

# Asymmetric syntheses of (+)- and (-)-collybolide enable reevaluation of *kappa*-opioid receptor agonism

Sophia L. Shevick,<sup>1,3</sup> Stephan M. Freeman,<sup>1,3</sup> Guanghu Tong,<sup>†</sup> Robin J. Russo,<sup>2,3</sup> Laura M. Bohn<sup>2</sup> and Ryan A. Shenvi<sup>1\*</sup>

<sup>1</sup>Department of Chemistry, Scripps Research, 10550 North Torrey Pines Road, La Jolla, California 92037, United States.

<sup>2</sup>Department of Molecular Medicine, Scripps Research, 130 Scripps Way, Jupiter, FL 33458

<sup>3</sup>Skaggs Graduate School of Chemical and Biological Sciences

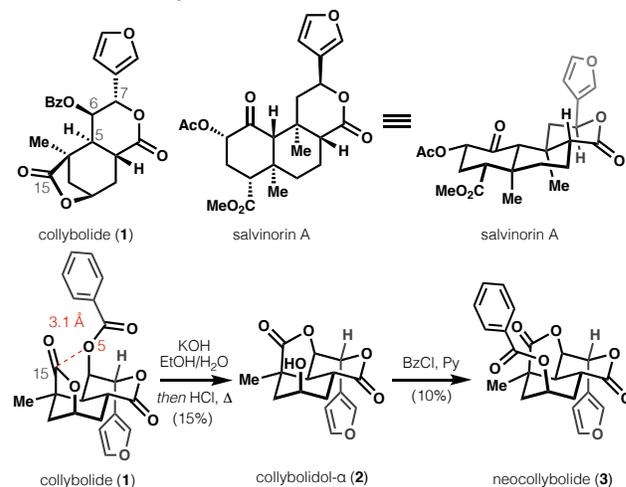
The fungal metabolite collybolide attracted attention as a non-nitrogenous, potent and biased agonist of the *kappa*-opioid receptor (KOR). Here we report a 10-step asymmetric synthesis of this complex sesquiterpene that enables facile access to either enantiomer. The synthesis relies on a diastereoselective  $\alpha$ -benzoyloxylation to install the buried C6 benzoate and avoid irreversible trans-lactonization of the congested, functionally dense core. Neither enantiomer, however, exhibited KOR agonism, indicating that collybolide has been mischaracterized as a KOR agonist. Given the pharmaceutical, medical and societal interest in collybolide as a next-generation antipruritic and analgesic, this refutation of KOR activity has important ramifications for ongoing studies. Excitement over identification of a new non-nitrogenous, KOR-selective, potent agonist with the same clinical potential as salvinorin A seems to have been misplaced.

**Introduction** The freckled mushroom *Collybia maculata* (*nom. alt. Rhodocollybia maculata*) clusters in lignan-rich soils of conifer forests of Europe and North America. Bitter and tough to the point of inedibility, no references mention its use in traditional medicine nor its activity in mammals. In 2016, however, a metabolite of *C. maculata*<sup>1</sup> was reported to exhibit potent and biased agonism of the *kappa*-opioid receptor (KOR).<sup>2</sup> This sesquiterpene, collybolide, had been singled out for testing due to the similarity of its furano- $\delta$ -lactone motif to that of the potent KOR agonist diterpene salvinorin A (SalA). In side-by-side assays with SalA, collybolide exhibited typical agonist behavior selective for the KOR. In the GTP $\gamma$ S binding assay, the collybolide-induced response was submaximal compared to SalA; while in the ERK1/2 MAP kinase and Akt phosphorylation studies, collybolide produced more stimulation than SalA. This change in relative rank-order efficacy led the authors to conclude that collybolide displays biased agonism at KOR.<sup>3</sup> Collybolide also exhibited anti-pruritic activity in mice, a known consequence of KOR agonism.<sup>4</sup>

The translational potential of collybolide attracted attention across the biomedical community<sup>5</sup> due to indications for the KOR as a target in next-generation analgesics, antipruritics and antidepressants.<sup>6</sup> However, many questions remained: the unusual dose-response curves and the lack of a  $\beta$ -arrestin recruitment assay, specifically, required greater pharmacological scrutiny,<sup>7</sup> and structural homology with SalA was non-obvious in three dimensions (Figure 1). Synthetic access to collybolide might address these questions, clarify the absolute stereochemistry of **1**,<sup>8</sup> provide material with a different impurity profile than *C. maculata* isolates (*vide infra*) and establish means for deep-seated modifications of the scaffold. Here, we report the first synthesis of collybolide (**1**) and the biochemical profiles of the pure enantiomers, (+)-**1** and (-)-**1**. In contrast to a pre-

vious report,<sup>2</sup> our data does not support collybolide as a KOR agonist.

