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(−)-FR182877 Is a Potent and Selective Inhibitor of Carboxylesterase-1**

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Natural products constitute a rich source of structurally diverse compounds that display exceptional biochemical and cellular activities. Although many natural products bind reversibly to their protein targets to alter protein–protein interactions[1] and/or catalytic activity,[2–4] others produce their biological effects through the covalent modification of specific proteins and/or protein families. Members of this latter class of natural products are typically endowed with electrophilic functionalities that react with nucleophilic residues in structured sites of proteins, such as the active

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sites of enzymes. Key examples of protein-reactive natural products include fumagillin,[5] lipstatin,[6] microcystin,[7] and wortmannin,[8] which label the active sites of methionine aminopeptidases (MetAPs), pancreatic lipases, serine/threonine phosphatases, and lipid kinases, respectively.

Within the domain of natural products bearing electrophilic functional groups, the structure of FR182877 (**1**) is particularly provocative in that it displays two potential sites for protein reactivity: a strained bridgehead olefin and a lactone carbonyl group.[9–11] The unprecedented architecture of **1** (Scheme 1) has attracted the attention of several groups in organic synthesis.[12–19] Additionally, preliminary biological studies of **1** have revealed that this molecule displays several interesting activities, including promotion of microtubule polymerization,[9] induction of cell-cycle arrest in human cancer cell lines,[9] and antitumor effects in mouse models.[10] Nonetheless, whether **1** produces its pharmacological effects through binding and/or modifying specific proteins in the proteome remains unknown. The recent establishment of efficient routes for the synthesis of **1** and derivatives thereof[14–16] has provided an opportunity to investigate the biological activity of this natural product. Toward this end, we report here the synthesis of a series of biotin/fluorophore-conjugated forms of both the natural (−) and unnatural (+) enantiomers of FR182877 and their application to mouse-tissue proteomes to identify specific sites of protein reactivity.

Reporter-group-tagged versions of (−)-**1** were synthesized in a two-stage sequence in which the natural product was first treated with 10-azidodecanoyl chloride (**2**) to create **3** (Scheme 1).[20] Reaction of **3** with an alkyne-derivatized rhodamine or rhodamine–biotin bifunctional agent in the presence of a copper(I) catalyst[21] provided **4** and **6**, respectively. The analogous tagged variant **5** of the unnatural enantiomer (+)-FR182877 was also synthesized in this manner.

The tagged derivative **4** was incubated at 0.1 μM for 1 h with a mouse-heart proteome (2 mg protein mL^{-1}) and found by SDS-PAGE analysis to specifically label a diffuse 70-kDa protein (Figure 1a, lane 2). Heat denaturation of the proteome prior to treatment with **4** completely blocked labeling of the 70-kDa target (Figure 1a, lane 1), suggesting that this

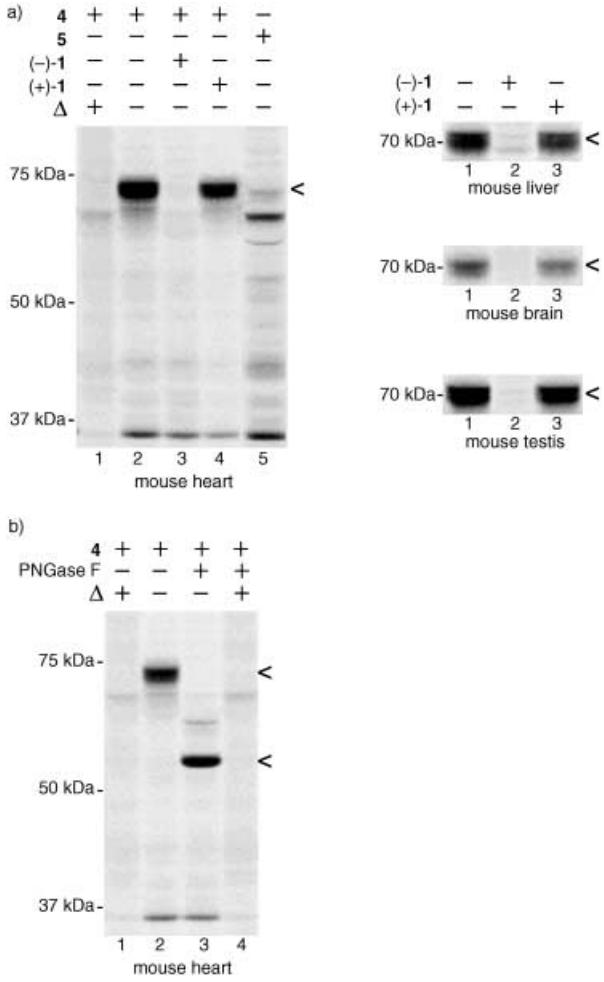
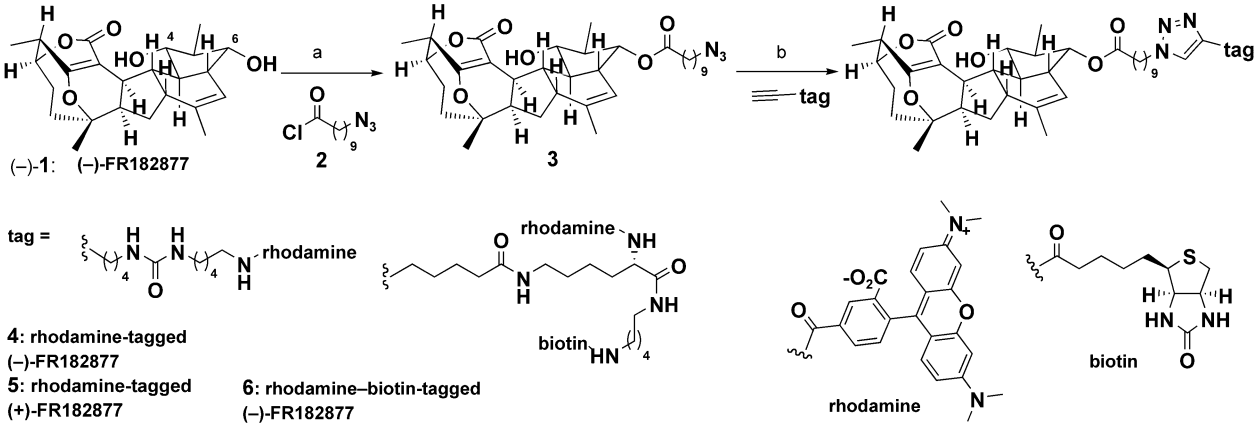


