Target Identification

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Probing the Anticancer Mechanism of (−)-Ainsliatrimer A through Diverted Total Synthesis and Bioorthogonal Ligation**

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Abstract: *Herein, we report an efficient approach for exploring the novel anticancer mechanism of (−)-ainsliatrimer A, a structurally complex and unique trimeric sesquiterpenoid, through a combined strategy of diverted total synthesis (DTS) and bioorthogonal ligation (TQ ligation), which allowed us to visualize the subcellular localization of this natural product in live cells. Further biochemical studies facilitated by pretarget imaging revealed that PPARγ, a nucleus receptor, was a functional cellular target of ainsliatrimer A. We also confirmed that the anticancer activity of ainsliatrimer A was caused by the activation of PPARγ.*

Historically, natural products and their derivatives have been an invaluable source for drug discovery.[1] However, identifying functional targets and clarifying the mechanisms of action of bioactive natural products have proven to be particularly challenging.[2] First, structural modifications of complex natural products for the synthesis of useful chemical probes are difficult to achieve due to the intrinsic architectural complexity of many natural products. Furthermore, target identification, which is the foundation of a chemical biology research program, remains challenging for several reasons including low target affinities, low abundance of targets in vivo, and limitations of bioanalytical methods.[3]

Despite these challenges, several excellent studies have been able to facilitate the target identification of complex natural products.[4] A number of remarkable functionalization strategies aimed at modifying natural products directly have been developed to tackle synthetic challenges.[2a,5] However, diverted total synthesis (DTS) remains an effective approach for the syntheses of natural product analogues or natural product-based chemical probes,[6] because DTS can expand the flexibly of the chemical space around the natural products from advanced intermediates. It can thus provide many valuable analogues for structure–activity relationship (SAR) studies and affinity-tag labeling. The emerging bioorthogonal ligation approach affords a new avenue for cellular studies of natural products,[7] as the incorporation of small chemical reporters into complex scaffolds minimally affects their original bioactivity. Subsequent target localization and identification studies can thus be performed by completing bioorthogonal reactions with a cognate tag. Recently, our research group developed a novel bioorthogonal ligation method that was enabled by a click hetero-Diels–Alder cycloaddition between a thio vinyl ether and an *ortho*-quinolinone quinone methide (TQ ligation).[8] We envisioned that TQ ligation might provide us with a useful means to study the subcellular localization of complex natural products in live cells, which would facilitate subsequent target identification.

Enabled by total synthesis,[9] we recently not only confirmed that (−)-ainsliatrimer A (**1**; Figure 1), an architecturally complex and unique trimeric sesquiterpenoid, displayed potent cytotoxicity against several cancer cell lines (see Table S1 in the Supporting Information), but also demonstrated that the activity resulted from the induction of apoptosis.[10] However, the exact mode of anticancer action remained elusive. Herein, we introduce a combined strategy of diverted total synthesis (DTS) and bioorthogonal ligation (TQ ligation) to facilitate identification of the target of this complex natural product.

To perform the pretarget imaging and target identification of **1** using the TQ ligation, it was first necessary to prepare **1** modified with a thio vinyl ether group. Initially, we attempted to modify the natural product directly to obtain a pendant functional group that could react with a thio vinyl ether fragment. However, the similar properties of four α,β-unsaturated ketones can lead to selectivity issues, and one or more of them might act as a Michael acceptor in reactions with biomolecules. We therefore proposed that other feasible approaches might be possible including alkylation and hydroxylation at C2″, reduction of the C3″-carbonyl group, allylic oxidation of the C9″-OH group, and cross-metathesis and hydroboration of the exo-methylene group at C14″

