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Semisynthesis and Kappa-Opioid Receptor Activity of Derivatives of Columbin, a Furanolactone Diterpene

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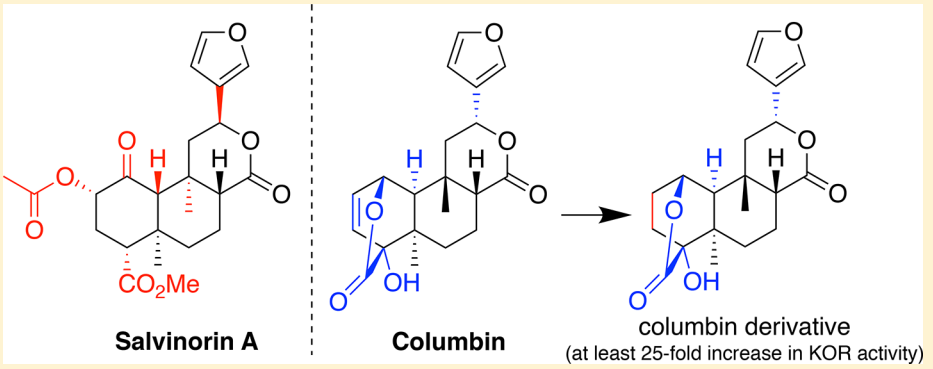
Supporting Information



ABSTRACT: Columbin (**1**) is a furanolactone diterpene isolated from the roots of *Jateorhiza* and *Tinospora* species. These species generally grow in Asia and Africa and have been used in folk medicine for their apparent analgesic and antipyretic activities. Columbin (**1**) is of particular interest due to its structural similarity to the known kappa-opioid receptor (KOR) agonist salvinorin A. Given that the KOR is of interest in the study of many serious diseases, such as anxiety, depression, and drug addiction, obtaining natural or semisynthetic molecules with KOR activity recently has gained much interest. For this reason, in the present study, derivatives of **1** were designed and synthesized using known structure−activity relationships of salvinorin A at KORs. The structures of the columbin analogues prepared were elucidated by NMR spectroscopy and mass spectroscopy, and their KOR activity was investigated in vitro by inhibition of forskolin-induced cAMP accumulation. Slight improvements in KOR activity were observed in columbin derivatives over their parent compound. However, despite the structural similarities to salvinorin A, neither columbin (**1**) nor its derivatives were potent KOR ligands. This work represents not only the first evaluation of columbin (**1**) at the KOR but also one of the first works to explore synthetic strategies that are tolerated on the columbin core.

Columbin (**1**) is a cytotoxic *cis*-clerodane diterpene isolated from the roots of *Jateorhiza columba*, *Tinospora capillipes*, and *Tinospora sagittata*.[1−3] The genera *Tinospora* and *Jateorhiza* belong to the Menispermaceae family and are found most widely in Asia and Africa. Characteristic components of these genera are neoclerodane diterpenoids,[4,5] representative of an important chemical class with a variety of demonstrated biological activities including cytotoxic, analgesic, and antimicrobial activities.[6−9] The roots of *J. columba*, *T. capillipes*, and *T. sagittata* containing columbin (**1**) have been used in folk medicine as analgesic and antipyretic agents.[2] *Tinospora sagittata*, which is in the *Pharmacopeia of the People's Republic of China*, demonstrates anti-inflammatory, analgesic, antibacterial, antiulcer, antitumor, and antistress effects,[10−13] and its roots have been used in traditional medicine to ease sore throats, stop diarrhea, and treat upper respiratory tract infections.[14] Pharmacological studies have shown that ethanol extracts of the roots of *T. sagittata* and *T. capillipes* have antibiotic, anti-inflammatory, and antiviral activities.[15] Columbin (**1**) itself has been demonstrated to possess cytotoxic and anti-inflammatory activities.[2,16]

Salvinorin A, a *trans*-neoclerodane diterpene, is the main component of *Salvia divinorum* and is a highly selective κ-opioid receptor (KOR) agonist. KORs are important targets for several diseases such as anxiety, depression, and drug addiction.[17] Although much work has been done to develop KOR ligands for the treatment of these disease targets, few compounds have progressed to human clinical trials to date.[18] Through these studies, several structure−activity relationships (SARs) of salvinorin A at the KORs have been elucidated.[19−24]

Structural comparisons of columbin (**1**) and salvinorin A reveal high similarity between the B and C rings, with differences in the conformations of the C-10 proton, the C-19 methyl, and the C-12 furan ring (Figure 1). The largest structural distinctions can be seen by comparing the A rings. While salvinorin A has a carbonyl at C-1, an acetate at C-2, and

