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Activity-Based Proteome Profiling of Potential Cellular Targets of Orlistat – An FDA-Approved Drug with Anti-Tumor Activities

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Abstract: Orlistat, or tetrahydrolipstatin (THL), is an FDA-approved antiobesity drug with potential antitumor activities. Cellular off-targets and potential side effects of Orlistat in cancer therapies, however, have not been extensively explored thus far. In this study, we report the total of synthesis of THL-like protein-reactive probes, in which extremely conservative modifications (i.e., an alkyne handle) were introduced in the parental THL structure to maintain the native biological properties of Orlistat, while providing the necessary functionality for target identification via the bio-orthogonal click chemistry. With these natural productlike, cell-permeable probes, we were able to demonstrate, for the first time, this chemical proteomic approach is suitable for the identification of previously unknown cellular targets of Orlistat. In addition to the expected fatty acid synthase (FAS), we identified a total of eight new targets, some of which were further validated by experiments including Western blotting, recombinant protein expression, and site-directed mutagenesis. Our findings have important implications in the consideration of Orlistat as a potential anticancer drug at its early stages of development for cancer therapy. Our strategy should be broadly useful for off-target identification against quite a number of existing drugs and/or candidates, which are also covalent modifiers of their biological targets.

Introduction

Drug discovery is a long and costly process, yet most drugs have side effects, ranging from simple nuisances to life-threatening complications.[1] Unanticipated effects of a drug, often revealed either during clinical trials or sometimes after the drug enters the market, could lead to termination of a drug development program/recall of the drug, or, in some rare cases where the effects are beneficial, new drug applications.[1b,c] Therefore, one of the most critical steps in the drug discovery process is the effective identification of the so-called off-targets and anticipation of their potential side effects a priori.

Orlistat, or tetrahydrolipstatin (THL), is an FDA-approved antiobesity drug, which works primarily on pancreatic and gastric lipases within the gastrointestinal (GI) tract.[2] Recently, Orlistat was found to inhibit the thioesterase domain of fatty acid synthase (FAS), an enzyme essential for the growth of cancer cells but not normal cells.[3,4] By effectively blocking the cellular FAS activity, Orlistat induces endoplasmic reticulum stress in tumor cells, inhibits endothelial cell proliferation and angiogenesis, and consequently delays tumor progression on a variety of cancer cells, including prostate, breast, ovary, and melanoma cancer cells.[3] As a result, this compound (as well as other Orlistat-like analogues with improved potency and bioavailability[5]) has been proposed as a promising anticancer drug. Cellular off-targets and potential side effects of Orlistat in cancer therapies, however, have not been extensively explored thus far.[6] Our long-term research goals focus on developing novel chemical proteomic strategies that enable large-scale studies of

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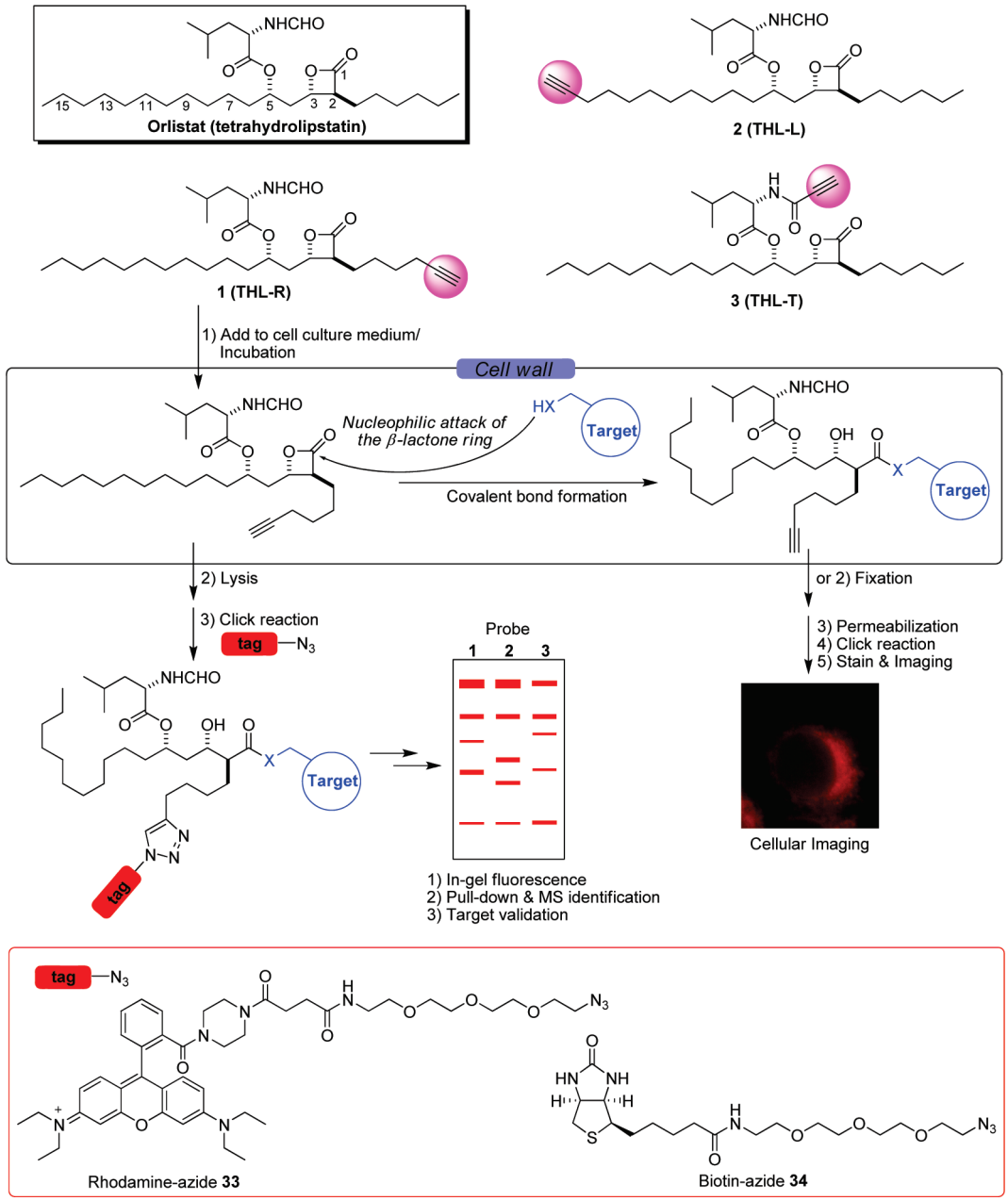

Figure 1. Overall strategy for activity-based proteome profiling of potential cellular targets of Orlistat using alkyne-containing, cell-permeable THL analogues (**1**, **2**, and **3**). The alkyne handles in the probes are shaded (purple circle). The "Click"-based reporters used in the manuscript are shown (in red rectangle).

therapeutically relevant enzymes, as well as small molecules (i.e., potential drug candidates) that can modulate these enzymes' cellular activities.[7] In the current study, we set out to look for new cellular targets, including off-targets, of Orlistat at its early stages of development for cancer therapy. Herein, we report, for the first time, the identification and putative validation of several previously unknown cellular targets of Orlistat by using a natural product-based, chemical proteomic approach.

Results and Discussion

Design and Synthesis of Orlistat-like Probes. Our strategy is based on the well-established activity-based protein profiling (ABPP) approach,[8] by making use of THL-like protein-reactive probes **1**, **2**, and **3** (i.e., **THL-R**, **THL-L**, and **THL-T**, respectively, in Figure 1). We took advantage of several key properties known to THL in the design of our activity-based probes:[3,5] (1) THL (being derived from a natural product) is