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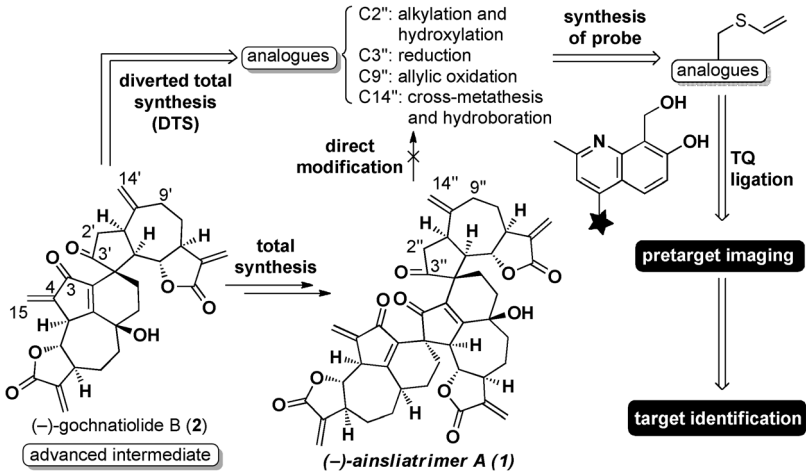
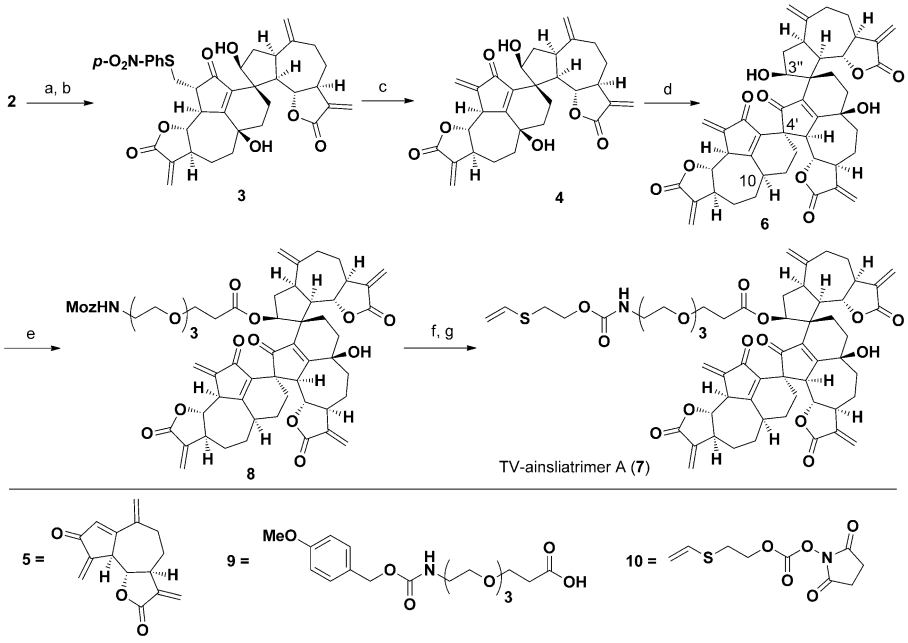


Figure 1. Target identification of (−)-ainsliatrimer A (**1**) enabled by diverted total synthesis (DTS) and bioorthogonal ligation (TQ ligation).

(Figure 1). However, none of these methods provided satisfactory results because of the lability of **1** and the poor reactivity and selectivity of the proposed procedures. Inspired by the remarkable diverted total synthesis strategy, we envisioned that (−)-gochnatiolide B (**2**), an advanced intermediate in the synthesis of **1**,[9b] could be modified. In turn, the modified **2** could be used in the syntheses of analogues of ainsliatrimer A.

Therefore, we aimed to modify **2** by using approaches similar to those described above. Although unsuccessful results were obtained, we noticed that the 3′-carbonyl group in **2** could be reduced under treatment with $NaBH_4/CeCl_3{\cdot}7H_2O$ concomitantly with the 1,4-reduction of the exo-methylene group at C4. We next decided to protect this methylene moiety. Thiophenol is commonly applied for the protection of α,β-unsaturated ketones;[11] however, in this case, its regioselectivity was poor due to concomitant addition to the enone moieties in the lactone rings. To our delight, after fine-tuning the nucleophile, the selective Michael addition proceeded smoothly using 4-nitrothiophenol, and the thioether was produced in high yield as a single diastereoisomer (Scheme 1). Luche reduction of the thioether provided **3** in moderate yield without the detection of other diastereoisomers.[12] However, the elimination of thiophenol from **3** proved nontrivial, and extensive experimentation with different bases and oxidative/β-elimination and alkylative thioether elimination procedures led to either low yields or recovery of the starting material.[10,13] Ultimately, we were pleased to discover that treatment of **3** with Ag_2O resulted in the successful elimination to afford 3′-hydroxy-gochnatiolide B (**4**) in high yield.[14] Treatment of **4** with the freshly prepared diene **5** under anaerobic conditions afforded **6** in moderate yield as a single isomer. The anaerobic conditions prevented autoxidation at C10 in **6**, and pyridine was added to inhibit the polymerization of **5**, both of which resulted in a higher yield of **6** than previously reported.[9b] Moreover, alcohol **6** could be converted into (−)-ainsliatrimer A (**1**) by Dess–Martin oxidation,[10] thereby demonstrating that the newly generated configurations at C4′ and C10 fully coincided with those in **1**. Fortunately, the reduction of the C3″-carbonyl group in **1** did not compromise the antitumor activity (see Figure S1 in the Supporting Information),[10] which allowed for the further attachment of thio vinyl ether fragments.