Figure 1. Profiling mouse-tissue proteomes with rhodamine/(−)-FR182877 (**4**). a) Left panel: **4** but not **5** labeled a diffuse 70-kDa protein in mouse-heart proteome (lanes 2 and 5, respectively) (reaction conditions: 0.1 μM **4** or **5**, 2 mg mL^{-1} protein, 50 mM Tris, pH 8.0, 60 min). This protein labeling event was heat sensitive (lane 1) and blocked by pretreatment with 10 μM unlabeled (−)-**1** (lane 3). Right panel: The 70-kDa **4**-labeled protein was found in many tissues. b) Treatment of the **4**-labeled mouse-heart proteome with PNGaseF reduced the size of the 70-kDa target to 55 kDa, indicating that this protein is glycosylated.



Scheme 1. Synthesis of reporter-tagged versions of (−)-**1**. a) **2** (4 equiv), 2,4,6-collidine (10 equiv), CH_2Cl_2, 77%; b) alkyne-tag (rhodamine or rhodamine–biotin) (0.5 equiv), cat. $CuSO_4$, cat. sodium ascorbate, $tBuOH/H_2O$, ca. 50%.

event occurs in a structured binding site in the protein. Interestingly, the 70-kDa protein was not labeled by **5** (Figure 1a, lane 5), indicating that it is a specific target of the natural enantiomer. Consistent with this premise, labeling of this protein was completely blocked by preincubation with 10 μM untagged (−)-**1**, but was unaffected by (+)-**1** (Figure 1a, lanes 3 and 4, respectively). A few additional protein targets of **4** were also detected, but the labeling of these proteins was also observed with **5** and was not competed by untagged forms of either enantiomer (e.g., 35-kDa band, lanes 2–6, Figure 1a). Treatment of the mouse-heart proteome with higher concentrations of **4** (5–20 μM) did not uncover additional protein targets that were selectively labeled by this natural product (data not shown). Collectively, these initial data suggest that (−)-**1** specifically labels a single protein in the mouse-heart proteome.

The 70-kDa target of (−)-**1** was detected in several other mouse tissues, including brain, liver, and testis (Figure 1b). The target was glycosylated as determined by its conversion to a 55-kDa band following treatment with the glycosidase PNGaseF (Figure 1c). To identify this protein, a version of (−)-**1** coupled to both rhodamine and biotin was employed (derivative **6**, Scheme 1) This type of "trifunctional" probe permits the affinity isolation of labeled proteins by avidin chromatography and direct visualization of affinity-purified proteins by in-gel fluorescence scanning.[22] The avidin-purified 70-kDa protein was excised from an SDS-PAGE gel, digested with trypsin, and identified by mass spectrometry (MS) analysis as carboxylesterase-1 (CE-1) (for MS data, see Supporting Information).