Degradation studies<sup>1a</sup> of collybolide revealed the synthetic challenges that lay ahead. Like SalA, collybolide (**1**) epimerizes under acidic conditions, scrambling C7 stereochemistry. Treatment of **1** with alkali causes benzoate solvolysis and lactone cleavage. The nucleophilic oxygen (O5) of **1** is positioned 3.1 Å from C15 and the isomer, neocollybolide (**3**), is calculated (MM2) to be 1.2 kcal/mol more stable than **1**, resulting in kinetic and thermodynamic preference for the formation of **2** and **3**.<sup>9</sup>

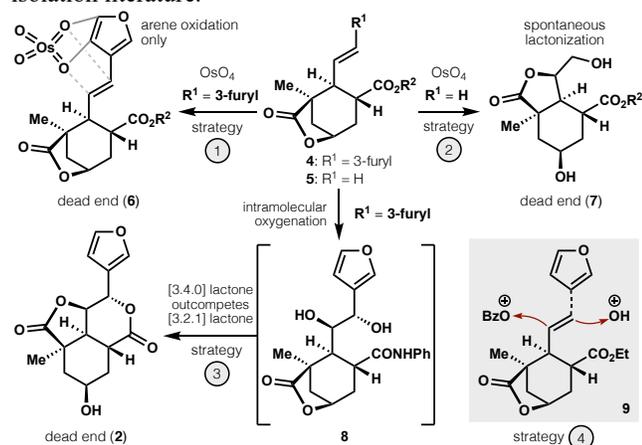


**Figure 1.** Collybolide and salvinorin A are suggested to share the  $\kappa$ -opioid receptor as a target, despite prominent differences in structure.

Treatment of **2** with benzoyl chloride delivers neocollybolide (**3**); rearrangement to the collybolide scaffold does not occur. The preference for **3** over **1** contrasts the reactivity of

other sesquiterpenes like bilobalide where benzylation of a hindered alcohol promotes scaffold rearrangement (e.g. **2** to **1**).<sup>10</sup> Synthesis of the collybolide scaffold, therefore, cannot rely on lactone rearrangement of **2** to favor the desired scaffold; the preference for **2** would prove detrimental to our first-, second- and third-generation routes and dictate our ultimately successful approach to synthesize **1**.

These three unsuccessful strategies targeted dioxygenation of [3.2.1]-bicyclic lactone **4** or **5** (Figure 2). However, steric shielding of vinylfuran **4** led to exclusive arene oxidation by OsO<sub>4</sub> (strategy 1). In the absence of the furan, dihydroxylation favored the [4.3.0] bicycle (strategy 2)—another dead end due to the intractability of this fused lactone, as noted above. A third-generation approach (strategy 3) accessed **8** via internal radical dioxygenation (see SI) and similarly led to a dead end translactonization, mirroring observations in the isolation literature.



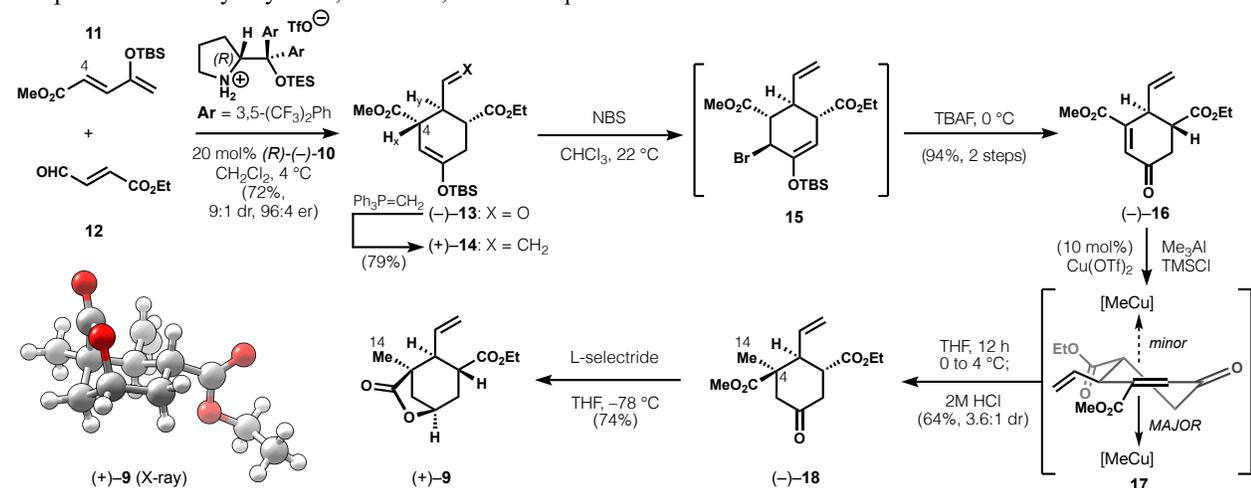
**Figure 2.** Three generations of routes reveal the difficulty of C6-OH intermediates.

