JOURNAL OF NATURAL PRODUCTS

Article

Cite This: J. Nat. Prod. XXXX, XXX, XXX−XXX

pubs.acs.org/jnp

An Electrophilic Natural Product Provides a Safe and Robust Odor Neutralization Approach To Counteract Malodorous Organosulfur Metabolites Encountered in Skunk Spray

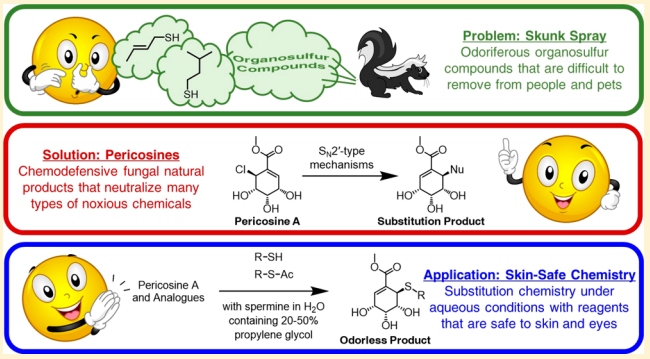
Lin Du,[†] Charissa Munteanu,[‡] Jarrod B. King,[†] Doug E. Frantz,[‡] and Robert H. Cichewicz*[,†]

[†]Natural Products Discovery Group, Institute for Natural Products Applications and Research Technologies, Department of Chemistry and Biochemistry, Stephenson Life Science Research Center, University of Oklahoma, 101 Stephenson Parkway, Norman, Oklahoma 73019, United States

[‡]Department of Chemistry, The University of Texas at San Antonio, San Antonio, Texas 78249, United States

Supporting Information

ABSTRACT: The anal secretions of skunks comprise several types of malodorous organosulfur compounds. The pungent metabolites are used defensively by skunks to repel threats posed by predators, and in many parts of the world, those perceived threats include humans and their pets. The extremely low thresholds for detection of the organosulfur metabolites make efforts to "de-skunk" people, animals, and clothing a process fraught with many challenges. The fungal-derived metabolite pericosine A (**4**) is a promiscuous yet stabile electrophilic compound that we propose is used by some fungi as a novel form of chemical defense. Our investigations have indicated that pericosine A readily reacts with skunk-spray secretions to transform them into odorless products. Mechanistic and computational studies suggested that pericosine A and its synthetic analogues react via S_N2'-type mechanisms with thiols and thioacetates under aqueous conditions to generate stable thioethers. Testing revealed that pericosine A did not cause skin or eye irritation and was highly effective at deodorizing skunk anal gland secretions when formulated to include adjunctive cosmetic ingredients.



Human−wildlife conflict is a growing global problem resulting from adverse interactions between humans and nondomesticated plants and animals.[1,2] The outcomes of these interactions can range from unpleasant to beastly with humans and/or wildlife suffering a variety of consequences. One distasteful type of conflict that occurs with considerable frequency involves encounters between humans and skunks (Mephitidae). Skunks possess a unique anal gland system that has evolved to generate and store cocktails of extremely odoriferous metabolites. When threatened, skunks expel those fluids in the form of a spray, which serves to overwhelm predators and deter further attacks. Exposure to skunk defensive secretions (also known as skunk spray or musk) is a common and challenging problem for humans, pets, and livestock throughout North and South American countries as well as portions of the Malay Archipelago. Skunk sprays are generally composed of organic thiols, including (*E*)-2-butene-1-thiol and 3-methyl-1-butanethiol (Figure 1A) and their thioacetate derivatives. The two aforementioned thiols are the major (>50%) volatile components of skunk sprays and are largely responsible for their extraordinarily repellent odors.[3] Although the thioacetates tend to be less or nonodoriferous, their presence can contribute to the continual emanation of odors from affected humans, animals, and objects as the compounds undergo autohydrolysis or are subjected to microbial biotransformation into their thiol forms.[3] The pungent odor of skunk thiols is highly persistent (e.g., compounds are oily and easily adhere to clothing, hair, and skin), and they are readily detectable by humans and other animals at low concentrations (e.g., the human nose can detect concentrations as low as ∼10 ppb).[4] Animals unfortunate enough to be directly exposed to skunk sprays can develop a variety of physical symptoms including ocular edema, conjunctivitis, drooling, and squinting. In some instances, the thiols in skunk sprays can induce oxidative damage to hemoglobin and cause Heinz body anemia in affected animals.[5]

It is rather difficult to fully "de-skunk" (i.e., remove the detectable scent from) sprayed objects and animals (e.g., pet dogs). The well-known Krebaum's formula[3] containing hydrogen peroxide and baking soda is a somewhat effective home remedy that converts the thiols to odorless sulfonic acids (Figure 1B). However, this highly oxidative combination of ingredients can cause dermal and ocular irritation as well as bleach hair, fur, and textiles. Several commercial products are

Received: May 3, 2019

Downloaded by UNIV AT BUFFALO STATE UNIV NEW YORK at 19:42:50:228 on July 05, 2019
from https://pubs.acs.org/doi/10.1021/acs.jnatprod.9b00415.