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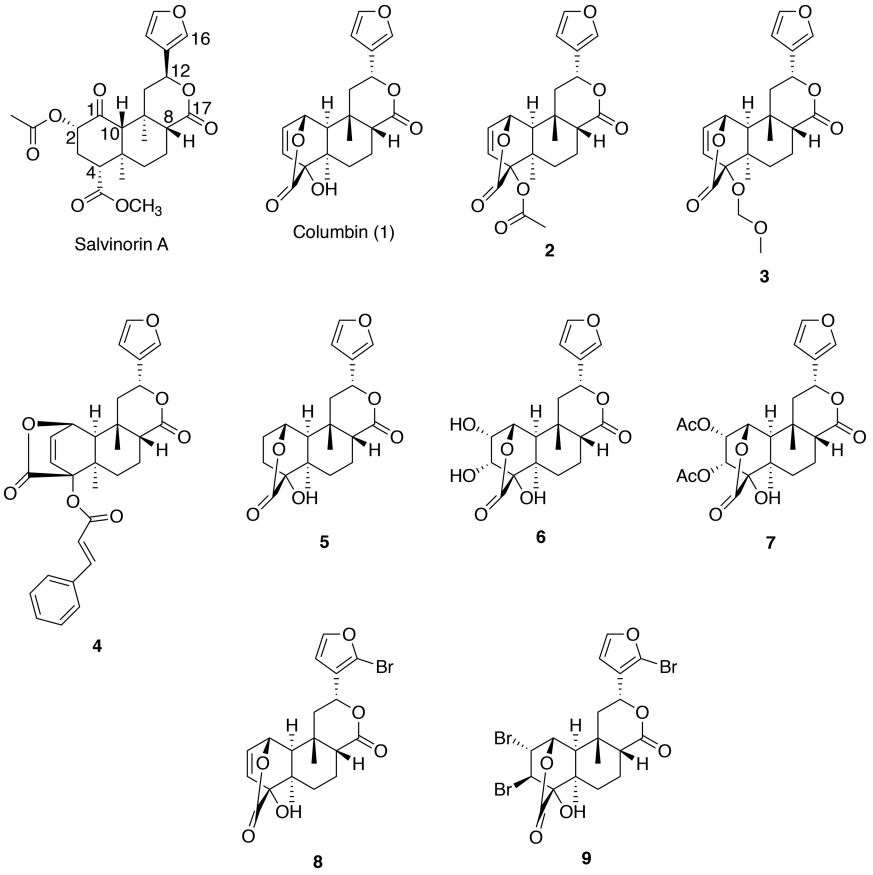

Figure 1. Structures of compounds **1**–**9** and salvinorin A.

a carbomethoxy group at C-4, columbin (**1**) has a lactone ring between C-1 and C-4, resulting in a more strained A ring. Compound **1** also has a hydroxy group at the quaternary C-4 and a double bond between C-2 and C-3 on ring A, unlike salvinorin A.

Although there are some isolation and bioactivity studies on columbin (**1**) in the literature, few synthetic studies on columbin (**1**) have been published to date.[25,26] The high structural similarity of these two diterpenoid furanolactones, columbin (**1**) and salvinorin A, led us to investigate whether **1** possesses the same KOR activity as salvinorin A. Additionally, it was sought to determine if SAR known to affect the KOR activity of salvinorin A could be applied to the structurally similar **1**. Therefore, in the present study, several derivatives of columbin (**1**) were synthesized and evaluated for KOR activity.

RESULTS AND DISCUSSION

Synthesis of Columbin (1) Analogues. Considering the functional groups of columbin (**1**) and their similarity to those of salvinorin A as well as known transformations and SAR of salvinorin A, several analogues of **1** were generated (Figure 1). The C-2 acetoxy group of salvinorin A plays a significant role in the activity at KORs.[22] While salvinorin A is very potent at KORs, cleavage of the C-2 acetate to form salvinorin B significantly reduces potency.[27] As this ester cleavage is rapid in vivo, many modifications on C-2 of salvinorin A have been done to date to improve both activity and metabolic stability.[28−31] While the replacement of the C-2 acetoxy group with an ethoxymethyl ether gave a more potent neoclerodane at KORs,[30] replacement of the C-2 acetoxy group with a benzoate group yielded herkinorin, a very potent diterpenoid at μ-opioid receptors.[29] For this purpose, the double bond of **1** was dihydroxylated and the hydroxy groups at C-2 and C-3 were acetylated in order to obtain a clerodane with an acetoxy group at C-2 similar to salvinorin A. The role of the 4-position carbomethoxy group of salvinorin A has also been investigated, and it has been demonstrated that reduction of this carbomethoxy group to the primary alcohol reduces affinity for KORs.[32,33] Columbin (**1**) has a hydroxy group at C-4 that could be utilized to introduce ester and alkoxy functionalities to determine if these changes on the molecule would affect KOR activity. Compound **1** is unsaturated between C-2 and C-3, unlike salvinorin A, so reduction of this double bond was envisioned to make a molecule more like salvinorin A. Bromination of the furan ring of salvinorin A results in a KOR agonist that successfully attenuates the drug-induced reinstatement of cocaine in an animal model of drug abuse without causing sedation.[23] Thus, brominated analogues of columbin (**1**) were synthesized to see if a similar trend in SAR was observed.

Columbin (**1**) in dichloromethane was treated with acetic anhydride (Ac_2O) in the presence of 4-dimethylaminopyridine (DMAP) at room temparature for 3 days to give 4-acetoxycolumbin (**2**) in good yield (73%) (Scheme 1). Columbin (**1**) in dichloromethane was reacted with diisopropylethylamine (DIPEA) and chloromethyl methyl ether (MOM-Cl) in the presence of DMAP at room temparature for 2 days to give 4-methoxymethylcolumbin (**3**) in good yield (73%). To cinnamic acid, *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and DMAP in dichloromethane was added columbin (**1**), and the reaction

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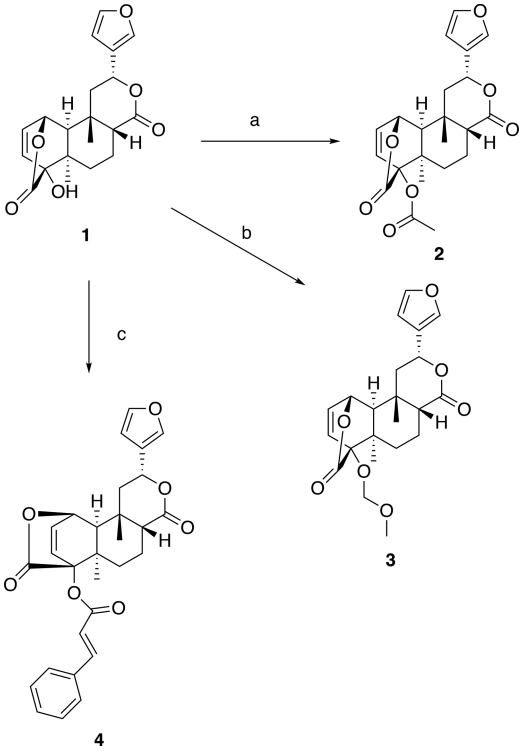
Scheme 1. Modifications on the Hydroxy Group of Columbin (1)[a]



