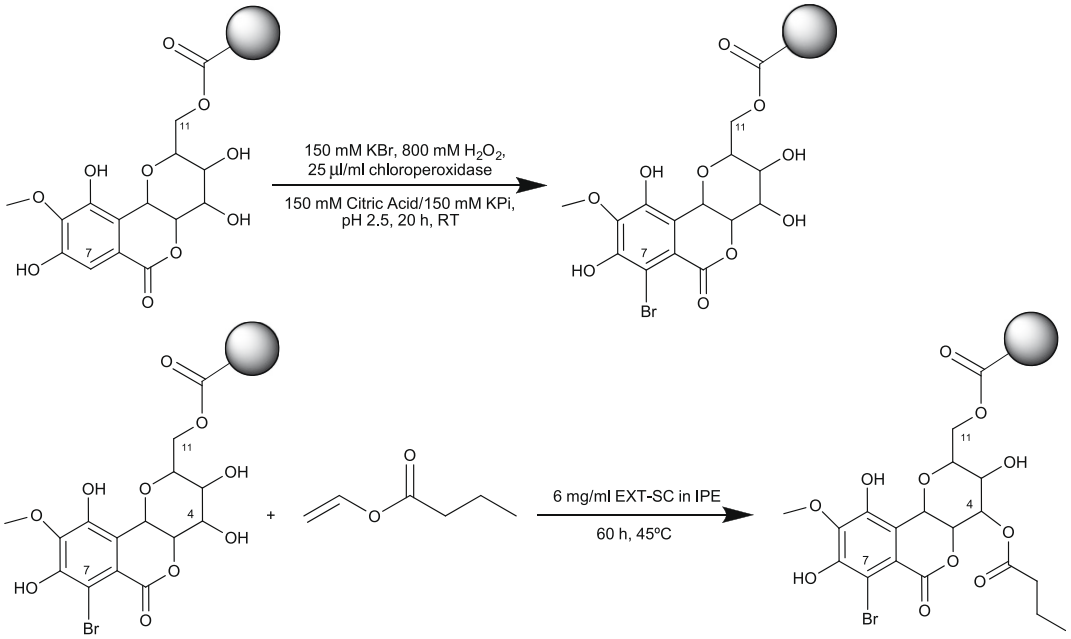
Reaction conditions	Yield (%)
1 M H_2SO_4 (aq)	48.9
20 mg/ml SC in aqueous buffer	4.7
20 mg/ml CT in aqueous buffer	9.4

A major advantage of solid-phase chemistry and biocatalysis over solution-phase reactions is the ability to perform sequential reactions without purification of intermediate products from the reaction mixture. To demonstrate that solid-phase biocatalysis can be used to carry out sequential reactions on immobilized substrates in multiple solvents, the halogenated bergenin derivative was subsequently acylated by EXT-SC in diisopropyl ether (IPE) with 500 mM excess vinyl butyrate. EXT-SC was selected over its DS counterpart based on its higher solution phase activity (nearly twofold higher initial rate) in IPE, the solvent in which the highest overall EXT-SC activity was observed (Table 3). The resulting product was cleaved from the CPG via concentrated LipB dissolved in aqueous buffer in both 0% and 10% acetonitrile (ACN) co-solvent (Table 4), resulting in a ~10% post-cleavage recovery yield of 7-bromo-4-butyrylbergenin under optimal cleavage conditions (10% ACN). To our knowledge, this represents the first example of a two-step aqueous:organic biocatalytic reaction sequence for modifying a solid-supported natural product. This approach can likely be expanded to produce libraries of solid-supported natural products in both aqueous and non-aqueous media, which could serve to be a valuable tool for compound generation and lead optimization in drug discovery.

Extraction: Native protein (i.e., taken directly from the bottle without further modification) was dissolved in aqueous buffer (SC: 10 mM bis-Tris Propane with 2 mM $CaCl_2$, pH 7.8; LipB: 10 mM bis-Tris Propane with 300 mM NaCl, pH 6.0) at 1 mg/mL and then added to an equivalent volume of 2 mM sodium bis(2-ethylhexyl) sulfosuccinate (AOT) dissolved in isooctane. The vial was vortexed for 10 s and placed in an orbital shaker at 30 °C and 250 rpm for 45 min. The biphasic mixture was then immediately centrifuged at 2700*g* for 5 min to separate the two phases. Following phase separation, the organic phase was removed and evaporated to dryness by a flowing air stream and the concentration of the enzyme/surfactant complex in the organic phase was determined spectrophotometrically by scanning from 220 to 320 nm, recording the peak absorbance at 280 nm, and subtracting a baseline absorbance reading at 310 nm. The extinction coefficient for protein solubilized in organic solvent was experimentally determined to be the same as for protein dissolved in H_2O.