We then set out to synthesize the chemical probe of **1**. To enhance the hydrophilicity of the chemical probe and facilitate the reaction between the labeled *ortho*-quinolinone quinone methide (*o*QQM) and the thio vinyl ether fragment, a tri(ethyleneglycol) linker was designed to be inserted between the thio vinyl ether and **6** to form the chemical probe **7**. However, direct esterification between the thio vinyl ether modified linker acid and **6** was unsuccessful under



Scheme 1. Synthesis of the chemical probe TV-ainsliatrimer A (**7**). Reagents and conditions: a) *p*-nitrothiophenol, $CHCl_3$, RT, 97%; b) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, MeOH/CH_2Cl_2 (1:1), −78 °C, 46% (68% brsm); c) Ag_2O, MeOH, RT, 87%; d) **5**, toluene/pyridine (100:1), glove box, 35 °C, 62–76%; e) 1. **9**, 2,4,6-trichlorobenzoyl chloride, *i*Pr_2EtN, toluene, 30 °C then 2. DMAP, benzene, 35 °C, 56%; f) 3% TFA/CH_2Cl_2, RT; g) **10**, Et_3N, DMF, 35 °C, 82% over 2 steps. DMAP = 4-dimethylaminopyridine, TFA = trifluoroacetic acid, DMF = *N*,*N*-dimethylformamide, Moz = [(*p*-methoxybenzyl)oxy]carbonyl, brsm = based on recovered starting material.

various conditions because of the lability of alcohol **6** and the low solubility of the acid fragment. Therefore, we focused our attention on a stepwise procedure, and the Moz-protected linker **9** was attached to **6** in moderated yield.[15] After deprotection, the thio vinyl ether fragment was assembled to furnish the desired TV-modified ainsliatrimer A (**7**) in a facile manner.[16] Furthermore, we observed that **7** was stable in DMSO solution for two weeks under air at room temperature, thus indicating that the potential occurrence of inter- or intramolecular Diels–Alder reactions between the thio vinyl ether and the dienes in the ainsliatrimer A could be ruled out.

With probe **7** in hand, we further demonstrate that **7** possesses equipotent activity at approximately double the concentration of **1**, and retained its apoptosis-inducing ability (see Figure S1 in the Supporting Information).[10] Further cell-cycle analysis experiments revealed that both **1** and **7** could cause cell-cycle arrest in the G_2/M phase (see Figure S2 in the Supporting Information).[10] Collectively, these studies demonstrated that **7** is a suitable chemical probe for the anticancer natural product **1** for subsequent bioimaging studies in live cells.

Encouraged by this result, we concentrated our efforts on live-cell imaging studies. HeLa cells were first treated with TV-ainsliatrimer A (**7**) under conditions that were expected to produce high intracellular levels without killing the cells. After extensive exploration, we chose to treat the HeLa cells with 1.2 μM **7** for 2 h at 37 °C. After washing the cells with DMEM media three times, they were exposed to the media containing the 10 μM fluorescein-modified *o*OQM precursor (**11**) for 18 h. After thorough washing of the cells to remove the excess labeling mixture and subsequent co-staining with the nuclear dye DAPI, the cells were imaged with a confocal fluorescence microscope. Clearly stained, intracellular structures of the nucleus were distinguishable in the group treated with **7**, and the **7**-associated fluorescence fully merged with DAPI (Figure 2; see also Figure S3 in the Supporting Information). In contrast, in the DMSO-treated group, whereby the cells were incubated with **11** in the absence of **7**, the fluorescence from **11** alone generated extremely low levels of cellular nuclear labeling, and the majority of the signals were enriched in the cytoplasm. This indicated that **7** was specific for the cellular targets of interest. As an additional control, we performed tagging with **11** under identical conditions, but in cells treated with **1**; in this case, nuclear labeling was not observed, again indicating that successful labeling was enabled by TQ ligation. These results definitively show that **7** accumulates primarily in the nuclei of HeLa cells, further suggesting that the potential target(s) of **1** are localized in the nucleus.