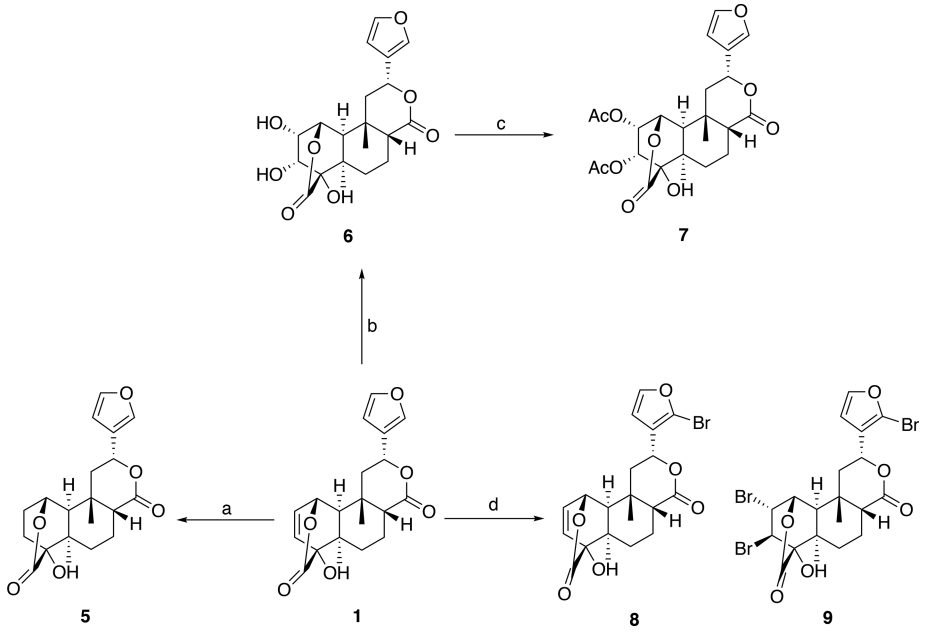
[a]Reagents and conditions: (a) Ac_2O, DMAP, CH_2Cl_2, rt, 3 d; (b) DIPEA, DMAP, MOM-Cl, CH_2Cl_2, rt, 2 d; (c) cinnamic acid, EDC, DMAP, CH_2Cl_2, rt, 6 d.

mixture was stirred at room temparature for 6 days. This reaction yielded columbin-4-cinnamate (**4**) (yield 16%) (Scheme 1). While the alkylation of the C-4 hydroxy group with small acyclic reagents, such as Ac_2O and MOM-Cl, proceeded easily, the alkylation with large and cyclic alkyl groups, such as 1,1′-thiocarbonyldiimidazole (Im_2CS) and cinnamic acid, either gave a low yield or did not form any product. It can be deduced that steric hindrance on ring A of columbin (**1**) is responsible for this low yield and unsuccessful alkylation reactions.

Columbin (**1**) in methanol was stirred under hydrogen in the presence of 10% Pd/C at room temparature for 6 h. Hydrogenation of the double bond of **1** was straightforward and yielded 2,3-dihydrocolumbin (**5**) (yield 77%) (Scheme 2). Columbin (**1**) in acetone (degassed) was treated with a 4% solution of osmium tetroxide (OsO_4) in water and a 1 M solution of 4-methylmorpholine *N*-oxide (NMO) in deionized water. This dihydroxylation reaction also succeeded and gave 2*α*,3*α*-dihydroxycolumbin (**6**) in 47% yield. Stereochemistry of the hydroxy groups was determined using NMR coupling constants, with the $^3J_{H1,H2}$ of 1.1 Hz indicating a *trans*-correlation and the $^3J_{H2,H3}$ of 8.3 Hz indicating a *syn*-interaction, both of which designate the stereochemistry of the hydroxy groups at C-2 and C-3 in the alpha position.

Treatment of **6** in dichloromethane with triethylamine (TEA) and acetyl chloride in the presence of DMAP at room temperature overnight yielded 2*α*,3*α*-diacetoxycolumbin (**7**) (46% yield) (Scheme 2). Derivative **6** is trihydroxylated, and a possible result of the acetylation reaction is a triacetylated analogue, but only two hydroxy groups were acetylated under these conditions. The hydroxy group bound to a quaternary carbon atom was not acetylated, presumably due to steric hindrance. If this hydroxy group had been acetylated, two downfield signals at 5.55 ppm (1H, d, J = 8.3 Hz) and 5.14 ppm (1H, dd, J = 8.3, 1.3 Hz) would not be observed. The COSY correlation via three bonds of the signals at 5.55 and 5.14 ppm also showed that the hydroxy groups bonded to C-2 and C-3 were acetylated.

Scheme 2. Modifications on the Double Bond of Columbin (1)[a]



[a]Reagents and conditions: (a) 10% Pd/C, $H_{2(g)}$, MeOH, rt, 6 h; (b) OsO4, NMO, acetone, rt, 24 h; (c) NEt_3, DMAP, acetyl chloride, CH_2Cl_2, rt, 1 d; (d) NBS, Br_2, CH_2Cl_2, rt, 45 min.