Direct solubilization: Directly solubilized proteins were prepared according to the procedure described by Akbar et al.[30] In particular, a small amount of nano-filtered water (0.25% v/v for SC, 0.70% v/v for LipB) was added to a flame-dried vial, followed by addition of 2 mM AOT dissolved in isooctane. Native protein was added to the isooctane/water mixture to yield a final concentration of 1 mg/mL suspended enzyme and the contents were magnetically stirred for ca. 45 min. After stirring, the vial was centrifuged at 2700*g* for 5 min and the supernatant was transferred to a separate vial and evaporated to dryness before being dissolved in the final reaction solvent.

Bergenin immobilization and cleavage on CPG: In a flame-dried vial, 30 mM bergenin was dissolved in a 1:1 (v/v) DMF:DCM solution, followed by addition of 1 equiv 1-hydroxybenzotriazole (HOBT), 0.2 equiv 4-dimethyl aminopyridine (DMAP), and 0.25 equiv carboxy-CPG. After allowing the resin to settle to the bottom of the vial, the liquid above the resin was sampled for LC analysis of initial bergenin concentration and 1 equiv 1,3-diisopropyl carbodiimide (DIC) was added to initiate the immobilization



Scheme 3. Two-step enzymatic modification of solid-supported bergenin via halogenation by chloroperoxidase in aqueous solution followed by acylation via EXT-SC in IPE.

Table 3
Initial rates of product formation for solution phase bergenin transesterification with vinyl butyrate catalyzed by directly solubilized (DS), extracted (EXT), and native subtilisin carlsberg (SC) in isooctane or diisopropyl ether

Enzyme preparation	Reaction solvent	Initial rate (mM min^{-1})
DS-SC	Isooctane	0.088
DS-SC	Diisopropyl ether	0.62
EXT-SC	Isooctane	0.0035
EXT-SC	Diisopropyl ether	1.2
Native SC	Isooctane	0.88
Native SC	Diisopropyl ether	0.36

Table 4
Cleavage yields for multiple products resulting from cleavage reaction of 7-bromo-4-butyrylbergenin immobilized on carboxy-CPG using either aqueous Lipase B or Lipase B in 10% ACN co-solvent

Cleavage product	Yield (%) in aqueous buffer	Yield (%) in 10% ACN (v/v)
7-Bromo-4-butyryl bergenin	5.0	9.6
7-Bromobergenin	10.5	7.8
4-Butyrylbergenin	17.7	16.6
Total cleavage yield (%)	94.7	48.0

reaction. Reaction vials were incubated for ~50 h at 25 °C, 100 rpm.

After 50 h, the liquid above the resin was again sampled for LC analysis and the reaction was quenched by vacuum filtration of the solvent, followed by four washes/filtrations each with excess ACN, MeOH, DMF, and DCM. Immobilized substrate-containing resins were stored under vacuum at room temperature prior to further use. Additional control experiments were also carried out to verify the absence of bergenin side reactions and to quantify the amount of bergenin physically adsorbed to the carboxy-CPG surface. Bergenin was coupled to Boc-L-phenylalanine in solution using 80 mM each of bergenin and Boc-L-phenylalanine and the same molar equivalents of all remaining reagents. The reaction conditions for bergenin immobilization were modified from conditions for alcohol attachment onto carboxylic acid resins found in the 2003–2004 Advanced ChemTech combinatorial chemistry handbook.[42]