We next target identification of the functional target(s) of **1**. Since we assumed that some of the extracted nuclear proteins might be unstable at 37 °C for 12 h, we decided to complete the TQ ligation in vitro beforehand. Accordingly, **7** was treated with the biotin-tagged *o*QQM precursor **12** to afford biotin-labeled probe **13** (Figure 3A).[8] In parallel, we also synthesized biotin-labeled **14** as the negative control from saturated **6** by using the same synthetic strategy.[10] Examination of the bioactivity showed that **13** retained its original activity, whereas **14** was biologically inactive (Figure 3B), thereby indicating that the unsaturated enone moieties were essential for the biological activity of **1** (see Figure S4 in the Supporting Information).[10]

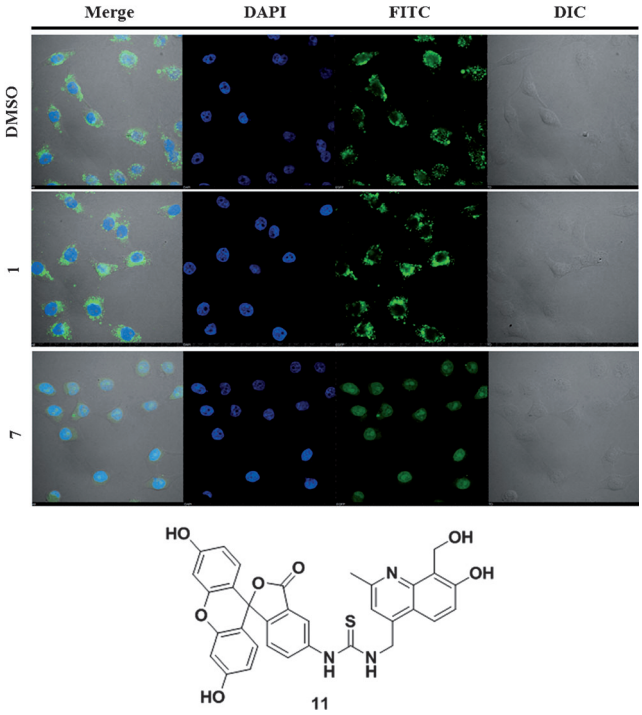


Figure 2. Pretarget imaging studies using **7** in live cells. Live HeLa cells were treated with fluorescein-modified tag **11** after treatment with DMSO, **1**, and **7**. All cells were treated with the nuclear dye DAPI before being imaged on a confocal microscope. DAPI = 4′,6-diamidino-2-phenylindole, FITC = fluorescein isothiocyanate, DIC = differential interference contrast.

With the functional chemical probes **13** and **14** in hand, we performed pull-down experiments by incubating HeLa cell nuclear extracts with **13** and **14**. The resulting lysates were subsequently incubated with streptavidin-labeled beads. After washing the beads, the proteins precipitated were resolved by SDS-PAGE and stained with silver (Figure 3C). The purity of the nuclear protein extraction was also examined by detecting for contamination from α-tubulin and the nuclear marker histone H1, which were easily detected (see Figure S5 in the Supporting Information).[10] Finally, we chose the gel bands preferentially pulled down by probe **13** rather than **14**, and cut down both of them for MS analysis. Several proteins were identified by peptide-mass fingerprinting analysis. The proteins included in the list were represented by more than 6 unique peptides (see Table S2 in the Supporting Information).[10] After excluding the proteins present in the **14** group from those of the **13** group, we finally focused on two potential target proteins: peroxisome proliferator activated receptors γ (PPARγ) and histone deacetylase 2 (HDAC2; see Tables S2 and S3 in the Supporting Information).[10]

We next evaluated the functional roles of these two candidate targets in the anticancer activity of ainsliatrimer A (**1**). Initially, we performed target-knockdown experiments using siRNA in HeLa cells, and measured the cell survival