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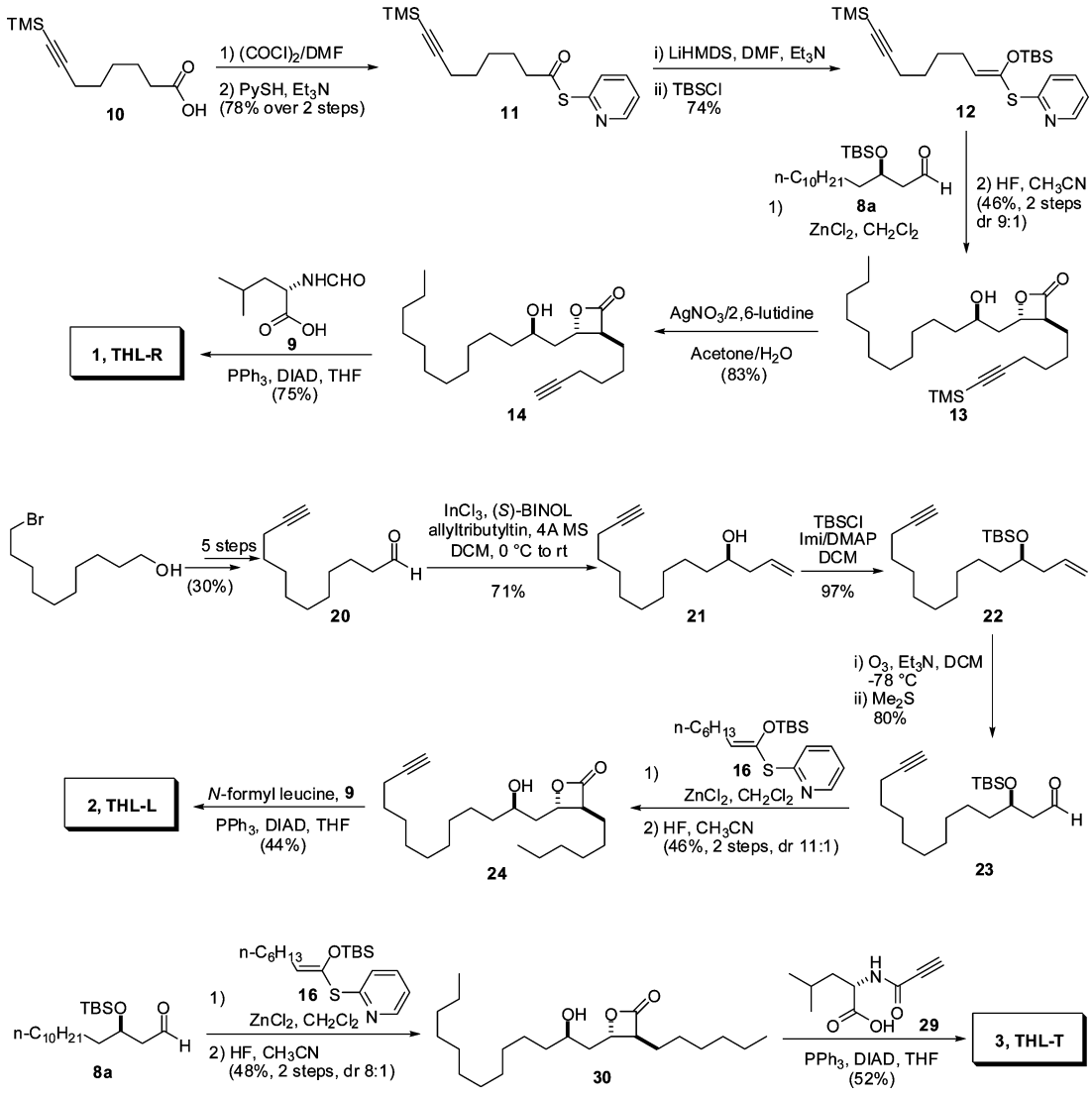
Scheme 1. Total Synthesis of the Three Probes



cell-permeable, making our probes applicable for direct in situ cell-based screening; (2) THL reacts with its known cellular targets via a covalent reaction through its reactive β-lactone moiety and the nucleophilic active-site residue (typically Ser/Cys residues, e.g. Ser[2308] in FAS[3b]) of the target protein, resulting in the formation of an isolatable protein/THL complex; (3) previous minor structural modifications at either the 16-carbon or 6-carbon aliphatic chains of THL did not significantly alter its native biological activities.[5b] Accordingly, probes **1** and **2**, in which a C−C triple bond was introduced into THL to replace the terminal C−C single bond of the aliphatic chains, were synthesized. We also synthesized probe **3** by substituting the CHO group in the *N*-formyl-L-leucine moiety of THL with a propiolic acyl group (Figure 1, shaded in purple). These extremely conservative modifications of introducing an alkyne handle in the parental THL structure were aimed at maintaining the native biological properties of Orlistat, while providing the necessary functionality for identification and characterization (i.e., imaging) of previously unknown cellular targets by downstream conjugation of the protein/probe complex to reporter tags via the bio-orthogonal click chemistry.[8,9]

Numerous strategies for the total synthesis of THL have been reported.[5] Our own efforts for the preparation of these probes followed similar tracks (Scheme 1). The synthesis of **1** (**THL-R**) commenced with the tandem Mukaiyama aldol-lactonization (TMAL) reaction between the optically pure aldehyde, **8a** (obtained in five steps involving separation of a diastereomeric mixture of racemic homoallylic acohols; Scheme S1 in the Supporting Information), and TMS-protected thiopyridyl ketene acetal **12** (prepared in four steps from 6-bromohexanoic acid; Scheme S3 in the Supporting Information), giving the desired β-lactone as a mixture of diastereomers (~9:1 anti/syn) with complete selectivity for the *trans*-β-lactone. Following *O*-desilylation and silica gel separation, the enantiomerically pure γ-hydroxy β-lactone **13** was isolated. The trans configuration of the β-lactone core was unambiguously confirmed by coupling constant analysis ($J_{H2,\ H3}$ = ~4.4 Hz).[5a] Subsequent *C*-desilylation with $AgNO_3$/2,6-lutidine gave terminal acetylene **14**, which was subjected to Mitsunobu conditions with *N*-formyl-L-leucine to give the configurationally inverted product **1**. For the synthesis of **2** (**THL-L**), an alternative route to obtain the

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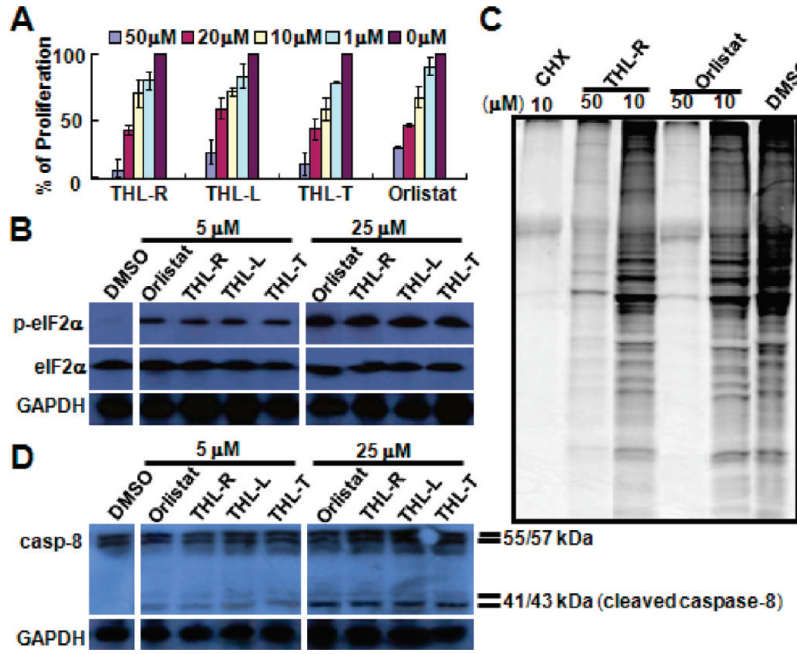

Figure 2. (A) Dose-dependent inhibition of HepG2 cell proliferation by Orlistat and three analogues (**1**, **2**, and **3**) using XTT assay. Data represent the average ±s.d. for two trials. (B) Western blot analysis of eIF2α phosphorylation in PC-3 cells upon treatment with the four compounds. GAPDH was used as a loading control. (C) Inhibition of protein synthesis in HepG2 cells treated with the indicated concentrations of Orlistat, **THL-R**, or CHX (cycloheximide, an inhibitor of protein biosynthesis) for 12 h and then pulsed with AHA (L-azidohomoalanine) for 4 h. Cell lysates were prepared and subjected to click chemistry with rhodamine-alkyne (provided with the AHA kit) following vendor's protocols, SDS-PAGE analysis, and in-gel fluorescence scanning (fluorescent gel is shown in grayscale). (D) Activation of caspase-8 in MCF-7 cells treated with the indicated concentrations of Orlistat/analogues.

optically active aldehyde **23** was explored. First, 10-bromo-1-decanol was converted to the chiral homoallylic alcohol **21** in five steps (Scheme S5 in the Supporting Information) involving asymmetric allylation with allyltributyl stannane in the presence of chiral (*S*)-BINOL-$InCl_3$ complex.[10] The absolute configuration of **21** was unequivocally determined using Mosher ester analysis (Scheme S6 in the Supporting Information).[11] Subsequently, a two-step reaction gave **23**, which reacted with **16** under TMAL conditions to give **24** and eventually **2**. For the synthesis of **3** (**THL-T**), the known hydroxyl-β-lactone **30** was first prepared based on published procedures,[5a] then reacted with **29** (obtained in four steps from Fmoc-Leu-OH; Scheme S7 in the Supporting Information) under Mitsunobu conditions, giving **3** in 52% yield.