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Columbin (1) in dichloromethane was treated with *N*-bromosuccinimide (NBS) in the presence of bromine at room temparature for 45 min. Bromination of **1** gave two products, 16-bromocolumbin (**8**) (yield, 45%) and 2α,3β,16-tribromocolumbin (**9**) (yield, 29%) (Scheme 2). Similar to determining the stereochemistry of the hydroxy groups in **6**, the stereochemistry of the bromo groups was determined using NMR coupling constants. According to the ^{1}H NMR spectrum of compound **9**, $^3J_{H1,H2}$ and $^3J_{H2,H3}$ are 0 and 3.9 Hz, respectively, indicating that the stereochemistry of H-2 and H-3 should be beta and alpha, respectively. The bromination of furan rings of similar natural compounds such as salvinorin A gave a range of yields in the previous reactions.[27,34] Bromination of columbin (1) gave two products, which differed from a similar reaction with salvinorin A, as columbin (1) has a double bond on ring A in addition to a furan ring.

Some reactions attempted on columbin (1) did not succeed (Scheme S1, Supporting Information). Natural product semisynthesis requires the operation of selective chemical transformations in the presence of a complex chemical functionality provided by Nature, and it is often challenging to predict the success of a reaction without prior experimental work. Columbin (1) in dichloromethane was treated with *meta*-chloroperoxybenzoic acid (mCPBA) in varying amounts in the presence of sodium bicarbonate for differing reaction times at room temperature. However, epoxidation of the A ring could not be accomplished, and the starting material decomposed each time. The A ring of columbin (1) is already strained because of the bicyclic lactone system, and thus the introduction of an epoxide to carbons 2 and 3 would make the product more strained. This added strain and inherent instability were likely why the reactions were unsuccessful.

Columbin (1) in dichloromethane was treated with Im_2CS in the presence of DMAP at room temperature overnight, but the starting material did not react and the coupling reaction failed. The introduction of Im_2CS to the hydroxy group failed presumably because ring A of columbin (1) is sterically hindered. Most of the previous alkylation reactions of the hydroxy group of columbin (1) with large alkyl groups also failed because of this steric hindrance. Columbin (1) in THF was treated with sodium hydride (NaH, 60% dispersion in mineral oil) at 0 °C and allyl bromide at room temperature, respectively. The reaction mixture was stirred at room temperature for 2 days, but the starting material remained and the reaction failed.

In the literature, there are several publications related to the synthesis and biological evaluation of C-12 derivatives of salvinorin A. The furan ring bound to C-12 was converted by ruthenium-catalyzed oxidation to a carboxylic acid, and then heterocycles were introduced. In this way, various C-12 derivatives of salvinorin A were obtained.[35,36] The same approach was attempted on columbin (1). Columbin (1) in solvent mixture (CCl_4/CH_3CN/H_2O) was treated with sodium periodate ($NaIO_4$) in the presence of ruthenium(III) chloride hydrate ($RuCl_3$) catalyst at room temparature for 3.5 h. The reaction went to completion, but the product decomposed during acid−base workup each time. This decomposition is presumably due to the fact that columbin (1) has two lactone rings that more than likely did not tolerate the acid workup. Previous reports have suggested ozonolysis to be more effective.[25]

KOR Activity of Columbin (1) Analogues. Currently, there is no published work related to the KOR activity of columbin (1) or any studies of its analogues. To begin the present study, columbin (1) was evaluated initially for KOR activity using an in vitro functional assay to assess the inhibition of forskolin-induced cAMP accumulation in CHO cells stably expressing the hKOR, as described previously.[23] Columbin (1) displayed no activity in this assay at the highest dose tested, 50 μM. As indicated above, columbin (1) and salvinorin A are very structurally similar (Figure 1); thus columbin's lack of activity at KOR was somewhat surprising, as salvinorin A is a full KOR agonist with a potency of 0.042 ± 0.005 nM. Therefore, we sought to determine if the SAR known from previous salvinorin A studies could be applied to the columbin (1) scaffold to identify an active KOR analogue from an inactive parent compound. Through this strategy, columbin analogues with KOR activity were identified (Table 1). Compounds with

Table 1. In Vitro KOR Activity Results of Columbin Analogues[a]

compound name	potency EC_{50} ± SEM[b,c] (μM)	fold increase over columbin (1)
columbin (1)	>50[d]	NA[e]
4-acetoxycolumbin (2)	8.0 ± 2.0	≥6.3
4-methoxymethylcolumbin (3)	12.0 ± 4.0	≥4.2
columbin-4-cinnamate (4)	6.0 ± 4.0	≥8.3
2,3-dihydrocolumbin (5)	2.0 ± 0.8	≥25.0
2α,3α-dihydroxycolumbin (6)	16.0 ± 6.0	≥3.1
2α,3α-diacetoxycolumbin (7)	>50[d]	NA[e]
16-bromocolumbin (8)	>50[d]	NA[e]
2α,3β,16-tribromocolumbin (9)	>50[d]	NA[e]

[a]Evaluation of forskolin-induced cAMP accumulation. In this assay salvinorin A has an EC_{50} value of 0.042 ± 0.005 nM and an E_{max} = 100%. [b]Means ± standard error of the mean; $n \geq 2$ individual experiments run in triplicate. [c]KOR E_{max} = 100% unless otherwise noted. [d]KOR E_{max} = 0% up to 50 μM. [e]Not applicable.