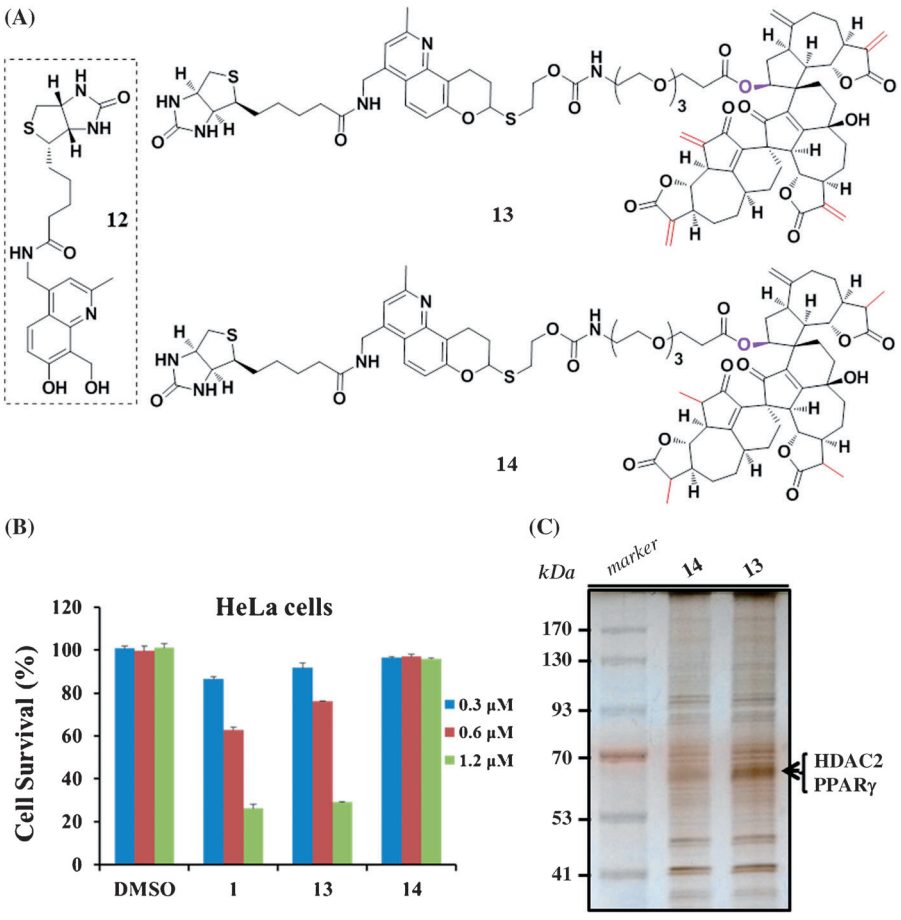


Figure 3. A) Chemical structures of **12** as well as probes **13** and **14**. B) HeLa cells were preincubated with the indicated concentrations of **1**, **13**, or **14** for 48 h and the cell viability was determined by MTT methods. C) The nuclear extracts of HeLa cells were incubated with **13** or **14** at 4 °C overnight, followed by pull-down with streptavidin-agarose beads. The precipitates were resolved by SDS-PAGE, and the gel was stained with silver. The indicated silver-stained protein bands were analyzed by mass spectrometry. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

To determine whether ainsliatrimer A directly binds to PPARγ, we generated recombinant Flag-tagged PPARγ protein. We first immobilized PPARγ on Flag affinity beads, then incubated the immobilized PPARγ with an increasing concentration of probe **13**. Subsequently, the precipitates were blotted for biotin or Flag with a short exposure. As shown in Figure 4C, a strong interaction could be observed between **13** and the Flag-tagged PPARγ. Moreover, this binding could be out-competed by an excess of unlabeled **1** (Figure 4D). We further confirmed that **1** cannot bind to other PPAR subtypes, including PPARα or PPARδ/β (see Figure S8 in the Supporting Information).[10] Collectively, these results demonstrate that **1** can efficiently interact with PPARγ.

Finally, we observed that the death of HeLa cells induced by **1** could be significantly reduced when cells were pretreated with GW9662, a previously reported PPARγ-selective antagonist. This suggested that **1** might act as an agonist of PPARγ (see Figure S9 in the Supporting Information).[10] Consistent with this result, we further demonstrated that **1** induced the transcriptional activity of PPARγ (see Figure S10 in the Supporting

rate. We found that the knockdown of PPARγ, a nuclear receptor protein that functions as a transcription factor, resulted in a significant decrease in the cytotoxicity caused by **1** (Figure 4A). This finding indicated that PPARγ might be a functional target of **1**. Knockdown at the protein level following siRNA treatment was confirmed by immunoblotting analysis (Figure 4B). In contrast, knockdown of HDAC2 did not rescue the cell death caused by **1**, thus demonstrating that HDAC2 was not a functional target (see Figure S6 in the Supporting Information).[10] Subsequently, we selected the melanoma cell line SK-MEL-28 for further testing because of the relatively low PPARγ expression level in HeLa cell.[17] SK-MEL-28 is known to have a high PPARγ expression level[18] and has been proven to be very sensitive to treatment with ainsliatrimer A (see Table S1 in the Supporting Information).[10] Pull-down experiments using chemical probes **13** and **14** with the nuclear extracts of SK-MEL-28 revealed only one observable band that was specifically precipitated by **13**, but not by **14**. We confirmed that this band was PPARγ by MS analysis (see Figure S7 in the Supporting Information).[10]