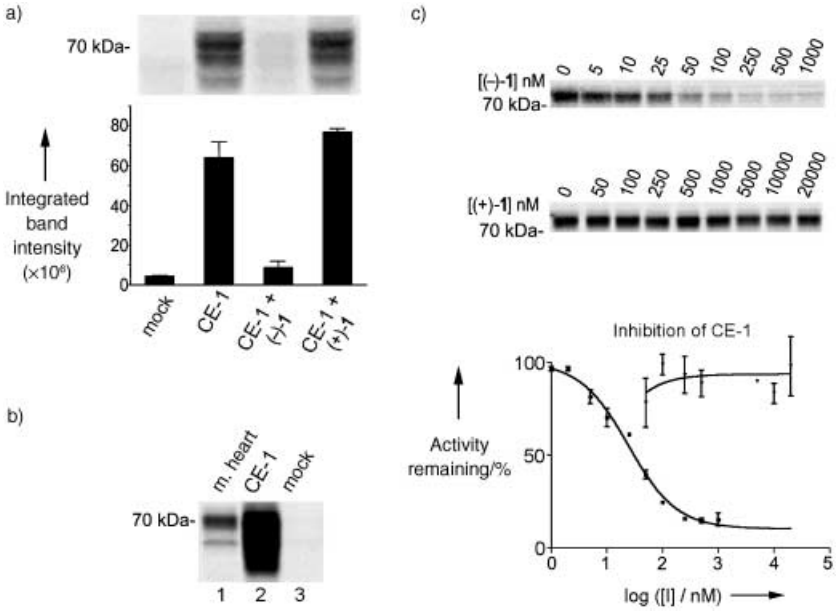


Figure 2. Evidence that (−)-**1** is an active site-directed inhibitor of mouse CE-1. a) Derivative **4** labels recombinant CE-1 expressed by transient transfection in COS-7 (lane 2) and this labeling event was blocked by pretreatment with 10 μM unlabeled (−)-**1** (lane 3), but not (+)-**1** (lane 4). Mock-transfected COS-7 cells did not contain CE-1 (lane 1). Proteomes shown represent soluble extracts from homogenized COS-7 cells; the conditioned media of CE-1-transfected COS-7 cells also contained this enzyme (data not shown). b) Native and recombinantly expressed CE-1 (lanes 1 and 2, respectively) were labeled by the serine hydrolase-directed activity-based probe, FP-rhodamine[28] (0.1 μM, 10 min). c) Upper panel: Inhibition of FP-rhodamine labeling of CE-1 by increasing concentrations of unlabeled (−)- and (+)-**1** [preincubated with heart proteomes for 10 min prior to addition of FP-rhodamine (0.1 μM, 10 min)]. Lower panel: Plotting of these competitive profiling data determined an IC_{50} value of 34 nM (28–41 nM, 95% confidence limits) for (−)-**1** inhibition of CE-1. No inhibition of CE-1 by (+)-**1** was detected at concentrations up to 20 μM.

CE-1 is a secreted serine hydrolase implicated in lipid metabolism and detoxification of xenobiotics.[23–25] To confirm that CE-1 is the target of modification by (−)-**1**, the cDNA for the enzyme was subcloned into the vector pcDNA3.1 and expressed in COS-7 cells by transient transfection following previously described procedures.[26] CE-1-transfected COS extracts, but not mock-transfected cell extracts (transfected with empty vector), were found to possess a diffuse 70-kDa protein that was labeled by derivative **4** (Figure 2a). Importantly, the labeling of recombinant CE-1 by **4** was blocked by pretreatment with untagged (−)-**1**, but not (+)-**1** (Figure 2a), verifying that this enzyme is a specific target of the natural enantiomer.

To determine whether (−)-**1** acted as an inhibitor of CE-1, an assay for the activity of this enzyme was needed. Although CE-1 has been hypothesized to participate in the metabolism of lipids in vivo,[23–25] its natural substrates remain unknown. Thus, few assays to measure CE-1 activity have been developed, other than those that employ a nonspecific substrate such as *p*-nitrophenyl acetate,[27] which are complicated to perform in cell/tissue extracts because of the presence of contaminating esterases. Considering that CE-1 is a member of the serine hydrolase superfamily, the activity of this enzyme should be measurable in whole proteomes by using a rhodamine-tagged fluorophosphonate probe (FP-rhodamine).[28] Previous studies have shown that FP-rhodamine serves as a general activity-based profiling tool for serine hydrolases[29] and can been used to evaluate the potency and selectivity of inhibitors that target this enzyme class directly in complex proteomes.[30,31]