These three unsuccessful strategies called for an alternative approach that avoided the C6 alcohol entirely and resisted the reflexive benzylation transform (+Bz<sup>+</sup>) suggested by **1**. A fourth-generation approach (Figure 2, gray panel) would install the C6 benzyloxy group directly (+BzO<sup>+</sup>), averting undesired translactonization and, ultimately, enabling the first chemical synthesis of **1**. All four generations proceeded through the same densely functionalized, enantioenriched core **9**. Like our second-generation route, furan installation would occur last, subsequent to alkene oxidation, to avoid furan decomposition. Benzyloxylation, however, would require an

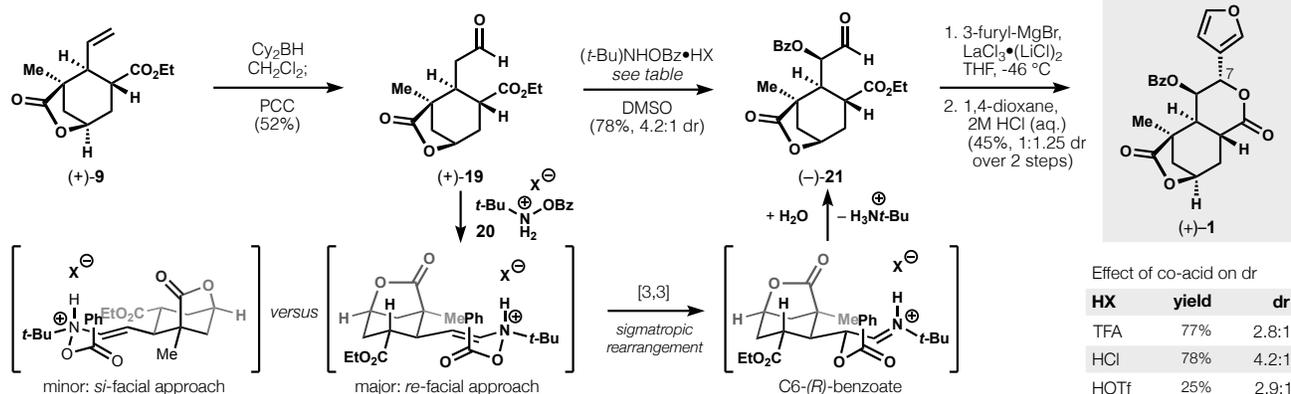
electrophilic source of benzoate and its stereoselective addition to one alkene face – a high risk tactic that ultimately paid off.

**Results and Discussion** Synthesis commenced with an asymmetric, organocatalyzed cycloaddition, utilizing the Hayashi-Jørgensen diarylprolinol triethylsilyl ether ammonium catalyst (*R*)-(-)-**10**, a more soluble variant of the commercially available trimethylsilyl ether.<sup>11,12</sup> The electron-deficient diene **12** necessitated extended incubation with **11** (4 days at 4 °C) but yielded cycloadduct (-)-**13** (80%, 92% *ee*) in large quantity (12 g) after careful optimization. In contrast to previous work in which the ammonium salt was formed *in situ*, we pre-formed the air-stable triflate salt (-)-**10**, as small excesses of triflic acid promoted Mukaiyama-aldol side reactions. The *exo*-adduct (-)-**13** was assigned definitively based on the homodecoupled <sup>1</sup>H NMR (homodec) coupling constant of 9.23 Hz between H<sub>x</sub> and H<sub>y</sub>.<sup>13</sup> Attempts to effect the cycloaddition with C4 methyl (C14) in place were unsuccessful and instead favored a Mukaiyama-Michael adduct likely due to a severe *syn*-pentane interaction in the cycloaddition transition state. Addition of the methyl group at the expense of additional steps was compensated for by the excellent yield and enantiomeric ratio. The incorrect relative stereochemistry of the C4 carboxylate proved inconsequential as it was inverted during this methyl addition step.