Effects on Cell Proliferation, Phosphorylation of eIf2α and Activation of Caspase-8. Next, the three probes were evaluated against Orlistat (as a positive control) for potential biological activities. Three different types of cellular assays based on previously established Orlistat biology were used.[3a,c−e] First, the antiproliferation activity of the four compounds (**1**, **2**, **3**, and Orlistat) against HepG2 cells (a human hepatocellular liver carcinoma cell line) was evaluated using the XTT assay (part A of Figure 2);[3a] all four compounds showed a dose-dependent inhibition of tumor cell proliferation over a 72 h time period with comparable potency. Second, we carried out comparative analysis of the compounds in their ability to induce phosphorylation of eIf2α in the prostate cancer PC-3 cells.[3c] Previous studies had shown that inhibition of FAS by Orlistat induces endoplasmic reticulum (ER) stress and results in the phosphorylation of the translation initiation factor eIF2α. As shown in part B of Figure 2, PC-3 cells treated with different amounts of each compound showed similarly elevated eIF2α phosphorylation. Because the phosphorylation of eIF2α is known to inhibit cellular protein synthesis,[3c] we carried out metabolic labeling experiments with azidohomoalanine (AHA, pioneered by Dieterich et al.[12]) to measure the level of newly synthesized proteins in cells treated with Orlistat, **THL-R** or cycloheximide (a well-known protein synthesis inhibitor). As shown in part C of Figure 2, greatly reduced levels of protein synthesis were observed in cells treated with each of the three compounds. The inhibition of protein synthesis was dose-dependent, which is consistent with previous findings carried out using radiolabeled ^{35}S-methionine.[3c] Lastly, inhibition of FAS by Orlistat was previously shown to induce tumor cell apoptosis by activating caspase-8.[3e] We therefore tested the compounds against the invasive human breast cancer MCF-7 cells (part D of Figure 2); similar degrees of caspase-8 activation (as evidenced by the appearance of the p41/43 bands corresponding to cleaved caspase-8) were observed against all four compounds (at either 5 or 25 μM). Collectively, these data show the introduction of a terminal alkyne handle at various designated locations in Orlistat scaffold did not noticeably affect its biological activities, and **1**, **2**, and **3** were indeed suitable chemical probes for cell-based proteome profiling and identification of previously unknown cellular targets of Orlistat.

In Situ and in Vitro Proteome Profiling. We next compared the in situ proteome reactivity profiles of the three probes to identify proteins which were covalently labeled by the probes in live HepG2 cells.[13] Probes (1−20 μM) were directly added to the cell culture medium, either alone or in the presence of

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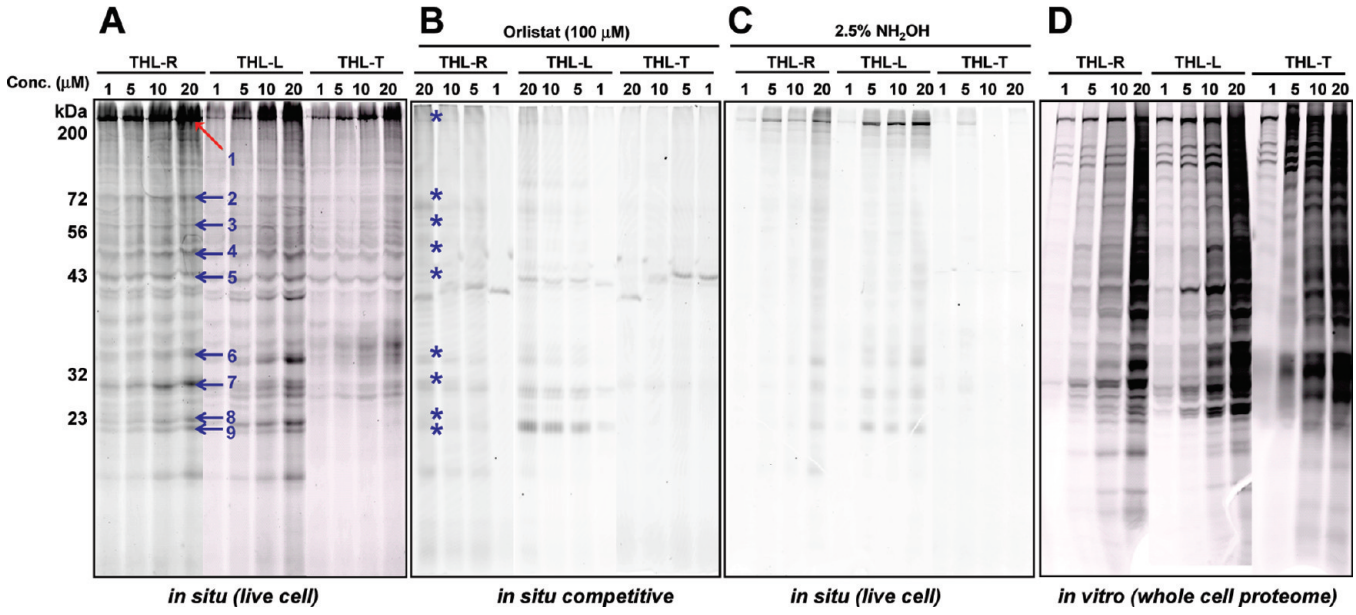

Figure 3. Proteome profiling of HepG2 cells using THL analogues **1**, **2**, and **3** (over a concentration range of 1−20 μM). Both in situ (live cell) and in vitro (whole-cell lysates) labelings were carried out. (A and B) In situ proteome labeling of HepG2 cells, with or without Orlistat (100 μM), followed by click chemistry with rhodamine-azide (**33**), SDS-PAGE analysis, and in-gel fluorescence scanning. The labeled FAS (marked with red arrow) and multiple Orlistat-sensitive targets (marked with blue arrows) were isolated and identified subsequently using biotin-azide (**34**), and summarized in Table 1. In (B), asterisks show the expected locations of FAS and other Orlistat-sensitive bands. (C) showed the fluorescent profile of the gel from part A of Figure 3 upon treatment with hydroxylamine. The fluorescence labeling of most proteins (but not FAS) were reversed by hydroxylamine, indicating a thioester/ester linkage. (D) In vitro labeling of HepG2 whole-cell proteome lysates (for in vitro competitive ABPP profile, part D of Figure S2 in the Supporting Information).

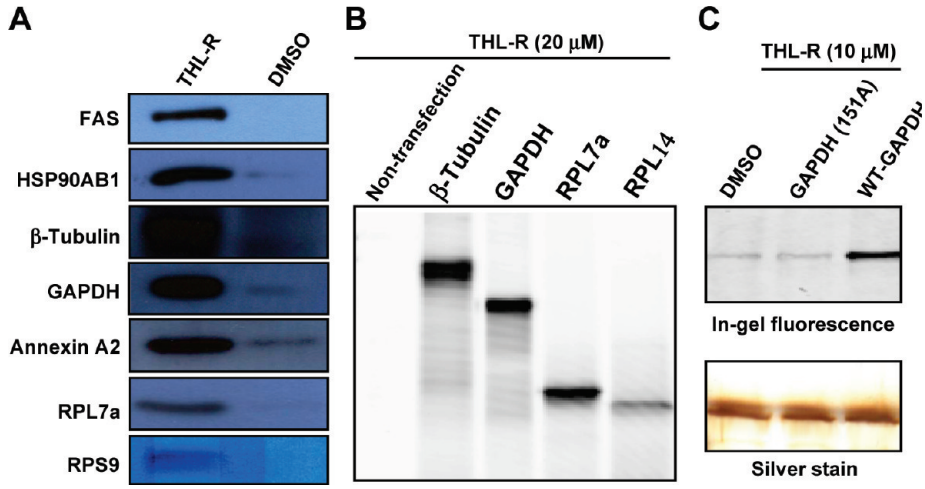

Figure 4. Target validation of the identified "hits". (A) Western blotting analysis of pulled-down fractions of HepG2 live cells treated with **THL-R** (or DMSO as negative controls; right lanes) with their respective antibodies. Biotin-azide **34** was used in the click chemistry with avidin-agarose beads for pull-down experiments. (B) In situ labeling of recombinantly expressed β-tubulin, GAPDH, RPL7a, and RPL14 by **THL-R** (fluorescent gel shown in grayscale). (C) Comparative labeling analysis of wild-type GAPDH and $Cys^{151}Ala$ mutant (upper panel, fluorescent gel shown in grayscale); (lower panel, silver-stained gel) comparable amounts of protein loading were demonstrated in all three lanes.