ACS Publications

© XXXX American Chemical Society and American Society of Pharmacognosy

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

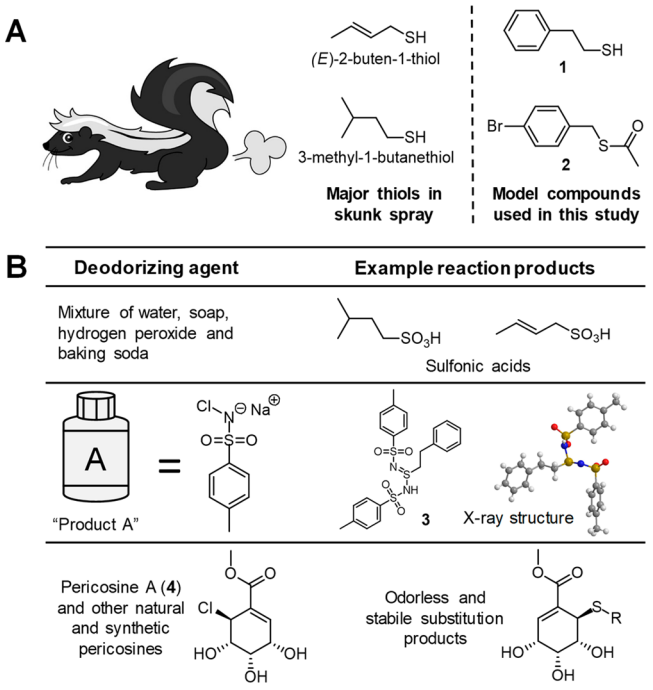


Figure 1. (A) Structures of the major odoriferous thiols in skunk defensive secretion and the test agents [2-phenylethanethiol (**1**) and 4-bromo-α-toluene thioacetate (**2**)] that were used as model compounds in this study. (B) Current and proposed chemical approaches to deodorization discussed in this report.

marketed as "de-skunking" agents (refer to the Supporting Information for examples), which are purported to break down or eliminate skunk spray components. However, in our testing of these products by LCMS monitoring using the model compound 2-phenylethanethiol (**1**) (Figure 1A), five of the six formulas that we tested failed to provide evidence that the target thiol had been chemically altered (passive exposure of members of our research team to the samples treated with these products confirmed that the thiol remained readily detectable to the nose). Several of the products did appear to contain surfactant-like molecules, which implied their intended functions might be the removal of organosulfur compounds from affected surfaces. Interestingly, one product (Product A) was found to readily convert **1** to a water-insoluble, crystalline product (**3**) (Figure 1B). Despite the manufacturer's claim that the product was "ECO friendly" and contained a "non-toxic mineral-based deodorizer," our analyses of the odorless reaction product [NMR and X-ray diffraction data (Figures 1B and S2)] as well as the product ingredients revealed that Product A contained chloramine-T, which has been identified as a potentially toxic and irritating oxidant that is primarily used as an industrial biocide and disinfectant.[6]

Considering the problems associated with existing "de-skunking" strategies, we set out to identify a safer and more effective skunk-spray-deodorizing solution. One of the lead substances to emerge from our search was the chemoreactive fungal metabolite pericosine A (**4**) (Figure 2), which had been previously reported by our group to protect fungi from nucleophilic antibiotics/toxins.[7] On the basis of those findings, we hypothesized that pericosine A and its synthetic analogues might offer an appealing solution to the neutralization of thiols and even thioacetates via S_N2' substitution mechanisms under aqueous conditions. Herein, we describe the unique thiol-reactive properties of pericosine A and its analogues. In addition, we report on the development and testing of reaction conditions using pericosine A that could be employed to "de-skunk" sensitive surfaces (i.e., skin and eyes) as well as present a concise summary of preliminary toxicological assay data aimed at assessing the dermal and ocular safety of pericosine A.