Wittig olefination of (-)-**13** proceeded smoothly to access terminal alkene (+)-**14**. Whereas this alkene served as a substrate for a carboxylate-directed Heck reaction<sup>11b</sup> in earlier routes, it would later serve as a masked, homologated aldehyde (*vide infra*). Attempts to oxidize silyl enol ether (+)-**14** to enone (-)-**16** using Saegusa oxidation conditions led to adequate yields, but only at stoichiometric palladium loading (40% yield); attempts to render the oxidation catalytic in palladium were unsuccessful.  $\alpha$ -Bromination en route (-)-**16** led to unexpected silyl enol ether transposition (**15**, verified by <sup>1</sup>H NMR and COSY).<sup>14</sup> Despite the potential for facile aromatization, **15** underwent concomitant deprotection and elimination when treated with tetrabutylammonium fluoride to yield (-)-**16** in 94% yield. We did not observe isomerization of the 1,4-diene terminal alkene to the internal position of a conjugated 1,3-diene. Low substrate concentration (0.03 M in **15**) proved crucial for clean conversion to (-)-**16**, potentially to disfavor olefin-to-olefin bromonium transfer.<sup>15</sup>



**Scheme 1.** Short asymmetric synthesis of the collybolide core.



**Scheme 2.** Completion of collybolide avoids C6–OH intermediate via stereoselective C–OBz bond formation.

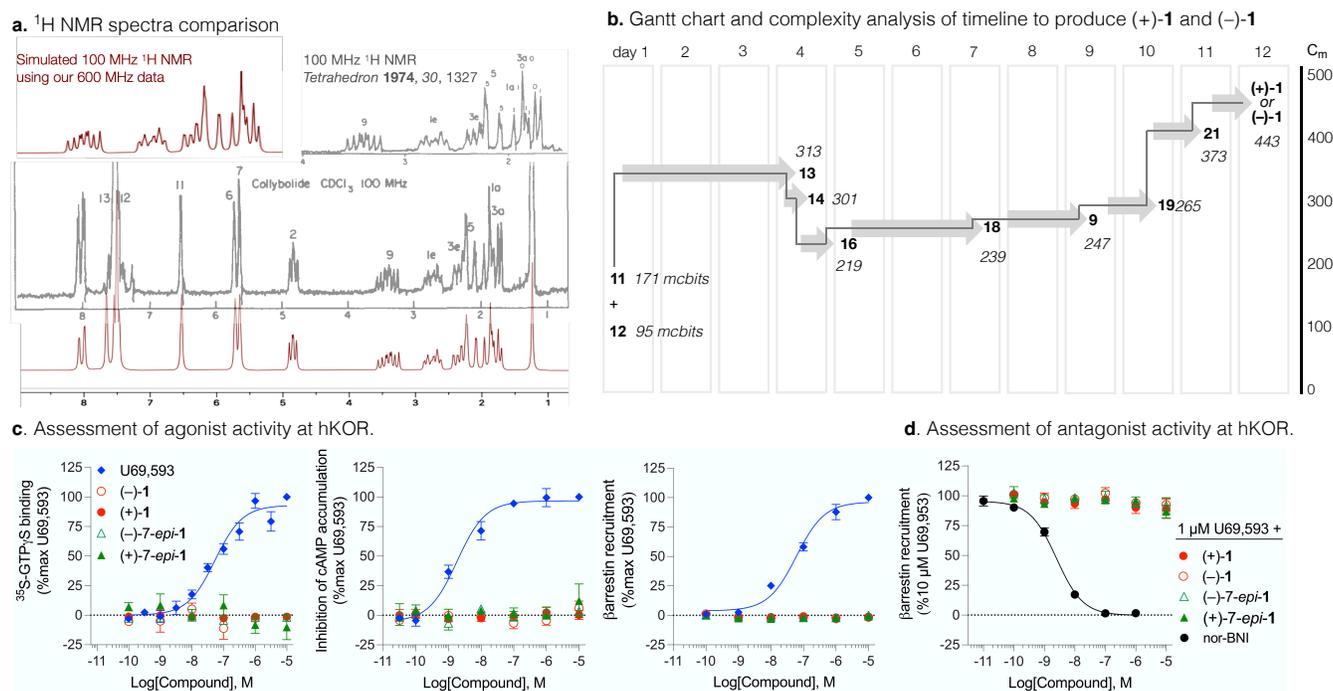
The challenging C4 quaternary stereocenter could be accessed via conjugate addition (see Scheme 1), but not using standard approaches (MeLi or MeMgBr in combination with Cu(I) salts), which either failed to convert (–)-16 or formed 1,2-addition products. To lessen the basicity and increase the Lewis acidity of the methyl pro-nucleophile, we turned to Me<sub>3</sub>Al<sup>16</sup> in the presence of (10 mol%) Cu(I)Br•SMe<sub>2</sub>, and TMSCl, which furnished the desired conjugate addition product in a modest 1.7:1 dr favoring diastereomer (–)-18 (60% yield, diastereomers assigned by NOE). The diastereomeric ratio could be improved to 3.6:1 dr with catalytic Cu(II)(OTf)<sub>2</sub> (10 mol%)<sup>17</sup> in favor of the desired diastereomer (64% yield).<sup>18</sup> Diastereoselectivity may depend on copper aggregation or oxidation states, which could perturb steric interactions with the vinyl substituent en route to Cu<sup>3+</sup> insertion products.<sup>19</sup> Addition of TMSCl prevented further reactivity of the aluminum enolate intermediate; its absence resulted in complex reaction mixtures. Deprotection of the newly formed trimethylsilyl enol ethers occurred upon work-up with 2M HCl. Diastereoselective reduction of cyclohexanone (–)-18 proceeded smoothly with L-Selectride®, resulting in a single diastereomer, which lactonized under the reaction conditions to form (+)-9 (75% yield, relative and absolute stereochemistry assigned by X-ray crystallography, see Scheme 1).