100 μM of the competing Orlistat. After two hours, the cells were washed (to remove excessive probes), homogenized, incubated with rhodamine-azide (**33**) under click-chemistry conditions, separated by SDS-PAGE gel, and analyzed by in-gel fluorescence scanning (Figure 3). In addition to the expected FAS band (265 kDa, marked with red arrow; part A of Figure 3), confirmed by treatment with an anti-FAS antibody (part A of Figure 4), we also observed a number of Orlistat-sensitive targets, that is, those labeled bands that were competed away by treatments with excessive Orlistat (marked with blue arrows and asterisks in parts A and B of Figure 3). Most of these labeled bands were clearly visible even at a low probe concentration (e.g., 1 μM of **THL-R**; lane 1 in part A of Figure 3). Both probe **1** and **2** gave similar proteome labeling profiles, whereas probe **3** (**THL-T**) consistently produced weaker labeled bands (possibly caused by inefficient click-chemistry conjugation due to inaccessibility of the alkyne handle located at the *N*-formyl-L-leucine end, which, based on X-ray structure of FAS/Orlistat complex, was buried deep into the protein[3b]). To assess which nucleophilic residue of the labeled proteins might have been covalently modified by our probes, SDS-PAGE gels from the in situ labeling were subjected to in-gel treatment with hydroxylamine (NH_2OH), which preferentially cleaves thioesters, and esters to a lesser extent, under neutral *p*H conditions.[15] As shown in part C of Figure 3, the labeled FAS and most other Orlistat-sensitive bands showed a much reduced fluorescence

Table 1. Proteins Identified by Pull Down and Mass Spectrometry

#	protein name	calcd/obsd mass Da/kDa	localization	protein function
2	Hsp90AB1	75 088/72	cytoplasm, nucleus	molecule chaperone with ATPase acitivity, stress response
3	unnamed	56 156/56	cytoplasm, nucleus	protein biosynthesis, translational elongation
4	β-tubulin	49 671/52	nucleus	GTPase acitivity and cell division cell division
5	ANXA2	38 808/39	cytoplasm, nucleus	calcium binding, cell division
6	GAPDH	36 201/36	cytoplasm, nucleus	glycolysis, energy production
7	RPL7a	30 148/30	cytoplasm, ribosome	biogenesis, protein synthesis
8	RPL14	23 902/23	cytoplasm, ribosome	biogenesis, protein synthesis
9	RPS9	22 635/22	cytoplasm, ribosome	biogenesis, protein synthesis

signal upon treatment with NH_2OH, suggesting likely involvement of a cysteine/serine residue in the labeling between these proteins and THL (vide infra). It is interesting to note that the labeled FAS band was to some degree resistant to NH_2OH treatment, clearly indicating the formation of a more chemically stable ester linkage between Ser^{2308} in FAS[3b] and **THL-R**. In a related experiment, we also performed competitive ABPP with Cerulenin, a known FAS inhibitor which irreversibly inactivates the β-ketoacyl-ACP synthase domain but not the thioesterase domain of FAS;[14] as expected (Figure S2 of the Supporting Information), Cerulenin did not abolish the labeling of the THL probes toward FAS as well as most other Orlistat-sensitive proteins. This indicates that the labeling of our probes is both target- and domain-specific, and they may in the future be used to distinguish different domains of FAS. As shown in part D of Figure 3, when compared to in situ (live cell) labeling, similar proteome labeling profiles, albeit with significantly higher background labeling, were obtained when whole-cell lysates were used instead. This shows the importance of the in situ labeling (made possible by the cell-permeable property of the THL probes) as a prerequisite for accurate and specific target identification.

Target Identification and Validation. Subsequently, the labeled protein extract was enriched (following click-chemistry conjugation with biotin-azide **34**) by avidin-agarose beads, separated by SDS-PAGE gel, confirmed by strepavidin blotting, subjected to in-gel trypsin digestion, and identified by MS/MS analysis. In addition to FAS, eight new proteins were identified (numbered **2**−**9**, in part A of Figure 3 and Table 1), of which one is an unnamed protein. Two of the proteins, GAPDH and β-Tubulin, are house-keeping genes constitutively expressed in most cell lines but are known to be expressed more highly in cancer cells.[16] Both GAPDH (a dehydrogenase) and β-tubulin (a hydrolase) possess nucleophilic active-site cysteine residues. It is therefore not surprising they were targets of Orlistat and their labeling was reversed by NH_2OH treatment. It should be noted that tubulins are well-known targets of anticancer drugs (e.g., taxol[17]). Three other proteins identified, RPL7a, RPL14, and RPS9, are ribosomal proteins. They are known to be implicated in protein synthesis, control of cellular transformation, tumor growth, aggressiveness and metastasis. Overexpression of these proteins had previously been reported in colon, brain liver, breast and prostate carcinoma.[18a−d] In retrospect, our earlier findings that protein synthesis was greatly inhibited in HepG2 cells treated with Orlistat, as shown in part C of Figure 2, may also imply that these ribosomal proteins are probable cellular targets of the drug. The remaining two proteins, Annexin A2 and Hsp90AB1, are involved in cell proliferation/division and protein degradation, respectively.

In an effort to further validate the MS results, we carried out pull-down and Western blotting experiments with the respective antibodies (part A of Figure 4);[19] results confirmed all proteins tested (including FAS) were indeed positively labeled by **THL-R**, and thus likely are true cellular targets of Orlistat. Four of the proteins (β-tubulin, GAPDH, RPL7a, and RPL14) were taken for additional validation experiments. First, the c-Myc fusions of these proteins were transiently expressed in HEK-293T cells (Figure S3 in the Supporting Information), labeled (by **THL-R** in situ), immune-purified (with c-Myc agarose beads), subjected to click chemistry (with rhodamine-azide **33**), and analyzed by SDS-PAGE (part B of Figure 4); results unambiguously confirmed that all four proteins were fluorescently labeled by the probe. To confirm whether labeling of the probe is active site-directed, GAPDH, whose active site residue Cys^{151} had previously been characterized,[16b] was taken as an example for further site-directed mutagenesis experiments. GAPDH is a multifunction protein and well known for its primary role as a glycolytic enzyme. Recently, increasing evidence has suggested that this enzyme is also involved in a variety of activities which are unrelated to energy production, including membrane fusion, microtubule bundling, DNA repair, and apoptosis.[16b] An active site (Cys^{151} to Ala) mutant of GAPDH was therefore generated, transiently expressed and purified from HEK-293T cells, labeled with **THL-R**, reacted with rhodamine-azide **33**, then finally analyzed by SDS-PAGE followed by in-gel fluorescence scanning (part C of Figure 4); as expected, probe **THL-R** only labeled the wild-type GAPDH and not the Cys^{151}Ala mutant, thus confirming Cys^{151} as the residue in GAPDH being covalently labeled by **THL-R**. This also confirms the active site-directed nature of the probe. It should be noted that, in a series of recent reports,[20] Sieber et al. had developed activity-based probes based on small molecule libraries containing a reactive β-lactone moiety. The authors concluded that β-lactones are promising privileged structures and could be used to identify a variety of mechanistically distinct enzymes. Our Orlistat-based probes, though structurally much

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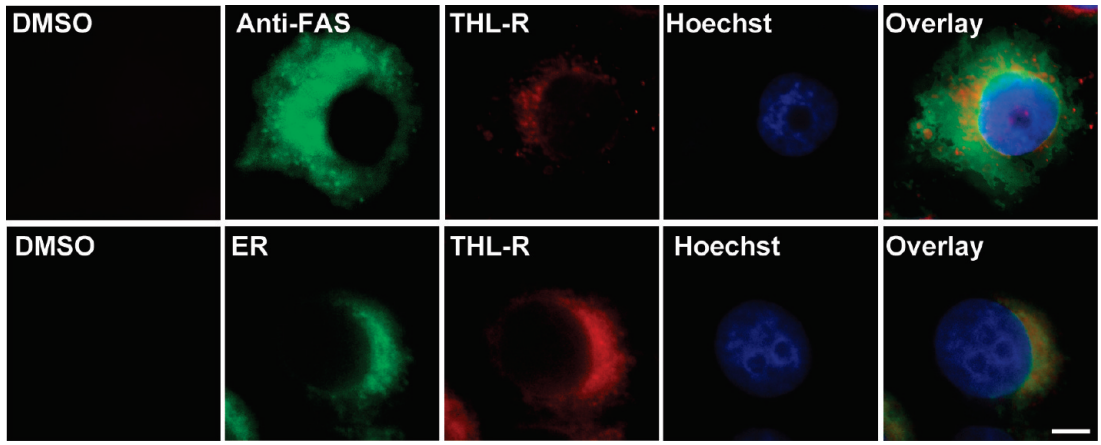

Figure 5. Cellular imaging of HepG2 cells with probe **1** (for **2** and **3**, see Figure S5 in the Supporting Information). Live HepG2 cells were treated with **1** (20 μM) for 2 h, fixed, permeabilized, reacted with rhodamine-azide (10 μM, false-colored in red) under click-chemistry conditions, stained with Hoechst (blue), anti-FASN primary antibodies, followed by FITC-conjugated antimouse IgG antibody (green), or ER-Tracker Green (glibenclamide BODIPY FL), mounted, and imaged. Samples were imaged with an Olympus IX71 inverted microscope, equipped with a 60X oil objective (NA 1.4, WD 0.13 mm) and CoolSNAP HQ CCD camera (Roper Scientific, Tucson, AZ, USA). Images were processed with *MetaMorph* software (version 7.1.2.; Molecular Devices, PA, USA). Scale bar = 8 μm. All images were acquired similarly. Cells treated with DMSO (negative controls) are also shown.

more complex, appear to behave similarly and are capable of targeting a number of other cellular proteins in addition to FAS. Even though the exact physiological roles of these proteins in connection with Orlistat and its pharmacological effects have not been established from this study, we believe these putative cellular targets of Orlistat should be carefully evaluated when one considers using Orlistat in cancer therapy.