substitutions to the A ring seemed to be the most promising compounds, with the analogues 2,3-dihydrocolumbin (**5**), columbin-4-cinnamate (**4**), 4-acetoxycolumbin (**2**), 4-methoxymethylcolumbin (**3**), and 2α,3α-dihydroxycolumbin (**6**) showing at least 25-, 8.3-, 6.3-, 4.2-, and 3.1-fold higher KOR activity than columbin (**1**), respectively. This result is not surprising, as the biggest structural differences between columbin (**1**) and salvinorin A are in the A ring, and attempts to make it more salvinorin A-like via A-ring modifications were successful in achieiving KOR activity. However, 2α,3α-diacetoxycolumbin (**7**) had the most similar substitution pattern to the A ring of salvinorin A, yet it was the only A-ring-substituted analogue synthesized that did not demonstrate KOR activity. Additionally, substitution to the furan ring of columbin (**1**) did not appear to be tolerated at the KOR, with 16-bromocolumbin (**8**) and 2α,3β,16-tribromocolumbin (**9**) having no activity at 50 μM. Although the most potent analogue (**5**) did not approach the potency of salvinorin A, with a potency value of 2.0 ± 0.8 μM, the increased activity from its parent columbin (**1**) is a promising result, suggesting that this strategy may lead to the development of other clerodane-based KOR agonists.

EXPERIMENTAL METHODS

General Experimental Methods. Columbin (1) was purchased from BOC Sciences (Shirley, NY, USA). All other chemical reagents were purchased from commercial suppliers and used without further purification. All solvents were obtained from a solvent purification

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system in which solvent was passed through two columns of activated alumina under argon. Reactions performed in standard glassware were performed under an atmosphere of argon using glassware dried overnight in an oven at 120 °C and cooled under a stream of argon. Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm Analtech GHLF silica gel plates and visualized using a UV lamp (254 nm) and vanillin solution. Flash column chromatography was performed on silica gel (4−63 mm) from Sorbent Technologies. ^{1}H and ^{13}C NMR spectra were recorded on a 500 MHz Bruker AVIII NMR spectrometer equipped with a cryogenically cooled carbon observe probe or 400 MHz Bruker AVIIIHD NMR spectrometer with a broadband X-channel detect gradient probe using tetramethylsilane as an internal standard. Chemical shifts (δ) are reported in ppm, and coupling constants (J) are reported in Hz. High-resolution mass spectra (HRMS) were obtained on an LCT Premier (Micromass Ltd., Manchester, UK) time-of-flight mass spectrometer with an electrospray ion source in either the positive or negative mode. Melting points were measured with a Thomas Capillary melting point apparatus and are uncorrected. HPLC was carried out on an Agilent 1100 series HPLC system with diode array detection at 209 nm on an Agilent Eclipse XDB-C18 column (250 × 10 mm, 5 mm). Compounds were identified as ≥95% pure by HPLC before all in vitro and in vivo analyses unless otherwise noted. UPLC/HRMS analyses were carried out on a Waters Acquity UPLC with a photodiode array UV detector and an LCT Premiere TOF mass spectrometer. Purification was carried out by mass-directed fractionation on an Agilent 1200 instrument with a photodiode array detector, an Agilent 6120 quadrupole mass spectrometer, and an HTPAL LEAP autosampler. The data from UPLC/HRMS analyses were used to choose narrow preparative gradients and triggering thresholds for both MS and UV signals.

Synthesis and Characterization. *4-Acetoxycolumbin (**2**).* To a 5 mL round-bottomed dry flask purged with argon were added columbin (**1**) (36 mg, 0.1 mmol, 1 equiv), dry dichloromethane (1 mL), DMAP (catalytic amount), and Ac_2O (15.3 mg, 0.15 mmol, 1.5 equiv), respectively. The reaction mixture was left to stir at room temperature overnight. The reaction mixture was monitored by TLC after 1 day, and there was still was some starting material in the reaction mixture, so extra Ac_2O (30.6 mg, 0.3 mmol, 3 equiv) was added and the resulting mixture was left to stir at room temperature for 2 days more. The reaction mixture was diluted with 25 mL of dichloromethane and washed with 1 N hydrochloric acid (HCl) (3 × 10 mL), water (10 mL), a saturated aqueous solution of sodium bicarbonate (10 mL), and brine (20 mL), respectively. Then, it was dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was purified via column chromatography (0−15% ethyl acetate/dichloromethane) to yield a product as a white crystalline solid (29.4 mg, 0.073 mmol, 73% yield). The purity of the product was determined according to purity method 1 given below (retention time: 2.64 min, purity: 97.4%): mp 176−181 °C (dec); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48 (1H, m), 7.44 (1H, t, J = 1.7 Hz), 6.55 (1H, dd, J = 8.1, 1.8 Hz), 6.45 (1H, m), 6.44 (1H, m), 5.41 (1H, dd, J = 11.9, 4.3 Hz), 5.14 (1H, m), 2.70 (1H, m), 2.42 (1H, dd, J = 11.2, 2.1 Hz), 2.29 (1H, dd, J = 14.9, 4.4 Hz), 2.24 (3H, s), 2.09 (1H, m), 1.96 (1H, dd, J = 14.9, 11.9 Hz), 1.85 (1H, dd, J = 14.5, 7.7 Hz), 1.62 (1H, m), 1.60 (1H, brs), 1.26 (3H, s), 1.08 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4, 169.5, 168.7, 143.9, 139.6, 134.3, 126.5, 124.6, 108.3, 84.3, 73.3, 70.7, 46.6, 44.5, 41.7, 37.2, 35.1, 28.1, 25.7, 24.5, 21.1, 17.1; HRESIMS m/z 423.1398 $[M + Na]^+$, calcd for $C_{22}H_{24}O_7Na$, 423.1420.