RESULTS AND DISCUSSION

Pericosine A is composed of a methyl shikimate skeleton with a chlorine atom attached to the C-6 position, which gives this

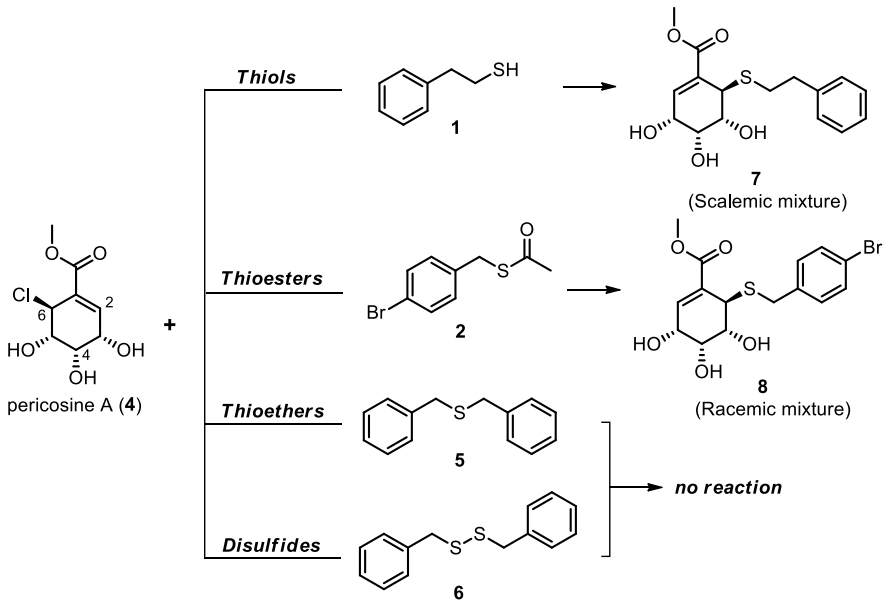


Figure 2. Reactivity of pericosine A (**4**) with selected sulfur-containing compounds (**1**, **2**, **5**, and **6**) in 50% MeOH with Et_3N as the catalyst. Reactant/reagent ratios for all reactivity tests, pericosine A (1 equiv)/sulfur-containing compound (1 equiv)/Et_3N (2 equiv). Products **7** (scalemic) and **8** (racemic) were generated in large scale under modified reaction conditions [pericosine A (1 equiv)/**1** (1.5 equiv)/Et_3N (2 equiv) for making **7** and pericosine A (1 equiv)/**2** (1.5 equiv)/Et_3N (10 equiv) for making **8**].

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

molecule a unique set of electrophilic properties and makes it an excellent substrate for reactions with a wide range of nucleophiles (Figures 2 and S3).[7] Our studies began by testing the reactivity of pericosine A against four classes of sulfur-containing model compounds: thiol (2-phenylethanethiol, **1**), thioester (4-bromo-α-toluene thioacetate, **2**), thioether (dibenzyl sulfide, **5**), and disulfide (dibenzyl disulfide, **6**) (Figure 2). Pericosine A reacted with thiol **1** under both neutral and basic conditions to generate product **7** (Figure S4A,B). The catalytic base (Et_3N in this case) significantly improved the rate of the reaction. Notably, compound **7** and similar thioether addition products did not produce noticeable odors; when reaction vessels were removed from the hood, no significant foul smells were encountered. Similarly, thioester **2** readily reacted with pericosine A under basic conditions to form **8**. Neither thioether **5** nor disulfide **6** reacted with pericosine A under the experimental conditions we investigated (Figures 2 and S4).

To understand (i) the spectrum of reactivity for pericosine A with organosulfur compounds under different reaction conditions, (ii) opportunities that existed to improve its thiol-neutralizing capabilities, and (iii) possible approaches for using pericosine A in real-world situations to neutralize odoriferous skunk metabolites, we set about determining how the natural product mechanistically functioned with different sulfur-containing substrates as well as explored alternative reagents that would be appropriate for topical use. As an entry point to these studies, we began by examining the structures of products **7** and **8**, which were solved through analyses of their 1D (^{1}H and ^{13}C) and 2D [^{1}H–^{1}H COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear mulitple bond correlation)] NMR data (Tables 1 and 2; Figures S25–S31) as well as high-resolution ESIMS (electrospray ionization mass spectrometry) data. Further chemical studies examining the absolute configurations of **7** and **8** and the reaction mechanisms leading to their formation revealed that the product distributions for these reactions were highly dependent on both the reaction conditions used and stoichiometric ratios of the substrates and reagents employed. During our initial synthesis of **7**, an excess of **1** (1.5 equiv) and the catalytic base Et_3N (2 equiv) was used to maximize product yield. Under these conditions, product **7** was identified as a scalemic mixture by chiral HPLC (high-performance liquid chromatography) (Figures 3F and S5E). The relative configurations of C-5 and C-6 were determined as 5*R**,6*R** via comparisons of calculated ^{13}C NMR data for the C-6 epimers **7a** (5*R*,6*R*) and **7b** (5*R*,6*S*) with experimentally derived data (Figure S5A,B). Interestingly, when the reaction was performed using an equal molar amount of **1** and pericosine A in the absence of Et_3N, it resulted in the formation of pure stereoisomer **7a** (Figures 3E and S5E). The absolute configuration of **7a** was confirmed as 3*R*,4*R*,5*R*,6*R* through an analysis of its experimental and calculated ECD (electronic circular dichroism) spectra and specific rotation values (Figures S5A,G). The reaction between pericosine A (1 equiv) and **2** (1.5 equiv) was also promoted when excess Et_3N (10 equiv) was used to form racemic **8** (Figures S5C,F). Enantiomers **8a** (3*R*,4*R*,5*R*,6*R*) and *ent*-**8a** (3*S*,4*S*,5*S*,6*S*) were separated by chiral HPLC, and their absolute configurations were determined by comparing their ECD spectra with data obtained for **7a** (Figure S5G).