Cellular Imaging Using THL-R. To demonstrate the utility of our cell-permeable probes for potential cellular imaging of Orlistat targets, we performed fluorescence microscopy to visualize probe-treated cells (Figure 5 and Figure S4 in the Supporting Information). Live HepG2 cells were first treated with **THL-R** (**1**), fixed with PFA, permeabilized with Triton X-100, conjugated to rhodamine-azide (**33**) by click chemistry, and imaged (colored in red). Immunofluorescence was also carried out on the same cells to visualize the localization of endogenous FAS (colored in green in top panels). Minimal fluorescence was observed in samples treated with only DMSO, whereas, in **THL-R** treated cells, fluorescence was mostly distributed in endoplasmic reticulum (ER, bottom panels in Figure 5). Note that endogenous FAS was mostly cytosolic (which includes ER, top panels). Thus, our imaging results are consistent with previous findings that inhibition of FAS with Orlistat induces ER stress specifically in tumor cells.[3c] We take note, however, that, at its current state, **THL-R** might not be the most suitable chemical probe for bioimaging of FAS, as it also labels a number of other cellular proteins (as evidenced from our studies herein). Work is in progress to develop other Orlistat analogues, which may confer much greater specificities, and results will be reported in due course.

Conclusions

We have developed a novel chemical proteomic approach that enabled, for the first time, identification and putative validation of several previously unknown cellular targets of Orlistat. The potential of these cell-permeable probes to be used as future imaging probes has also been explored. Whereas further studies are needed to better understand the exact relevance of Orlistat and its pharmacological effects in relation to these newly identified cellular targets, our findings point to a likely scenario that these proteins might be potential off-targets of Orlistat. It is also possible that the antitumor activities of Orlistat might have originated from the drug's ability to inhibit both FAS (as previously reported[3]) and some of these newly identified targets. In either case, our findings have important implications in consideration of Orlistat as a potential anticancer drug. Finally, our strategy should be broadly useful for off-target identification against quite a number of existing drugs and/or candidates, which are also covalent modifiers of their biological targets.[21]

Experimental Section

Chemicals and Antibodies. Orlistat (98%), Tris(2-carboxyethyl) phosphine (TCEP), and the click chemistry ligand, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine ("ligand"), were purchased from Sigma-Aldrich. Antibody against FAS (Cat No. 610963) was from BD Transduction Laboratories (San Diego, CA). Antibodies against eIF2α (#9722), phospho-eIF2α (#9721), and cleaved caspase-8 (#9746) were from Cell Signaling Technologies (Beverly, MA). Antibodies against HSP90b (sc-1057), Annexin A2 (sc-1492), RPL14 (KQ-16, sc-100826), β-tubulin (sc-58882), and c-Myc (9E10) were from Santa Cruz Biotechnology Inc. Antibodies against GAPDH (ab9484), RPL7a (ab70753), and RPS9 (ab74711) were from Abcam. (*S*)-(+)-*N*-formyl leucine **9**, thioketene acetal **16**, hydroxyl-β-lactone **30**, amino azide linker **31**, and rhodamine B acid **32** were prepared as described.[22−26] The AHA metabolic labeling kit was from Invitrogen.

Cell Culture. Cell lines were obtained from the National Cancer Institute Developmental Therapeutics Program (NCI60 cell line panel). HepG2 and HEK-293T were grown in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum

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(FBS, Gibco Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Scientific, Rockford, IL) and maintained in a humidified 37 °C incubator with 5% CO_2. MCF-7 and PC-3 were maintained in RPMI 1640 medium supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. To generate protein lysates, cells were washed twice with cold phosphate-buffered saline (PBS), and harvested with a cell scraper, and collected by centrifugation. Cell pellets were resuspended in PBS and lysed by sonication. Protein concentration was determined by the Bradford assay. Cell lysates were diluted with PBS to achieve final concentration of ∼1 mg/mL for labeling reactions.

Cell Proliferation Assay. Cell viability was determined using the XTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines. Briefly, cells were grown to 20−30% confluence (since they will reach ∼90% confluence within 48 to 72 h in the absence of drugs) in 96-well plates under the conditions described above. The medium was aspirated, and then washed with PBS, and then treated, in duplicate, with 0.1 mL of the medium containing different concentrations of THL analogues (1−50 μM) or Orlistat (1−50 μM, as a positive control). Probes were applied from DMSO stocks whereby DMSO never exceeded 1% in the final solution. The same volume of DMSO was used as a negative control. Fresh medium, along with THL analogues or Orlistat, were added every 24 h. After a total treatment time of 72 h, proliferation was assayed using the XTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines (read at 450 nm). Data represent the average ±s.d. for two trials.

Western Blotting. To monitor the effects of THL analogues on inducing phosphorylation of eIF2α, PC-3 cells were treated with indicated concentrations of Orlistat and THL analogues for 16 h. Samples from treated cells were then separated on 12% SDS-PAGE gel and further transferred to PVDF membranes. Membranes were blocked with 5% BSA in TBS. After blocking, membranes were incubated with anti-eIF2α (#9722 from Cell Signaling, 1/5000) or antiphospho-eIF2α (#9721 from Cell Signaling, 1/2000). After incubation, membranes were washed with TBST for three times and then incubated with an appropriate secondary antibody [anti-mouse conjugated HRP (1/5000) or antirabbit conjugated HRP (1/5000)]. After secondary incubation, blots were washed again with TBST before the development with SuperSignal West Pico kit (Pierce).

To monitor the effects of THL analogues on inducing activation of the caspase-8 pathway, MCF-7 cells were treated with indicated concentrations of Orlistat and THL analogues for 36 h. Samples from treated cells were then separated on a 12% SDS-PAGE gel and further transferred to PVDF membranes. Membranes were blocked with 5% BSA in TBS. After blocking, membranes were incubated with anticaspase 8 (#9746 from Cell Signaling, 1/2000). After incubation, membranes were washed with TBST for three times, and then incubated with antimouse conjugated HRP (1/5000). After secondary incubation, blots were washed again with TBST before the development with SuperSignal West Pico kit (Pierce).

Measurement of Protein Synthesis. Live HepG2 cells were treated with the indicated concentrations of Orlistat/**THL-R** or CHX (cycloheximide, an inhibitor of protein biosynthesis) for 12 h, washed twice with PBS, and then pulsed with AHA (L-azidohomoalanine, 20 μM) for 4 h. Cells were collected, washed, and cell lysates were prepared and subjected to click chemistry with rhodamine-alkyne (provided with the AHA kit), SDS-PAGE analysis, and in-gel fluorescence scanning.

In Vitro and In Situ Proteome Labeling and Analysis. For in vitro proteome labeling, probes were added to cell lysates (50 μg) in 50 μL of PBS at a final concentration of 1−20 μM in the presence or absence of excess Orlistat or Cerulenin competitor (a final concentration of 100 μM). Unless indicated otherwise, samples were incubated for 2 h with varying concentrations of probe at room temperature. After incubation, 10 μL of the freshly premixed click chemistry reaction cocktail in PBS [rhodamine-azide **33** (100 μM, 10 mM stock solution in DMSO), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in deionized water), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA) (100 μM, 10 mM stock solution in DMSO) and $CuSO_4$ (1 mM, 50 mM freshly prepared stock solution in deionized water)] was added and vortexed, then incubated for 2 h at room temperature with gentle mixing. The reactions were terminated by the addition of prechilled acetone (0.5 mL), placed at −20 °C for 30 min and centrifuged at 13000 rpm for 10 min at 4 °C to precipitate proteins. The supernatant was discarded and the pellet washed two times with 200 μL of prechilled methanol. The protein pellets were allowed to air-dry for 10 min, resuspended in 25 μL 1 × standard reducing SDS-loading buffer and heated for 10 min at 95 °C; ∼20 μg of protein was loaded per gel lane for separation by SDS-PAGE (12% or 8−16% gradient precast gel), then visualized by in-gel fluorescence scanning using a Typhoon 9410 Variable Mode Imager scanner.

For in situ labeling, cells were grown to 80−90% confluence in 24-well plates under the conditions described above. The medium was removed, and then cells were washed twice with cold PBS, and treated with 0.5 mL of DMEM-containing probe (1−20 μM), with or without Orlistat or Cerulenin (100 μM). Probes were applied from DMSO stocks whereby DMSO never exceeded 1% in the final solution. The same volume of DMSO was used as a negative control. After 2 h of incubation at 37 °C/5% CO_2, the growth medium was aspirated, and cells were washed twice with PBS to remove the excessive probe, trypsined, and pelleted by centrifugation. The cell pellet was resuspended in PBS (50 μL), homogenized by sonication, and diluted to ∼1 mg/mL with PBS. Probe targets were detected by click chemistry with rhodamine-azide **33**, SDS-PAGE analysis, and in-gel fluorescence scanning.

Hydroxylamine Treatment of Gels. After the proteins were separated by SDS-PAGE gel, the gel was soaked in 40% MeOH, 10% acetic acid, shaking overnight at room temperature, washed with deionized water (2 × 5 min), and scanned for the prehydroxylamine treatment fluorescence. The gel was then soaked in PBS, shaking 1 h at room temperature, followed by boiling in neutralized hydroxylamine (Alfa Aesar) (2.5% final concentration) for 5 min, washing with deionized water (2 × 5 min), and soaking in 40% MeOH, 10% acetic acid, shaking overnight at room temperature. The gel was washed with deionized water (2 × 5 min) and scanned for the posthydroxylamine fluorescence.