*4-Methoxymethylcolumbin (**3**).* To a 5 mL round-bottomed dry flask purged with argon was added columbin (**1**) (36 mg, 0.1 mmol, 1 equiv), dry dichloromethane (1 mL), DMAP (catalytic amount), and DIPEA (130 mg, 180 μL, 1 mmol, 10 equiv), respectively, and then MOM-Cl (94.54 mg, 100 μL, 1 mmol, 10 equiv) was added dropwise at room temperature. The resulting mixture was stirred at room temperature overnight. The reaction was monitored by TLC, and the starting material was not completely consumed, so 5 equiv more of DIPEA and MOM-Cl were added and the mixture was stirred at room temperature overnight. The reaction mixture was diluted with 25 mL of dichloromethane and washed with 1 N HCl (3 × 10 mL), water (10 mL), a saturated aqueous solution of sodium bicarbonate (10 mL), and brine (20 mL), respectively. Then, it was dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was purified via column chromatography (5−20% ethyl acetate/dichloromethane) to yield a product as a white crystalline solid (29.3 mg 0.073 mmol, 73% yield). The purity of the product was determined according to the purity method 1 given below (retention time: 3.07 min, purity: 98%): mp 163−167 °C (dec); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (1H, m), 7.44 (1H, t, J = 1.6 Hz), 6.83 (1H, dd, J = 8.2, 1.6 Hz), 6.53 (1H, dd, J = 8.2, 5.1 Hz), 6.44 (1H, m), 5.41 (1H, dd, J = 11.9, 4.3 Hz), 5.11 (1H, brd, J = 7.7 Hz), 5.07 (1H, d, J = 5.2 Hz), 4.94 (1H, brd, J = 7.7 Hz), 3.48 (3H, s), 2.64 (1H, m), 2.41 (1H, dd, J = 11.1, 2.1 Hz), 2.26 (1H, dd, J = 14.9, 4.4 Hz), 2.08 (1H, m), 1.92 (2H, m), 1.69 (1H, brs), 1.55 (1H, m), 1.26 (3H, s), 1.07 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.5, 172.7, 143.9, 139.6, 134.1, 130.1, 124.7, 108.4, 95.3, 86.8, 72.9, 70.9, 55.9, 47.5, 44.4, 41.8, 37.9, 35.2, 28.3, 26.1, 24.5, 17.4; HRESIMS m/z 425.1611 $[M + Na]^+$, calcd for $C_{22}H_{26}O_7Na$, 425.1576.

*Columbin-4-cinnamate (**4**).* To a 10 mL dry flask purged with argon were added cinnamic acid (30 mg, 0.2 mmol, 1.4 equiv), EDC (38 mg, 0.2 mmol, 1.4 equiv), DMAP (catalytic amount), and dry dichloromethane (4 mL) at 0 °C, and then columbin (**1**) (50 mg, 0.14 mmol, 1 equiv) was added at 0 °C. The resulting mixture was brought to ambient temperature and left to stir at room temperature overnight. TLC indicated that the starting material was not completely consumed, so cinnamic acid (3 equiv) and EDC (3 equiv) were added at 0 °C, and the resulting mixture was stirred at room temperature for 5 days more. The reaction did not go to completion. The reaction mixture was purified via column chromatography (5−25% ethyl acetate/dichloromethane) to yield a product as a white solid (11.2 mg, 0.023 mmol, 16% yield). Further purification was carried out by the preparative-HPLC method given below. The purity of the product was determined according to purity method 2 given below (retention time: 2.27 min, purity: 96% purity): mp 218−220 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (1H, brd, J = 15.9 Hz), 7.57 (2H, m), 7.50 (1H, d, J = 1.3 Hz), 7.45 (1H, t, J = 1.7 Hz), 7.42 (2H, m), 7.41 (1H, m), 6.64 (1H, dd, J = 8.1; 1.8 Hz), 6.57 (1H, d, J = 15.9 Hz), 6.49 (1H, dd, J = 8.1, 5.2 Hz), 6.45 (1H, m), 5.43 (1H, dd, J = 12.0, 4.3 Hz), 5.17 (1H, dd, J = 5.2; 1.8 Hz), 2.75 (1H, m), 2.45 (1H, dd, J = 11.2; 2.0 Hz), 2.31 (1H, dd, J = 14.8, 4.4 Hz), 2.13 (1H, m), 1.97 (2H, m), 1.74 (1H, brs), 1.69 (1H, m), 1.29 (3H, s), 1.16 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4, 168.7, 165.2, 146.6, 143.9, 139.6, 134.5, 134.0, 130.7, 128.9 × 2, 128.3 × 2, 126.5, 124.6, 117.0, 108.3, 84.4, 73.3, 70.7, 46.7, 44.5, 41.8, 37.5, 35.1, 28.2, 26.0, 24.7, 17.1; HRESIMS m/z 530.2156 $[M + ACN + H]^+$, calcd for $C_{29}H_{28}O_7C_2H_3NH$, 530.2173.