Prosecution of our fourth-generation strategy required oxidation of the terminal alkene to the corresponding aldehyde. Attempts to effect this transformation directly with nitrite-modified aldehyde-selective Wacker oxidation conditions failed to convert starting material, while Wacker oxidation of the alkene under substrate control yielded mixtures of aldehyde and methyl ketone.<sup>20</sup> Instead, successful oxidation was achieved via a one-pot hydroboration/oxidation approach. Efficient hydroboration of (+)-9 was accomplished exclusively with dicyclohexylborane, whereas BH<sub>3</sub>•SMe<sub>2</sub> or Sia<sub>2</sub>BH did not hydroborate efficiently and 9-BBN or BH<sub>3</sub>•THF did not allow efficient oxidation. The intermediate trialkylborane was oxidized directly to (+)-19 by PCC (52% yield). We attribute the difficulties of this alkene oxidation to steric crowding in (+)-9 from both the C4 methyl and the C9 ester that flank the terminal olefin. Given the difficulties of the oxidation of vinyl-furan **4** (Figure 2a), the steric hindrance of this alkene had proven to be a recurring challenge in this synthesis.

Access to (+)-19 allowed us to skirt this same steric hindrance and probe *intra*-molecular appendage of the benzyloxy group (Scheme 2). The intramolecularity of  $\alpha$ -oxidation would thus solve the problems of instability, inaccessibility and stereoselectivity in a single maneuver. To carry out the benzyloxylation, we turned to ammonium ion **20**, explored

extensively by the Tomkinson group in the chemoselective  $\alpha$ -oxygenations of aldehydes and ketones.<sup>21</sup> The mechanism proposed for this transformation involves condensation to the iminium, tautomerization to the enamine and [3,3]-sigmatropic rearrangement to give the  $\alpha$ -benzyloxy imine, which hydrolyzes *in situ* or upon workup. The development of this reagent hinges on literature precedent from House and Cummins on the spontaneous rearrangement of acylated oxime or nitrono intermediates, respectively.<sup>22</sup> A multi-hetero Claisen rearrangement proceeding through a chair-like transition state (i.e. (+)-19 to (–)-21) suggests two competing transition state conformers leading to the major and minor diastereomers.<sup>23</sup> Diastereoselectivity was improved by variation of acid co-reagents, with HCl leading to the highest dr (4.2:1) (see Table in Scheme 2). This dependence of dr on acid suggests that the acid either affects the equilibrium ratio of enamine conformations or changes the relative rates of rearrangement.<sup>24</sup> Although [3,3]-rearrangement is a precedented mechanism, we cannot rule out an alternative bimolecular benzoate transfer. Nevertheless, only furan addition and lactonization now separated (–)-21 from **1**.