Pull-Down and Mass Spectrometry Identification. To identify the in situ targets of **THL-R** in HepG2 cell line, pull-down followed by immunoblot or MS/MS identification experiments were carried out as described below. In situ labeling sample of HepG2 by **THL-R** were prepared as described previously. After in situ labeling, cells were washed with PBS, detached from T75 culturing flask and pelleted by centrifuge for 1000 rpm for 15 min. Five milligrams of the lysates were reacted by click chemistry with biotin-azide **34** under the conditions described above, acetone precipitated, and resolubilized in 0.1% SDS in PBS with brief sonication. This resuspended sample was then incubated with avidin-agarose beads (100 μL/mg protein) at room temperature for 30 min. After centrifugation, supernatant were removed and the beads were washed with 1% SDS in PBS for 4 times. After washing, the beads were boiled in elution buffer (200 mM Tris pH 6.8, 400 mM DTT, 8% SDS).

For immunoblotting analysis, this pulled-down sample was then separated on 8−16% gradient precast gel (Biorad), transferred to PVDF membrane and probed with purified mouse anti-FAS (Cat No. 610963, BD Transduction Laboratories).

For MS/MS identification, proteins from pulled-down fractions were separated on 12% SDS-PAGE gel, followed by silver staining. Trypsin digestion was performed with In-Gel Trypsin Digestion Kit from Pierce for respective visible protein bands. After digestion, digested peptides were then extracted from the gel with 50% acetonitrile and 1% formic acid. Tryptic peptide extracts were evaporated by speedvac and reconstituted with 10 μL of 0.1% TFA, a volume of 2 μL of the peptide extracts were manually spotted

onto a Prespotted AnchorChip MALDI target plate for MALDI-TOF Mass step with 10 μL of 10 mM ammonium phosphate in 0.1% TFA, and allowed to dry at ambient temperature. MALDI TOF mass spectra were recorded using Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics) with the *Compass 1.2* software package including *flexControl 3.0* and *flexAnalysis 3.0*, calibrated with PAC peptide calibration standards. MS/MS analysis for the major peaks in PMF spectra were carried out by autoLIFT on the MALDI-TOF/TOF instrument. MS and MS/MS Peak lists with intensity value were submitted to Matrix Science Mascot server (http://www.matrixscience.com/) through *BioTools 3.0* (Bruker Daltonics) using database NCBInr 090202 version, variable modifications of carbamidometyl on cysteine (C) and oxidation on methione (M), allowing maxium of trypsin missed cleavage, peptide mass tolerance at 200 ppm; MS/MS mass tolerance of 0.7 Da.

Target Validation by Western Blotting. Pull-down sample from labeled lysates was separated on 12% SDS-PAGE gel together with pull-down sample from DMSO-treated, unlabeled lysates (negative controls). After SDS-PAGE gel separation, proteins were then transferred to a PVDF membrane and subsequently blocked with 2.5% (w/v) BSA/PBST. Membranes were incubated for 1 h at room temperature with the respective antibodies (i.e., anti-RPL7a, anti-Annexin A2, anti-HSP90b, anti-RPS9, anti-GAPDH, and anti-β-tubulin). After three times of washes with PBST, blots were further incubated with the appropriate secondary antibody for 1 h at room temperature. After incubation, the blot was washed again with PBST for 3 times and the SuperSignal West Pico kit (Pierce) was used to develop the blot.

Target Validation by Recombinant Protein Expression in HEK-293T Cells. Mammalian expression vectors overexpressing each of the four targets were purchased from Origene (USA). Vectors were transfected with Lipofectamine reagent (Qiagen) at 80% confluence. After 24 h of transfection, either nontransfected and transfected cell were incubated with **THL-R**. After **THL-R** incubation, small fractions of the samples were analyzed by Western blotting with 1/2000 anti-c-Myc antibody (Santa Cruz). The rest of the cells were lysed and immunopurified with c-Myc agarose beads (Santa Cruz). Eluted fractions were clicked with rhodamine-azide **33** as previously described. After click chemistry, samples were concentrated, separated on a SDS-PAGE gel and scanned by in-gel fluorescence.

Identification of GAPDH Labeling Site. The mammalian expression vector overexpressing GAPDH was used as template for generating the GAPDH active-site mutant, using the Site-Direceted Mutagenesis System provided by Invitrogen (USA). Cys^{151} was mutated to Ala. Primers used for site-directed mutagenesis were designed following the vendor's protocols. They are shown below:

5′ -ATCAGCAATGCCTCCGCCACCACCAACTGC-3′

5′-GCAGTTGGTGGTGGCGGAGGCATTGCTGAT-3′

Vectors overexpressing both wild-type and mutant GAPDH were transfected to HEK-293T cell lines. Recombinant protein was purified, labeled and clicked as previously described. Further, samples were separated on SDS-PAGE gel and fluorescently scanned with Typhoon Scanner. After scanning, gel was fixed and silver stained to visualize the total protein bands.

Cellular Imaging Using THL-R. HepG2 cells were seeded onto 24-well plates containing sterile glass coverslips and grown until 70−80% confluence. Further, cells were treated with 0.5 mL of DMEM with 20 μM **THL-R** or DMSO. After 24 h, the growth medium was removed, and cells were washed twice with PBS. After fixation in 3.7% paraformaldehyde in PBS for 15 min at room temperature, cells were washed twice with cold PBS again. Cells were permeabilized with 0.1% Triton X-100 in PBS 10 min at room temperature, and blocked with 2% BSA in PBS for 30 min at room temperature, and then washed twice with PBS. Cells were then treated with a freshly premixed click chemistry reaction solution in a 250 μL volume at final concentrations of the following reagents: 1 mM of $CuSO_4$, 1 mM of TCEP, 100 μM of TBTA, and 10 μM of rhodamine-azide **33** in PBS for 2 h at room temperature with vigorous shaking. Cells were washed with PBS three times, and then with 20 mM of HEPES, pH 7.5, 500 mM of NaCl, 2% triton for overnight at room temperature, then washed again by PBS for three times. For colocalizing in situ targets of THL analogues with FAS, cells were further incubated with anti-FASN primary antibodies (1:200) for 1 h at room temperature (or overnight at 4 °C), and washed twice with PBS. The cells were incubated with FITC-conjugated antimouse IgG (1:500) for 1 h, washed again. Cells were stained with 1 μg/mL Hoechst for 10 min at room temperature, washed again before mounting. For colocalizing in situ targets of **THL-R** with ER, cells were further incubated with ER-Tracker Green (glibenclamide BODIPY FL) for 1 h at room temperature (or overnight at 4 °C), and washed twice with PBS. Cells were stained with 1 μg/mL Hoechst for 10 min at room temperature, washed again before mounting.

Chemical Synthesis. All chemicals were purchased from commercial vendors and used without further purification, unless otherwise noted. Tetrahydrofuran (THF) was distilled over sodium benzophenone and used immediately. Dichloromethane (CH_2Cl_2) was distilled over CaH_2. All nonaqueous reactions were carried out under nitrogen atmosphere in oven-dried glassware. Reaction progress was monitored by TLC on precoated silica plates (Merck 60 F254, 250 μm thickness) and spots were visualized by ceric ammonium molybdate, basic $KMnO_4$, UV light, or iodine. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker model Avance 300 MHz or DPX-300 MHz or DPX-500 MHz NMR spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CHCl_3$ = 7.26 ppm). ^{1}H NMR coupling constants (J) are reported in Hertz (Hz) and multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br s (broad singlet), dd (doublet of doublet), dt (doublet of triplet), dq (doublet of quartet), tq (triplet of quartet). Mass spectra were obtained on Shimadzu IT-TOF-MS or Shimadzu ESI-MS system.

(*E*)-2-(1-(*tert*-butyldimethylsilyloxy)-8-(trimethylsilyl)-oct-1-en-7-ynylthio)-pyridine (12). Thiopyridyl ester **11** (prepared in three steps from 6-bromohexanoic acid, Scheme S3 in the Supporting Information) (5.42 g, 17.9 mmol) was dried azeotropically with xylenes. After purging with N_2, anhydrous DMF (1.57 g, 21.5 mmol) and anhydrous Et_3N (3.02 g, 21.5 mmol) in dry CH_2Cl_2 (120 mL) was added and the mixture was cooled to −78 °C. After 10 min, LiHMDS (1.0 M, 41.3 mL, 41.3 mmol) was added dropwise. The reaction mixture was stirred for 30 min and TBSCl (5.4 g, 35.8 mmol) was added dropwise to the reaction mixture at −78 °C. The reaction mixture was stirred for 2 h at this temperature and then quenched with pH 7 phosphate buffer (20 mL × 2). The organic layer was separated, dried over Na_2SO_4 and evaporated. The resulting yellowish oil was purified by flash chromatography (10% EtOAc/hexane) to afford ketene acetal **12** (5.60 g, 74%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.40−8.50 (m, 1H), 7.54 (dt, J = 1.9, 7.6, Hz, 1H), 7.31−7.33 (d, J = 8.2 Hz, 1H), 6.98−7.01 (m, 1H), 5.39 (t, J = 7.0 Hz, 1H), 2.34 (dd, J = 6.9, 13.8 Hz, 2H), 2.20 (dd, J = 5.7, 12.6 Hz, 2H), 1.50−1.56 (m, 4H), 0.88 (s, 9H), 0.14 (s, 9H), 0.08(s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.1, 160.4, 149.4, 139.7, 136.5, 123.4, 121.5, 119.6, 107.3, 84.5, 35.9, 28.4, 28.3, 26.3, 25.7, 25.6, 25.0, 19.7, 18.1, 17.6, 0.2. −4.8; ESI-MS: $[M+1]^+$ calcd: 419.213, found: 420.244.