FP-rhodamine was found to label both native and recombinantly expressed CE-1 (Figure 2b). The effect of (−)-**1** on FP-labeling of CE-1 was tested by preincubating mouse-heart extracts with the natural product over a concentration range of 0.001–1 μM for 10 min, followed by addition of FP-rhodamine (0.1 μM) (Figure 2c). From these data, an IC_{50} value of 34 nM (28–41 nM, 95% confidence limits) was calculated for the inhibition of CE-1 by (−)-**1**. In contrast, (+)-**1** failed to inhibit the FP-labeling of CE-1, even at the highest concentration tested (20 μM). Notably, (−)-**1** completely blocked the FP-labeling of CE-1 at a concentration (1 μM) that was at least 20-fold lower than that required to

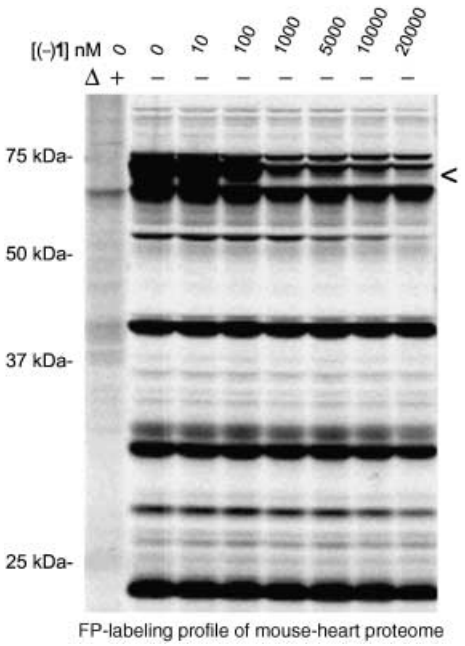


Figure 3. Evidence that (−)-**1** is a selective inhibitor of mouse CE-1. Preincubation of the mouse-heart proteome with increasing concentrations of (−)-**1** (0.01–20 μM) prior to treatment with FP-rhodamine (0.1 μM) identified CE-1 as a selective target of this natural product. FP-labeling of CE-1 was completely blocked by 1 μM (−)-**1** (for a lighter exposure of this profile, see upper panel in Figure 2c), while additional serine hydrolase targets of (−)-**1** were not detected until 20 μM of this natural product was added to the proteome [at this concentration, one additional serine hydrolase target of (−)-**1** was detected (60 kDa protein)].

reduce the FP-labeling of other heart hydrolases (Figure 3). Collectively, these inhibitor profiling data indicate that (−)-**1** is a potent and selective inhibitor of CE-1.

In summary, we have synthesized a series of rhodamine–biotin-tagged forms of (−)- and (+)-**1** and applied these reagents to identify the mouse enzyme carboxylesterase-1 (CE-1) as a specific target of this natural product. Considering that the association of (−)-**1** with CE-1 was stable to protein denaturation, this interaction appears to be covalent in nature. Activity-based profiling experiments with the serine hydrolase directed probe FP-rhodamine determined that (−)-**1** acts as a highly potent ($IC_{50} = 34$ nM) and selective inhibitor of CE-1. Notably, the inhibition of CE-1 by (−)-**1** occurs at much lower concentrations than the reported effects of this natural product on tubulin polymerization (~100 μM[9]). These findings raise the intriguing possibility that at least some of the pharmacological activities of (−)-**1** in vivo may be due to CE-1 inhibition. For example, the antitumor effects of (−)-**1** would not be inconsistent with CE-1 inhibition, as carboxylesterases are expressed in a variety of cancers.[32,33] However, establishing a direct link between the biochemical properties of (−)-**1** and its pharmacological activities in vivo will require more detailed investigations. In this regard, it is important to emphasize that (−)-**1** may also possess non-covalent protein targets that would have eluded detection by the methodologies employed in this study.

As mentioned previously, the complex and rigid molecular architecture of FR182877 (**1**) is distinguished by two electrophilic groups, a strained bridgehead olefin and a lactone carbonyl group. One or both of these functional groups may represent sites for protein modification. Although it remains unclear which, if either, of these moieties is responsible for covalent binding to CE-1, instances of lactone natural products serving as inhibitors of serine hydrolases are known. For example, lipstatin is a covalent inactivator of the pancreatic lipases,[6] and, in its tetrahydro form (Orlistat), it is in clinical use for the treatment of obesity.[34] Considering further that many synthetic serine hydrolase inhibitors suffer from poor target selectivity within this large enzyme class,[31,35] the apparent specificity that FR182877 and lipstatin display for their respective targets is provocative and suggests that lactone natural products, or natural product-like compounds, may be a fertile source for novel hydrolase inhibitors. Toward this end, the creation of diverse compounds based on the structure of FR182877,[36] coupled with an activity-based proteomic screen,[31] may provide a general strategy to discover potent and selective inhibitors of many members of the serine hydrolase superfamily.

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