3-Metallofuran addition to an aldehyde had proven to be an effective approach to the related aryl- $\delta$ -lactone motif of salvinorin A.<sup>25</sup> Organotitanium reagents, in particular, have exhibited high chemoselectivity and diastereoselectivity on similar scaffolds, in the presence of scalemic titanium Lewis acids.<sup>26</sup> In our hands, 3-furyltitanium reagents failed to deliver **1**, however, resulting instead in titanium alkoxide addition to the aldehyde. Furyllithium and magnesium halides were unsuccessful, either resulting in recovered starting material or complex reaction mixtures. Organolanthanides, however, proved effective, likely due to the high nucleophilicity and oxophilicity but low basicity relative to organolithium and -magnesium counterparts. We first explored organocerium addition, but operational difficulties of the hygroscopic and poorly soluble cerium(III) chloride salt led to irreproducible results between batches of reagent. The organolanthanum, however, could be formed by transmetalation of the corresponding Grignard reagent using THF solutions of LaCl<sub>3</sub>•2LiCl and reproducibly yielded collybolide (+)-1 and 7-*epi*-collybolide (7-*epi*-1) (1:1.25).<sup>27</sup> Attempts to vary diastereoselectivity by increasing the temperature were unsuccessful. Fortunately, Potier and coworkers found that trifluoroacetic acid established an equilibrium between collybolide and 7-*epi*-collybolide (1:1 at 72 °C), which allows material to be cycled toward collybolide. <sup>1</sup>H and <sup>13</sup>C NMR spectra of synthetic materials were identical



**Figure 3.** Neither enantiomer of collybolide (**1**) or *7-epi-1* activate or inhibit *kappa*-opioid receptor signaling. **a.** The  $^1\text{H}$  NMR data report of *synth*-(+)-**1** matches *nat*-(+)-**1** and its 600 MHz data can be simulated at 100 MHz to produce aligned spectra for visual comparison. **b.** The rapidity of complexity increase, depicted on a Gantt chart, allowed the synthesis of (-)-**1** in 12 days. **c.** Stimulation of hKOR using (+)-**1**, (-)-**1**, (-)-*7-epi-1* and (+)-*7-epi-1* ( $10^{-10}$ – $10^{-5}$  M) relative to the control agonist U69,593 in (left) a [ $^{35}\text{S}$ ]GTP $\gamma$ S binding assay performed in hKOR-CHO cell membranes; (center) inhibition of forskolin-stimulated cAMP accumulation in CHO-hKOR cells; (right)  $\beta$ -arrestin2 recruitment assay (U20S hKOR DiscoverRx PathHunter $^{\text{R}}$ ). **d.** Antagonism of 1  $\mu\text{M}$  U69,593 stimulated  $\beta$ -arrestin2 recruitment with the KOR antagonist, norBNI; collybolide does not antagonize the response. Experimental details can be found in Ref. 28.

in all aspects to those of naturally-occurring **1** and *7-epi-1* (see SI and Figure 3a). Optical rotation and circular dichroism spectroscopy correlated the absolute configuration imparted by (*R*)-(-)-**10** to synthetic (+)-**1** with the data reported for naturally-occurring collybolide, showing the materials were identical in all respects.