(3*S*,4*S*)-4-((*R*)-2-hydroxytridecyl)-3-(6-(trimethylsilyl)hex-5-ynyl)oxetan-2-one (13). TMAL Reaction. $ZnCl_2$ (796 mg, 5.84 mmol) was fused under vacuum and then allowed to cool to room temperature under a flow of nitrogen. Fifteen milliliters of dry CH_2Cl_2 was then added via syringe followed by a solution of **8a** (547 mg, 2.92 mmol) in 5 mL of CH_2Cl_2 and ketene acetal **12** (1.72 g, 4.08 mmol). The reaction mixture was stirred for 72 h at room temperature. Ten milliliters of pH 7 phosphate buffer were then added and the mixture was stirred vigorously for 45 min, filtered through a pad of Celite, and washed with CH_2Cl_2. The organic filtrate was dried over Na_2SO_4, filtered, and concentrated. The

residue was purified by flash chromatography on SiO_2 (10% EtOAc/hexane) to provide the mixture of silyloxy-β-lactone diastereomers as a white solid. ^{1}H NMR (500 MHz) analysis of the mixture of diastereomers indicated a diastereomeric ratio of 8.5:1. Without further purification, the mixture was used directly in the next step.

***O*-Desilylation.** To a stirred solution of the mixture of silyloxy-β-lactones in 48 mL of CH_3CN cooled to 0 °C, 4.8 mL of HF (48%) was added dropwise. The mixture was stirred at 0 °C for 2 h, then allowed to warm to room temperature and stirred for an additional 8 h. The reaction mixture was diluted with 150 mL of Et_2O, quenched carefully with cold saturated $NaHCO_3$ solution, and washed with brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (10% EtOAc/hexane) to provide the hydroxy-β-lactone **13** (360 mg, 46%) and a mixture of the two diastereomers (90 mg, 12%) as white solids (58% overall two steps). Spectroscopic data are reported for the major diastereomeric-β-lactone **13**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.50 (app quint., J = 4.4 Hz, 1H), 3.78−3.83 (m, 1H), 3.27 (dt, J = 3.8, 7.6 Hz,1H), 2.24 (t, J = 7.0 Hz, 2H), 1.77−1.96 (m, 4H), 1.49−1.57 (m, 8H), 1.26 (br s, 18H), 0.88 (t, J = 7.0 Hz, 3H), 0.15 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.3, 106.7, 85.0, 75.6, 68.5, 56.5, 41.8, 38.2, 31.9, 29.6, 29.5, 29.3, 28.1, 27.3, 26.9, 25.9, 25.4, 22.7, 19.6, 14.1.; ESI-MS: $[M+1]^+$ calcd: 422.32, found: 423.25.

***C*-Desilylation.** To a solution of (trimethylsily1)-acetylene **13** (134 mg, 0.5 mmol) in 20 mL of a mixture of acetone/H_2O/2,6-lutidine (1:1:0.l) was added solid $AgNO_3$ (850 mg, 5 mmol). The white suspension was stirred vigorously for 4 h, and then 5 mL of a 1.0 M aqueous KH_2PO_4 solution was added. The resulting yellow slurry was stirred for an additional 30 min. Filtration of the reaction mixture through Celite removed most of the yellow precipitate. The filtrate was extracted with Et_2O, and the combined organic extracts were washed once with brine and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure afforded a pale-yellow oil. Purification by chromatography on silica gel (15:l hexane/EtOAc) gave 81 mg of **14** (83%) as a sticky, colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.50 (app quint., J = 4.4 Hz, 1H), 3.78−3.83 (m, 1H), 3.28 (dt, J = 3.8, 8.2 Hz, 1H), 2.19−2.23 (m, 2H), 1.91−1.97 (m, 2H), 1.77−1.87 (m, 2H), 1.47−1.62 (m, 8H), 1.26 (br s, 18H) 0.88 (t, J = 6.3 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.4, 84.0, 75.6, 68.7, 68.5, 56.4, 41.8, 38.2, 31.9, 29.6(2), 29.5(2), 29.3, 28.0, 27.2, 25.8, 25.4, 22.7, 18.1, 14.1.; IT-TOF-MS: m/z $[M+1]^+$ calcd: 350.282, found: 351.288.

Mitsunobu Reaction. β-lactone **14** (70 mg, 0.2 mmol), PPh_3 (79 mg, 0.3 mmol), and *N*-formyl-L-leucine **9** (128 mg, 0.8 mmol) were placed in a RB-flask and azeotroped under vacuum with 0.5 mL of xylene for 30 min. Addition of 5.0 mL of dry THF was followed by cooling to 0 °C, DIAD (60 μL, 0.3 mmol) was then added via syringe. The mixture was stirred at 0 °C for 10 min, allowed to warm to room temperature. The reaction was monitored by TLC. After the reaction was complete, the mixture was then concentrated in vacuo and polar impurities were separated by flash chromatography (60:40, hexane/EtOAc). Further purification of the residue by flash chromatography (20% EtOAc/hexane) yielded β-lactone **1**. (74 mg, 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (s, 1H), 5.92 (d, J = 8.2 Hz, 1H), 5.01−5.06 (m, 1H), 4.68 (dt, J = 4.4, 8.8 Hz, 1H), 4.30 (q, J = 4.4 Hz, 1H), 3.23 (dt, J = 3.8, 7.6 Hz, 1H), 2.14−2.23 (m, 3H), 1.96 (t, J = 2.5 Hz, 1 H), 1.95−2.04 (m, 1H), 1.55−1.82 (m, 11H), 1.25−1.27 (m, 18 H), 0.96−0.98 (m, 6H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, CDCl3) δ 171.9, 170.5, 160.6, 83.9, 74.7, 72.7, 68.8, 56.9, 49.7, 41.5, 38.7, 34.1, 31.9, 29.6, 29.5, 29.4, 29.3(2), 28.0, 27.1, 25.7, 25.1, 24.9, 22.9, 22.7, 21.9, 21.7, 18.1, 14.1.; ESI-MS: m/z $[M+1]^+$ calcd: 491.4, found: 492.3.

(*R*)-Pentadec-1-en-14-yn-4-ol (21). (*S*)-BINOL (415 mg, 1.45 mmol) was added to a suspension of $InCl_3$ (292 mg, 1.32 mmol, azeotropically dried over THF (2 × 3 mL)) in CH_2Cl_2 (15 mL) and stirred for 2 h at room temperature. Allyltributyltin (4.37 g, 13.2 mmol) was added and stirred for 5 min, then the reaction mixture was cooled to −78 °C and stirred for 10 min. Aldehyde **20** (1.19 g, 6.6 mmol) in DCM (10 mL) was added dropwise and stirred at −78 °C for 4 h then warmed to room temperature overnight. Saturated $NaHCO_3$ solution (25 mL) was added to the reaction mixture and stirred for 30 min. The aqueous phase was extracted with DCM. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography (hexane/EtOAc, 95:5) to give homoallylic alcohol **21** (1.06 g, 71%, 62.4% ee) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.79−5.87 (m, 1H), 5.15 (br d, J = 3.2 Hz, 1H), 5.12 (s, 1H), 3.62−3.67 (m, 1H), 2.28−2.33 (m, 1H), 2.11−2.19 (m, 3H), 1.93 (t, J = 2.5 Hz, 1H), 1.29−1.57 (m, 16H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 134.9, 118.1, 84.8, 70.7, 68.0, 42.0, 36.8, 29.6, 29.5, 29.4, 29.1, 28.7, 28.5, 25.6, 18.4.

(*R*)-*tert*-Butyldimethyl(pentadec-1-en-14-yn-4-yloxy)silane (22). Imidazole (848 mg, 12.5 mmol), DMAP (51 mg, 0.42 mmol) and TBSCl (1.13 g, 1.47 mmol) was added to a solution of alcohol **21** (980 mg, 4.15 mmol) in DCM (20 mL) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 7.5 h. The organic phase was washed with water, brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product was then purified by flash column chromatography (hexane/EtOAc, 96:4) to provide **22** (1.35 g, 97%) as a colorless oil.^{1}H NMR (500 MHz, $CDCl_3$) δ 5.77−5.85 (m, 1H), 5.03 (br d, J = 8.8 Hz, 1H), 5.01 (s, 1H), 3.67 (quint., J = 5.7 Hz, 1H), 2.16−2.22 (m, 4H), 1.94 (t, J = 2.5 Hz, 1H), 1.54 (q, J = 7.6 Hz, 1H), 1.26−1.41 (m, 15H), 0.89 (s. 9H), 0.04 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 135.5, 116.5, 84.8, 72.0, 68.0, 41.9, 36.8, 29.7, 29.6, 29.4, 29.1, 28.8, 28.5, 25.9, 25.7(2), 25.3, 18.4, 18.2, 18.1, −2.9.