Bergenin immobilization and cleavage on Phe-CPG: To prepare aldehyde CPG (CPG-CHO), CPG was added to a solution of 5% (v/v) triethoxysilylpropanal or triethoxysilyldecanal in chloroform. The reaction vial was incubated at 50 °C and 250 rpm for 12 h. After 12 h, the supernatant was filtered out and the CPG was washed/filtered four times each in excess chloroform and then MeOH. CPG-CHO was dried under reduced pressure at room temperature prior to further use. For the Phe reaction, 1 equiv CPG-CHO and 5 equiv L-Phe in water and 5 equiv $NaBH_3(CN)$ in DMF (1:1 water/DMF (v/v)) were mixed together and the vial was incubated at 25 °C and 250 rpm for 12 h. After 12 h, the supernatant was filtered out and the Phe-CPG was washed/filtered four times each in excess DMF, DCM and MeOH. Phe-CPG was dried under a nitrogen stream and stored under reduced pressure at room temperature. Bergenin coupling to the Phe-CPG was carried out using HATU/DIPEA as coupling reagents. To a vial containing 1 equiv Phe-CPG, 5 equiv bergenin and 5 equiv DIPEA in a 1:1 DMF/DCM (v/v) solution was added and incubated at 25 °C and 250 rpm for 12 h. The supernatant was then filtered out and the Bergenin-Phe-CPG was washed/filtered four times each in excess DMF, DCM and MeOH. Each reaction step was monitored by the colorimetric detection of relevant functional groups on the surface of the CPG, using the reagent Purpald for aldehydes, Malachite Green for carboxyl acids, and chloranil for secondary amino groups.[43–47] For quantitative analysis of the immobilized phenylalanine and bergenin on CPG, elemental analysis was performed on CPG after drying under reduced pressure for 24 h. For solution phase reactions, 10 mM bergenin was coupled to 10 mM Boc-L-Phe using either DIC (1 equiv), HOBt (1 equiv), DMAP (0.2 equiv), or DIC (1 equiv), DMAP (0.2 equiv), or HATU (1 equiv), DIPEA (2 equiv), or BrOP (1 equiv), DIPEA (2 equiv), or PyBrOP (1 equiv), DIPEA (2 equiv). For bergenin cleavage reactions, bergenin immobilized on the Phe-CPG was added directly to a solution containing either 1 M aqueous sulfuric acid, 20 mg/mL of SC (220 U/mg) in 100 mM K_2HPO_4 at pH 7.5 or 20 mg/mL of CT (1600 U/mg) in 100 mM K_2HPO_4 at pH 8.0. For H_2SO_4 cleavage, the reaction was run at 45 °C and 100 rpm for 24 h, followed by quenching via 1 equiv base. When native enzyme in aqueous buffer was used, the

reaction was run at 45 °C and 100 rpm for 48 h (SC) or at 37 °C and 100 rpm for 24 h (CT). Following each cleavage reaction, the filtrate was analyzed via LC/MS for the presence of bergenin.

Solid-phase bergenin halogenation and cleavage: 0.5–0.7 mM immobilized bergenin (50 mg resin/mL, 14.5 μmol COOH/g CPG) was added to a 150 mM citric acid/150 mM potassium phosphate (pH 2.5) aqueous buffer solution containing 150 mM KBr (1.6 mL total reaction volume). Following addition of 25 μL/mL CPO (20.8 kU/mL, 1450 U enz/μmol bergenin), vials were pre-incubated at either 4 or 25 °C, 180 rpm for 20 h. Reactions were initiated by continuous infusion of 800 mM H_2O_2 (in buffer) at a flowrate of 20 μL/h (0.4 mmol/h H_2O_2/μL CPO) via a syringe pump. Reaction vials—left open to the atmosphere—were incubated at room temperature under orbital shaking at 180 rpm for 18–20 h. If additional rounds of reaction were desired, the original solution above the resin was removed, followed by addition of fresh CPO and KBr dissolved in aqueous buffer and overnight pre-incubation. Upon completion of the reaction, the contents were centrifuged at 2700*g* for 5 min, followed by removal of the supernatant. After gently drying the vial contents under a flowing air or N_2 stream, the resin was further dried under vacuum overnight. During optimization of the reaction conditions, enzyme/substrate ratios between 1000 and 3000 U CPO/μmol bergenin, co-factor/enzymes ratios of 0.2–3.3 mmol/h H_2O_2 per μL CPO, and pre-incubation times of 20–96 h were also tested. For cleavage via LipB in aqueous buffer, 40–60 mg/mL resin containing immobilized bergenin and its derivatives was added to a 50 mM potassium phosphate (pH 7.0) solution containing 75 mg/mL LipB and incubated at 45 °C, 180 rpm for ~30–40 h. After addition of DMF to help dissolve the cleaved bergenin/derivatives (~20–25% v/v DMF in final mixture) and re-incubation at 45 °C, 180 rpm for 30 min, the vials were centrifuged at 2700*g* for 5 min, followed by sampling of the supernatant for LC/MS analysis.