Access to (+)-**1** allowed its independent analysis in a battery of human *kappa*-opioid receptor (hKOR) experiments. Radioligand assays showed that collybolide displaced KOR-selective agonist [ $^3\text{H}$ ]-U69593 only above 1  $\mu\text{M}$  and with low selectivity among *mu*- and *delta*-opioid receptors (see SI),<sup>29</sup> whereas collybolide had previously been reported to have a  $K_i$  of 0.7 nM in the same assay with high selectivity over *mu*- and *delta*.<sup>2</sup> To our surprise, (+)-**1** demonstrated no increase in [ $^{35}\text{S}$ ]GTP $\gamma$ S binding over basal activity (Figure 3c), in contrast to the low nanomolar potency previously described.<sup>2</sup> To further investigate the pharmacological properties of (+)-**1**, we also tested whether agonism could be revealed in other assays; however, there was no evidence that (+)-**1** activated hKOR in assays measuring inhibition of adenylyl cyclase or the recruitment of  $\beta$ -arrestin2. Moreover, to assure that we were not missing low efficacy partial agonism, we tested whether (+)-**1** could antagonize  $\beta$ -arrestin2 recruitment induced by U69,593; however, again no efficacy for this system was observed. The lack of efficacy associated with synthetic (+)-**1** might have been caused by synthesis of the incorrect unnatural enantiomer. Although the optical rotation and CD spectra of *synth*-**1** agreed with isolated *nat*-**1**, values in either sample might be overridden by an impurity with large circular birefringence of the opposite rotation as **1**. Existing X-ray crystal structures of **1** and *9-epi-1* reported inconclusive Flack parameters<sup>8</sup> that

prevent confidence in the assignment.<sup>9</sup> In contrast, the structure parameters of (+)-**9** (X-ray) and others (see SI) allowed conclusive assignment of absolute stereochemistry in the series from (*R*)-(-)-**10**.

Given the brevity of our synthesis and the unexpected inactivity of *synth*-(+)-**1**, we decided to synthesize the opposite enantiomer, (-)-**1**. Normally a major undertaking, the entire synthesis from (*S*)-**10**•HOTf and **11** was performed in only 12 days. The Gantt chart (Figure 3b) depicts the ease with which the route attains the 443 molecular bits (mcbits) of **1** from low complexity building blocks **11** and **12** (95 and 171 mcbits, respectively). Access to *ent*-(+)-**1**, the enantiomer of the absolute configuration assigned to *nat*-**1**, allowed parallel assays of (+)-**1** and (-)-**1** to identify which antipode might be responsible for KOR agonism. To our dismay, neither enantiomer of **1** or *7-epi-1* indicated KOR agonism as evident through [ $^{35}\text{S}$ ]GTP $\gamma$ S functional assay or inhibition of cAMP accumulation.<sup>30</sup> These data significantly affect ongoing research and expectations from multiple laboratories involved in opioid research related to the collybolides.<sup>5, 31</sup>

**Conclusion** Whereas biological activity has been wrongly ascribed to natural products when assay readouts are uncertain or impurities are present,<sup>32</sup> the conflict described here represents an unusual case in which multiple, well-established biochemical assays are incorrectly assigned. The explanation we favor for this mischaracterization is error in the assays themselves or interpretation of the data;<sup>33</sup> the original dose-response data<sup>2</sup> did not fit a standard sigmoidal curve, both for **1** and the positive control, salvinorin A. Other, albeit unlikely, explanations might include the presence of an unidentified impurity in isolates (ergosterol partly co-eluted with collybolide),<sup>1a</sup> degradation of collybolide during storage to an active congener that

was not removed by purification, or degradation of collybolide to an active congener during sample preparation or under the assay conditions. Related to these two latter possibilities is the recent discovery that derivatives of columbin, another furano- $\delta$ -lactone terpenoid, agonize KOR even though columbin itself does not.<sup>34</sup> If degradation products of collybolide are KOR agonists, our ability to quickly reach collybolide chemical space (Figure 3b) positions us well to access, modify and improve these congeners. The simplest explanation, however, is an assay error leading to incorrect interpretations. Furthermore, the observed *in vivo* activity of collybolide in mouse studies raises the possibility that **1** may be antipruritic, but its target has been incorrectly assigned.<sup>35</sup> For the time being, excitement over identification of a new non-nitrogenous, KOR-selective agonist with the same clinical potential as Sala seems to have been misplaced. The independent syntheses and assays reported here have excluded collybolide as a KOR agonist and have begun a search for the true potential of this unusual fungal metabolite.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental procedures and spectra are available (pdf).