(*R*)-3-(*tert*-Butyldimethylsilyloxy)tetradec-13-ynal (23). Ozone was bubbled through a solution of **22** (900 mg, 2.67 mmol) and Et_3N (540 mg, 5.34 mmol) in DCM (25 mL) cooled at −78 °C. After reaction was complete (monitored by TLC), Me_2S (332 mg, 5.34 mmol) was added to the reaction mixture and warmed to room temperature. After stirring for 2 h, the solvent was removed under reduced pressure. The crude aldehyde was then purified by column chromatography (hexane/EtOAc, 100:0 to 9:1) to furnish aldehyde **23** (720 mg, 80%) as a colorless oil.^{1}H NMR (500 MHz, $CDCl_3$) δ 9.81 (t, J = 2.6 Hz, 1H), 4.17 (quint., J = 5.7 Hz, 1H), 2.51 (dd, J = 2.6, 5.0 Hz, 2H), 2.18 (dt, J = 2.5, 6.9 Hz,, 2H), 1.93 (t, J = 2.5 Hz, 1H), 1.29−1.55 (m, 16H), 0.88 (s, 9H), 0.07 (s, 3H), 0.06 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 202.4, 84.8, 68.3, 68.0, 50.8, 37.8, 29.6, 29.5, 29.4, 29.0, 28.7, 28.5, 25.8, 25.1, 18.4, 18.0, −4.4, −4.7.

(3*S*,4*S*)-3-Hexyl-4-((*R*)-2-hydroxytridec-12-ynyl)oxetan-2-one (24). This was prepared according to the general TMAL reaction procedure using aldehyde **23** (544 mg, 1.61 mmol), $ZnCl_2$ (438 mg, 3.22 mmol), and ketene acetal **16** (740 mg, 2.42 mmol) in DCM (10 mL). The crude mixture was purified by column chromatography (hexane/EtOAc: 100:0 to 97:3) to provide a mixture of silyloxy-β-lactone diastereomers as a colorless oil. ^{1}H NMR (500 MHz) analysis of the mixture of diastereomers indicated that a diastereomeric ratio of 11:1. Without further purification, the mixture was used directly in the next step. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.41−4.44 (m, 1H), 3.83−3.85 (m, 1H), 3.16−3.24 (m, 1H), 2.18 (dt, J = 2.5, 6.9 Hz, 3H), 1.94 (t, J = 2.5 Hz, 1H), 1.28−1.85 (m, 27H), 0.90 (s, 9H), 0.89 (t, J = 7.0 Hz, 3H), 0.07 (s, 6H).

Prepared according to the general *O*-desilylation procedure using silyloxy-β-lactone **23** (409 mg, 0.9 mmol), and 40% HF (782 μL) in CH_3CN (12 mL). The crude mixture was purified by flash chromatography (hexane/EtOAc, 100:0 to 9:1) to provide the hydroxy-β-lactone **24** (173 mg, 32%) and a mixture of the two diastereomers (42 mg, 8%) as a colorless oil (40% overall two steps). Spectroscopic data are reported for the major diastereomeric β-lactone **24**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.49 (dt, J = 4.4, 8.8 Hz, 1H), 3.78−3.83 (m, 1H), 3.24−3.27 (m, 1H), 2.18 (dt, J = 2.5, 7.0 Hz, 2H), 1.93 (t, J = 2.5 Hz, 1H), 1.72−1.92 (m, 4H),

1.29−1.53 (m, 25H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.6, 84.7, 75.6, 68.5, 68.0, 56.6, 41.8, 38.1, 31.5, 29.4, 29.0, 28.9, 28.7, 28.4, 27.7, 26.7, 25.4, 22.5, 18.4, 14.0; IT-TOF-MS: m/z $[M+1]^+$ calcd: 350.281, found: 351.301.

(*S*)-((*S*)-1-((2*S*,3*S*)-3-Hexyl-4-oxooxetan-2-yl)tridec-12-yn-2-yl)-2-formamido-4-methylpentanoate (2). Prepared according to the general Mitsunobu reaction procedure using β-lactone **24** (11 mg, 0.03 mmol), DIAD (23 μL, 0.12 mmol), *N*-formyl-L-leucine (20 mg, 0.13 mmol), and triphenylphosphine (33 mg, 0.13 mmol) and in THF (1 mL). Purified by flash chromatography (hexane/EtOAc, 9:1 to 4:1) to give β-lactone **2** as a colorless oil (44%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (s, 1H), 5.91 (br d, J = 8.2 Hz, 1H), 5.00−5.03 (m, 1H), 4.66−4.71 (m, 1H), 4.27−4.34 (m, 1H), 3.22 (dt, J = 4.4, 7.6 Hz, 1H), 2.18 (dt, J = 2.5, 6.9 Hz, 2H), 2.00 (dt, J = 4.4, 14.5 Hz, 1H), 1.93 (t, J = 2.5 Hz, 1H), 1.28−1.85 (m, 30H), 0.96−0.99 (m, 6H), 0.89 (t, J = 7.6 Hz, 3H); IT-TOF-MS: m/z $[M+1]^+$ calcd: 491.361, found: 492.320.

(3*S*,4*S*)-3-Hexyl-4-((*R*)-2-hydroxytridecyl)oxetan-2-one (30).[5a] Prepared according to the general TMAL reaction procedure using aldehyde **8a** (432 mg, 1.27 mmol), $ZnCl_2$ (346 mg, 2.54 mmol) and ketene acetal **16** (652 mg, 1.85 mmol). Purification by flash chromatrography (hexane/EtOAc, 100:0 to 95:5) gave a mixture of silyloxy-β-lactone diastereomers as colorless oils, which was used directly in the next *O*-desilylation step.

Prepared according to the general *O*-desilylation reaction procedure using silyloxy-β-lactone (500 mg, 1.07 mmol) and 40% HF (2.5 mL). Purification by flash chromatrography (hexanes/EtOAc, 100:0 to 96:4) gave the hydroxy-β-lactone **30** (174 mg, 38%) and a mixture of the two diastereomers (47 mg, 10%) as white solids (48% overall, 2 steps): ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.50 (m, 1H), 3.81 (m, 1H), 3.26 (dt, J = 7.6, 3.8 Hz, 1H), 1.95−1.26 (m, 35H), 0.88 (t, J = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.6, 75.6, 68.5, 56.6, 41.8, 38.1, 31.9, 31.5, 29.63, 29.61, 29.55, 29.5, 29.3, 29.0, 27.7, 26.8, 25.4, 22.7, 14.1, 14.0; ESI-MS: m/z $[M+Na]^+$ calcd: 354.3, found: 377.2.

(*R*)-((*S*)-1-((2*S*,3*S*)-3-Hexyl-4-oxooxetan-2-yl)tridecan-2-yl)-4-methyl-2-propiolamidopentanoate (3). Prepared according to the general Mitsunobu reaction procedure using β-lactone **30** (71 mg, 0.20 mmol), PPh_3 (68 mg, 0.26 mmol), acid **29** (46 mg, 0.25 mmol) and DIAD (52 μL, 0.26 mmol) in THF (3 mL). Purification by flash chromatrography (hexane/EtOAc, 100:0 to 85:15) afforded **3** (54 mg, 52%) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.31 (br d, J = 7.6 Hz, 1H), 5.01−5.04 (m, 1H), 4.60 (dt, J = 4.4, 8.8 Hz, 1H), 4.26−4.30 (m, 1H), 3.21 (dt, J = 3.8, 7.6 Hz, 1H), 2.84 (s, 1H), 2.15 (dt, J = 7.6, 15.1 Hz, 1H), 1.99 (dt, J = 4.5, 15.7 Hz, 1H), 1.54−1.80 (m, 8H), 1.25 (br s, 25H), 0.96 (d, J = 6.3 Hz, 3H), 0.95 (d, J = 6.3 Hz, 3H), 0.874 (t, J = 7.0 Hz, 3H), 0.867 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.4, 170.7, 151.7, 83.3, 74.7, 74.1, 72.9, 57.0, 51.4, 41.2, 38.6, 34.0, 31.9, 31.4, 29.6, 29.5, 29.4, 29.3, 29.2, 28.9, 27.6, 26.7, 25.1, 24.9, 22.8, 22.6, 22.5, 21.7 14.1, 14.0; IT-TOF-MS: m/z $[M+1]^+$ calcd: 519.392, found: 520.391.

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Supporting Information Available: Details concerning the synthesis of **8a**, **9**, **11**, **16**, **29**, and additional figures containing in vitro competition studies with Orlistat, cellular imaging with probe **2** and **3**, Western blotting analysis of recombinant enzymes, mass spectrometry data, complete Table 1, and spectral characterization of new compoounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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