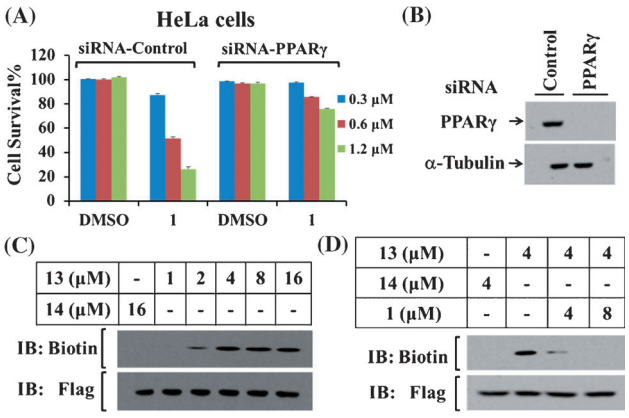


Figure 4. A) HeLa cells were transfected with the indicated siRNA. 48 h post-transfection, the cells were treated under the indicated conditions for an additional 48 h, and then the cell viability was determined by an MTT assay. B) The cell lysates were collected and harvested for Western blot analysis by using the indicated antibodies. C,D) The recombinant Flag-PPARγ proteins were incubated with **13**, **14**, or in the absence of **1** for 1.5 h at 37 °C. The mixtures were blotted for biotin or Flag. All experiments were repeated at least three times, with similar results obtained.

Information).[10] Furthermore, we detected that **1** activated the expression of COX-2, a downstream signaling target of PPARγ,[19] in the 184B5 cell line (see Figure S11 in the Supporting Information).[10,20] Taken together, these results establish that **1** acts as an agonist to PPARγ.

In summary, we used an efficient workflow to complete the target identification of the bioactive and structurally complex natural product (−)-ainsliatrimer A (**1**). This approach was facilitated by a combined strategy of diverted total synthesis and the pretarget imaging enabled by TQ ligation, which allowed us to visualize the subcellular localization of the natural product before pull-down experiments. By applying this strategy, we identified PPARγ as a functional target of **1**. Through subsequent biochemical studies, we also confirmed that the anticancer activity of ainsliatrimer A is caused by the activation of PPARγ. We also observed the occurrence of intriguing reactions during the synthesis, including the selective addition of α,β-unsaturated ketones by fine-tuning the nucleophile and Ag_2O-dependent thiophenol elimination, which should have potential synthetic utility in the transient protection of enones. Notably, PPARγ is regarded as a promising anticancer target in view of its crucial regulatory role in cell metabolism. To date, several anticancer mechanisms involving the activation of PPARγ have been reported, such as the induction of apoptosis and regulation of metabolic pathways.[21] Further studies towards the elucidation of how exactly the activation of PPARγ by ainsliatrimer A regulates cell proliferation through transcription level or protein–protein interactions are in progress and will be reported in due course.

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Communications

Target Identification

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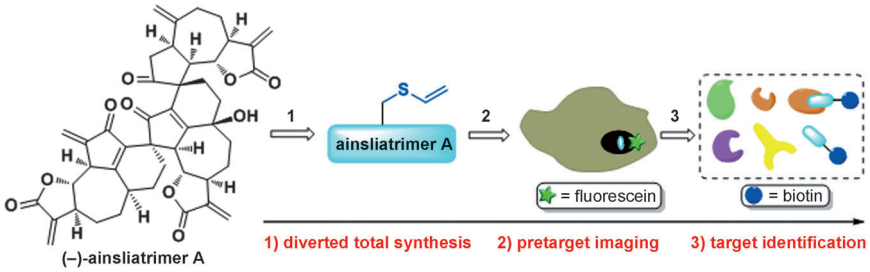

The target of the structurally complex natural product (−)-ainsliatrimer A has been identified by a systematic and efficient approach enabled by diverted total synthesis and bioorthogonal ligation. This approach enabled visualization of the subcellular localization of the natural product in live cells and identified activation of PPARγ as leading to the anticancer activity of ainsliatrimer A.