*2,3-Dihydrocolumbin (**5**).* To a 10 mL round-bottomed dry flask purged with argon were added columbin (**1**) (72 mg, 0.2 mmol, 1 equiv), 10% Pd/C (catalytic amount), and methanol (5 mL). The resulting mixture was stirred at room temperature under argon for 5 min. The flask was then fully purged with H_2 and was stirred rapidly at room temperature for 6 h. The mixture was subsequently passed through a plug of Celite, dried over anhydrous sodium sulfate, decanted, and concentrated. The residue was purified via column chromatography (15% ethyl acetate/dichloromethane) to yield a product as a white crystalline solid (55.3 mg, 0.15 mmol, 77% yield). The purity of this product was determined according to the purity method 2 given below (retention time: 1.50 min, 100% purity): mp 215−219 °C (dec); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (1H, m), 7.44 (1H, t, J = 1.7 Hz), 6.43 (1H, dd, J = 1.9, 0.9 Hz), 5.41 (1H, dd, J = 11.9, 4.4 Hz), 4.68 (1H, brd, J = 4.0 Hz), 2.69 (1H, m), 2.47 (1H, m), 2.37 (1H, dd, J = 11.2, 2.2 Hz), 2.24 (1H, m), 2.17 (1H, m), 2.05 (1H, m,), 1.89 (1H, dd, J = 14.8, 12.0 Hz), 1.84 (1H, m), 1.84 (1H, brs), 1.70 (1H, m), 1.66 (1H, m), 1.44 (1H, m), 1.26 (3H, s), 1.24 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.8, 173.4, 143.9, 139.5, 124.7, 108.3, 75.3, 75.2, 70.7, 47.8, 44.1, 41.6, 39.6, 34.4, 27.7, 27.0, 25.5, 25.2, 22.0, 16.6; HRESIMS m/z 402.1849 $[M + ACN + H]^+$, calcd for $C_{20}H_{24}O_6C_2H_3NH$, 402.1911.

*2α,3α-Dihydroxycolumbin (**6**).* To a 5 mL round-bottomed dry flask purged with argon were added columbin (**1**) (36 mg, 0.1 mmol, 1

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equiv) and acetone (degassed) (1 mL), and the mixture was stirred under argon at 0 °C. A 4% solution of OsO_4 in water (36 μL) and a 1 M solution of NMO in deionized water (158 μL) were added. After the mixture was stirred at room temperature overnight, a saturated aqueous solution of sodium sulfite (2 mL) was added, and the resulting mixture was stirred at room temperature for 1 h. The reaction mixture was extracted with ethyl acetate (20 mL × 4), and the pooled organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated. The residue was purified via column chromatography (50−100% ethyl acetate/dichloromethane) to yield a product as a white powder (18.6 mg, 0.047 mmol, 47% yield). The purity of the product was determined according to the purity method 1 given below (retention time: 1.45 min, 95% purity): mp 231−235 °C (dec); ^{1}H NMR (500 MHz, CD_3COCD_3) δ 7.47 (1H, m), 7.43 (1H, t, *J* = 1.7 Hz), 6.47 (1H, dd, *J* = 1.9, 0.9 Hz), 5.59 (1H, dd, *J* = 12.3, 4.1 Hz), 4.54 (1H, brs), 4.25 (1H, d, *J* = 8.3 Hz), 4.10 (1H, dd, *J* = 8.3, 1.1 Hz), 2.49 (1H, m), 2.41 (1H, dd, *J* = 11.3, 2.0 Hz), 2.34 (1H, dd, *J* = 14.8, 4.2 Hz), 1.89 (1H, brs), 1.87 (1H, m), 1.84 (1H, m), 1.50 (1H, dd, *J* = 14.5, 8.6 Hz), 1.34 (1H, m), 1.10 (3H, s), 1.00 (3H, s); ^{13}C NMR (125 MHz, CD_3COCD_3) δ 173.6, 173.1, 143.6, 139.9, 126.0, 108.8, 80.1, 79.5, 70.6, 67.9, 67.4, 43.7, 42.8, 41.0, 37.8, 34.3, 26.7, 26.0, 21.8, 16.1; HRESIMS *m*/*z* 415.1110 $[M + Na]^+$, calcd for $C_{20}H_{24}O_8Na$, 415.1369.

*2α,3α-Diacetoxycolumbin (**7**).* To a 5 mL round-bottomed dry flask purged with argon were added 2α,3α-dihydroxycolumbin (**6**) (20 mg, 0.05 mmol, 1 equiv), dry dichloromethane (1 mL), DMAP (catalytic amount), and TEA (76 mg, 100 μL, 0.75 mmol, 15 equiv), respectively, and then acetyl chloride (59 mg, 54 μL, 0.75 mmol, 15 equiv) was added dropwise at 0 °C. The resulting mixture was brought to ambient temperature and left to stir at room temperature overnight. The reaction was quenched with 1 mL of water and diluted with chloroform. The organic phase was separated and washed with a saturated aqueous solution of ammonium chloride (20 mL × 3), water, and brine, respectively. Then, it was dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was purified via column chromatography (25% ethyl acetate/dichloromethane) to yield a product as a white powder (11 mg, 0.023 mmol, 46% yield). Further purification was carried out by the preparative-HPLC method given below. The purity of the product was determined according to the purity method 2 given below (retention time: 1.67 min, purity: 100%): mp 137−139 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (1H, m), 7.44 (1H, t, *J* = 1.8 Hz), 6.43 (1H, brd, *J* = 1.8 Hz), 5.55 (1H, d, *J* = 8.3 Hz), 5.45 (1H, dd, *J* = 11.6, 4.5 Hz), 5.14 (1H, dd, *J* = 8.3, 1.3 Hz,), 4.68 (1H, brs), 3.31 (1H, s, −OH proton), 2.68 (1H, m), 2.42 (1H, dd, *J* = 11.2, 2.2 Hz), 2.19 (1H, dd, *J* = 14.9, 4.6 Hz), 2.11 (3H, s), 2.09 (3H, s), 2.03 (1H, m), 1.92 (1H, dd, *J* = 14.9, 11.6 Hz), 1.85 (1H, brs), 1.75 (1H, dd, *J* = 14.6, 8.2 Hz), 1.46 (1H, m), 1.27 (3H, s), 1.26 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.09, 173.05, 169.77, 169.75, 143.9, 139.7, 124.5, 108.3, 77.4, 76.6, 70.6, 68.5, 68.4, 44.4, 43.8, 41.5, 38.0, 34.4, 27.7, 25.5, 22.9, 20.5, 20.3, 16.2; HRESIMS *m*/*z* 518.1956 $[M + ACN + H]^+$, calcd for $C_{24}H_{28}O_{10}C_2H_3NH$, 518.2020.