## AUTHOR INFORMATION

### Corresponding Author

\*rshenvi@scripps.edu

### Present Addresses

†Chemistry Department, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139

## ACKNOWLEDGMENT

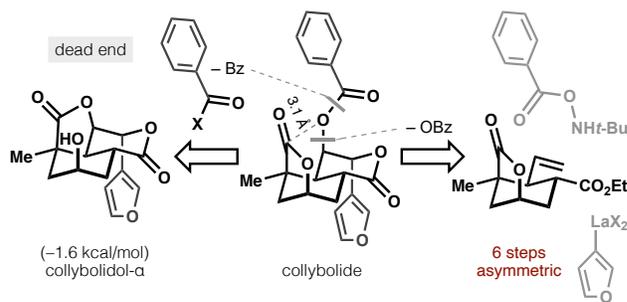
Radioligand  $K_i$  determinations at opioid receptors and confirmation of functional assay results were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2018-00023-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA. We are grateful to Professor Roth and Professor Xi-Ping Huang for expediting these assays. Arnold Rheingold, Milan Gembicky, Jack Bailey and Erika Samolova are acknowledged for X-ray crystallographic analysis. We thank Dr. Marcus Jaegar and the Kelly lab for use of their circular spectropolarimeter. We thank Dr. L. Pasternack and Dr. D.-H. Huang for NMR assistance. We gratefully acknowledge Dr. Thomas Munro for insightful comments regarding a possible connection between antipruritic activity and  $Ca^{2+}$  channel inhibitors. The Scripps Automated Synthesis Facility at Scripps performed separations and analysis of key intermediates. Support was provided by the National Institutes of Health (R35 GM122606, TL1TR002551, DA031927 and DA048490) and the Skaggs Graduate School (fellowship to S.M.F.). We thank the authors of Ref. 2 for transparency and extensive correspondence.

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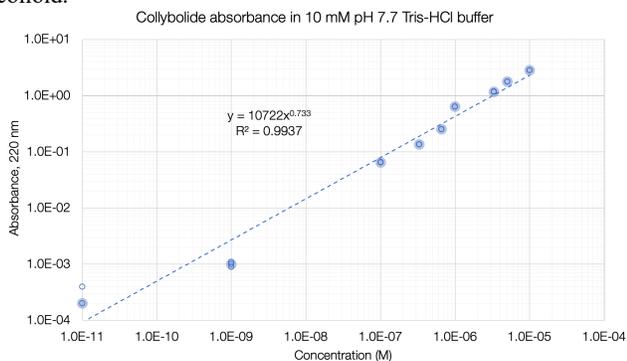
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33. We have corresponded with the authors of Ref. 2, who continue to interrogate the assays used for the characterization of collybolide. Originally, their sample of collybolide was described as sticky and led them to treat all glass and plastic with surface modifying agents to prevent adhesion. We did not observe adhesion of **1** to surfaces or precipitation of **1** in DMSO or in

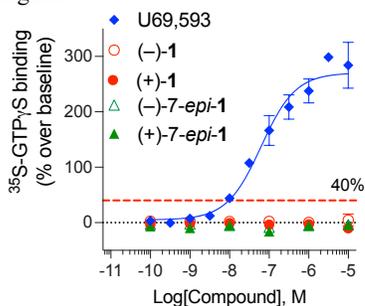
serial dilutions (pH 7.7 Tris•HCl buffer) at assay concentrations. When DMSO/buffer solutions of **1** sat in plastic eppendorf tubes for >12 h, **1** was recovered in high purity (<sup>1</sup>H NMR) with no apparent material loss. The ClogP (ChemOffice) is calculated to be 1.99, which correlates well with its extrapolated log K<sub>w</sub> of 3.8. See Supporting Information for this measurement of lipophilicity using reverse-phase HPLC. Further correspondence with the authors of Ref. 2 identified the turbidity of aqueous solutions of collybolide as another explanation for why we observed no activity. However, between the assay concentrations of 1x10<sup>-11</sup> and 1x10<sup>-5</sup> M ([collybolide] in 10 mM pH 7.7 Tris•HCl buffer) we observed no turbidity and instead saw a linear correlation between absorbance and concentration, indicating that no turbidity, heterogeneity or aggregation occurs at these concentrations (see log-log plot below). Only at 1x10<sup>-3</sup> M [collybolide] in 10% DMSO/ pH 7.7 Tris•HCl buffer did we observe an apparent colloid.



Synthesis of both enantiomers and biological assay



One major difference between the assays reported here and those of Ref. 2 involves the maximum %stimulation over basal in [<sup>35</sup>S]GTPγS binding assays. Whereas our data reached 300%, the same assay in Ref. 2 only reached 30-40% (indicated as a dashed line below). Low stimulation may cause background noise to be interpreted as signal.



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## Graphical Abstract