Solution phase bergenin transesterification: 500 mM of bergenin dissolved in DMF was added to a flame-dried glass vial in a 95:5 v/v ratio of organic solvent (either isooctane or diisopropyl ether) to DMF, yielding a final bergenin concentration of 25 mM. Solubilized SC and native SC in either isooctane or diisopropyl ether was added to the reaction so that the final enzyme concentration was 1.6 mg/ml. Reactions were initiated by adding 500 mM of vinyl butyrate and the contents were incubated at 45 °C and 250 rpm. Initial rates of product formation were measured by removing aliquots from the reaction vials over time and analyzing them for the presence of product via LC/MS. Prior to LC/MS analysis aliquots were first quenched by addition of an excess of acetonitrile and incubation on ice, followed by centrifugation at 2700*g* for 5 min. The resulting samples were then evaporated to dryness and re-dissolved in acetonitrile for immediate LC/MS analysis.

Solid-phase bergenin transesterification and cleavage: Immobilized bergenin (~1–3 mM, 100 mg/mL resin) was transesterified with excess vinyl butyrate (500 mM) using EXT-SC (6 mg/mL) in IPE. Solubilized enzyme initially prepared in isooctane was transferred to the reaction vial; the contents were then evaporated to dryness and re-dissolved in IPE. After incubation at 45 °C and 180 rpm for ~60 h, 1 vol equiv of EXT-SC (6 mg/mL) in IPE was added and the contents were incubated for an additional 50–60 h. This reaction time was chosen based on the observation that complete conversion of solution phase bergenin to the acylated product was achieved in 24 h for EXT-SC in IPE, and on the constant activity exhibited by EXT-SC in isooctane for at least 30 h (data not shown). After 50 h, the contents were centrifuged at 2700*g* for 5 min and the supernatant was removed, followed by two additional IPE washes. For each wash, 1 vol equiv IPE was added, followed by incubation at 45 °C and 180 rpm for 30 min, centrifugation, and supernatant removal. For complete removal of IPE, the vials were stored overnight under vacuum prior to cleavage. For cleavage with concentrated LipB (~75 mg/mL) dissolved in 50 mM potassium phosphate (pH 7.0) with 0–30% ACN co-solvent, 60–70 mg/mL resin was incubated at 45 °C 180 rpm for 20–30 h. Following completion of each enzymatic cleavage reaction, DMF was added to the reaction vials to help dissolve the cleaved bergenin/derivative (~20–25% v/v DMF in final mixture), followed by re-incubation at 45 °C and 180 rpm for 30 min, centrifugation at 2700*g* for 5 min, and sampling of the supernatant for LC/MS analysis. When analyzing the LC/MS results, the calculated yields of the 4-butyryl bergenin derivative were multiplied by 1.5 to account for the fact that approximately 33% of the bergenin initially immobilized had been attached at the 4-hydroxyl position—thereby precluding formation of the 4-acylbergenin derivative—instead of the 11-position (i.e., only 2/3 of the total immobilized bergenin could be acylated at the 4 position).

Liquid chromatography/mass spectrometry: Bergenin transesterification reactions were analyzed using LC/MS. Products were resolved using a reverse-phase wide-pore C18 column (JT Baker, 4.6 mm × 250 mm, 5 μm particles) with a 0.9 mL/min flow of an acetonitrile (ACN)/H_2O gradient (0–20 min: 0/100% ACN/H_2O—67/33% ACN/H_2O, linear gradient) and analyzed on a Hewlett Packard (Agilent) 1100 LC/MSD equipped with a diode array detector (DAD) and an atmospheric pressure electrospray ionization (API-ES) unit. The quadrupole ion analyzer MS was set to scan from 300 to 500 amu (0.10 amu increments), with a fragmentor setting of 70 V, 3000 V capillary, 35 psig nebulizer pressure, and with N_2 drying gas set to 300 °C and 9.5 L/min. Absorbances were averaged between 260 and 290 nm (340–380 nm baseline) and were used in conjunction with positive-ion MS to provide quantitative detection and unambiguous molecular identification.

Acknowledgments

This work was supported by the National Institutes of Health (GM66712), by the National Science Foundation (BES-0228145) and the Korea Research Foundation Grant funded by the Korean Government (KRF-2007-357-D00057). The authors thank Prime Synthesis for custom synthesizing the 12–13 carbon spacer CPG as well as Peter McDermott, I-Fan Lin, Brandon Gan, and Vincent Chan for assistance in obtaining both chemical and enzymatic bergenin cleavage and hydrolysis data.

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