*16-Bromocolumbin (**8**) and 2α,3β,16-tribromocolumbin (**9**).* To a 25 mL round-bottomed dry flask purged with argon were added columbin (**1**) (72 mg, 0.2 mmol, 1 equiv), NBS (50 mg, 0.28 mmol, 1.4 equiv), dry dichloromethane (10 mL), and bromine (2 drops). The resulting mixture was stirred at room temperature for 45 min. The reaction mixture was purified via column chromatography (0−25% ethyl acetate/dichloromethane) to yield products as white powder (38.7 mg, 0.09 mmol, 45% yield and 34.4 mg, 0.06 mmol, 29% yield, respectively). Further purification was carried out by the preparative-HPLC method given below. The purities of the products were determined according to the purity method 2 given below (retention time: 1.73 min, purity: 100% and retention time: 2.12 min, purity: 100%, respectively).

*16-Bromocolumbin (**8**):* mp 110−112 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (1H, d, 2.1 Hz), 6.48 (1H, m), 6.47 (1H, m), 6.39 (1H, dd, *J* = 7.9, 1.8 Hz), 5.30 (1H, dd, *J* = 12.4, 4.4 Hz), 5.17 (1H, d, *J* = 5.2 Hz), 3.47 (1H, s, −OH proton), 2.60 (1H, m), 2.44 (1H, dd, *J* = 11.1, 2.4 Hz), 2.14 (1H, dd, *J* = 15.1, 4.4 Hz), 2.09 (1H, m), 1.90 (1H, dd, 15.0, 12.4 Hz), 1.77 (1H, brs), 1.77 (1H, m), 1.46 (1H, m), 1.25 (3H, s), 1.09 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.5, 173.3, 144.9, 136.8, 128.7, 122.7, 120.6, 110.6, 80.3, 74.1, 70.6, 47.2, 44.5, 41.4, 37.1, 35.2, 28.2, 25.6, 24.5, 17.4; HRESIMS *m*/*z* 481.0492 $[M + HCOO]^-$, calcd for $C_{20}H_{21}BrO_6HCOO$, 481.0504.

*2α,3β,16-Tribromocolumbin (**9**):* mp 154−155 °C (dec); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (1H, d, *J* = 2.1 Hz), 6.48 (1H, d, *J* = 2.1 Hz), 5.54 (1H, dd, *J* = 12.5, 4.1 Hz), 4.77 (2H, m), 4.59 (1H, brd, *J* = 3.9 Hz), 3.49 (1H, s, −OH proton), 2.75 (1H, m), 2.45 (1H, dd, *J* = 11.3, 1.7 Hz), 2.08 (2H, m), 1.83 (2H, m), 1.55 (1H, m), 1.46 (1H, m), 1.29 (3H, s), 1.25 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.9, 171.5, 145.0, 122.6, 120.9, 110.5, 78.0, 77.8, 70.8, 54.0, 50.5, 43.8, 41.1, 40.6, 40.3, 34.0, 27.2, 26.3, 21.4, 16.6; HRESIMS *m*/*z* 592.8829 $[M - H]^-$, calcd for $C_{20}H_{20}Br_3O_6$, 592.8815.

Purity Method 1. For this procedure, HPLC analysis was used involving gradient elution of 60% CH_3CN to 40% H_2O over 8 min on an Agilent C_{18} 4.6 × 150 mm, 5 μm column with a flow rate of 1.0 mL/min. The crude samples were dissolved in CH_3CN (concentration: 1 mg/mL, sample size: 10 μL, pump: Agilent 1100 Series Quaternary).

Purity Method 2. For this procedure, UPLC/HRMS analysis was used involving gradient elution of 5% to 100% CH_3CN over 2.7 min on a Waters Acquity BEH C_{18} 2.1 × 50 mm, 5 μm column with a flow rate of 0.6 mL/min. Crude samples were dissolved to make DMSO stocks of 50 mg/mL, and then 8 μL was taken up in 400 μL of acetonitrile for analytical solution from which 2 μL injections were made (pump: Agilent 1200 Binary SL).

Preparative-HPLC Method. Preparative HPLC using narrow gradients chosen based on the analytical results were obtained, with elution increasing to 20% CH_3CN over 5 min. Injections of 1 mL each were made from DMSO stocks onto a Waters T3 C_{18} 20 × 150 mm, 5 μm column with a flow rate of 20 mL/min (gradient pumps: Agilent 1200 Prep G1361A w/gradient extension).

In Vitro Assay. All cell lines and assays were used as previously described.[23] Briefly, CHO-K1 cells stably expressing the human κ-opioid receptor were purchased from Discoverx Corp. (Fremont, CA, USA; OPRK1 catalogue no. 95-0088C2). These cells were used in the DiscoverX HitHunter cAMP assay for small-molecule assay according to the manufacturer's directions as described in detail.[23]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00327.

^{1}H NMR, ^{13}C NMR, mass spectra, and HPLC chromatograms of compounds **1**−**9**; 2D NMR spectra of compound **7**; scheme of unsuccessful attempts at synthetic modifications on columbin (**1**) (PDF)

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Notes

The authors declare no competing financial interest.

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