Table 1. ^{1}H NMR Data of 7, 8, and 23–28

no.	7[a]	8[a]	23[b]	24[b]	25[b]	26[b]	27[b]	28[b]
2	6.91, d (4.6)	6.91, d (4.6)	6.66, d (2.5)	6.59, dd (3.6, 1.4)	6.59, dd (4.2, 1.5)	6.65, d (2.5)	6.59, dd (3.6, 1.5)	6.61, d (3.8)
3	4.23, br s	4.21, t (4.8)	4.29, dd (2.5, 8.5)	4.26, dd (4.8, 3.6)	4.32, dd (4.2, 3.6)	4.26, dd (8.5, 2.5)	4.26, dd (5.0, 3.6)	4.32, dd (4.0, 3.8)
4	4.12, br s	4.06, br d (4.8)	3.86, dd (2.3, 8.5)	3.70, dd (4.8, 2.2)	3.67, dd (7.4, 3.6)	3.83, dd (8.5, 2.2)	3.61, dd (5.0, 2.2)	3.68, dd (6.9, 4.0)
5	4.18, br s	3.97, br s	4.13, t (2.3)	4.07, dd (5.1, 2.2)	4.19, dd (7.4, 4.9)	3.93, dd (3.0, 2.2)	3.91, m	4.11, dd (6.9, 4.3)
6	3.85, d (2.9)	3.76, m	3.74, dd (2.3, 1.2)	3.81, br d (5.1)	3.46, br d (4.9)	3.63, dd (3.0, 1.4)	3.73, br d (5.0)	3.43, br d (4.3)
8	3.81, s	3.76, s	3.77, s	3.77, s	3.75, s	3.68, s	3.78, s	3.75, s
1′	2.97, m	3.86, d (13.4)	2.94, m	2.90, m	2.88, m	3.89, d (13.5)	3.93, d (13.5)	3.89, d (13.5)
		3.81, d (13.4)				3.85, d (13.5)	3.89, d (13.5)	3.83, d (13.5)
2′	2.99, m		2.94, m	2.95, m	2.90, m			
	2.89, m			3.01, m	2.97, m			
3′		7.23, d (8.3)				7.30, d (8.4)	7.30, d (8.4)	7.28, d (8.4)
4′	7.22, d (7.3)	7.45, d (8.3)	7.24, d (7.3)	7.24, d (7.3)	7.23, d (7.3)	7.48, d (8.4)	7.46, d (8.4)	7.44, d (8.4)
5′	7.29, t (7.3)		7.28, t (7.3)	7.27, t (7.3)	7.26, t (7.3)			
6′	7.23, m	7.45, d (8.3)	7.18, t (7.3)	7.17, t (7.3)	7.17, t (7.3)	7.48, d (8.4)	7.46, d (8.4)	7.44, d (8.4)
7′	7.29, t (7.3)	7.23, d (8.3)	7.28, t (7.3)	7.27, t (7.3)	7.26, t (7.3)	7.30, d (8.4)	7.30, d (8.4)	7.28, d (8.4)
8′	7.22, d (7.3)		7.24, d (7.3)	7.24, d (7.3)	7.23, d (7.3)			

[a]Spectra measured in $CDCl_3$. [b]Spectra measured in methanol-d_4.

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 2. ^{13}C NMR Data of 7, 8, and 23−28

no.	7[a]	8[a]	23[b]	24[b]	25[b]	26[b]	27[b]	28[b]
1	130.9	130.6	130.3	134.3	134.3	130.0	134.0	133.6
2	136.4	136.9	141.1	137.1	136.8	141.4	137.7	137.6
3	65.7	65.7	70.1	69.8	66.8	70.1	69.7	66.8
4	66.5	66.6	72.2	75.4	72.1	72.3	75.4	71.8
5	74.5	74.2	75.9	69.4	73.9	75.4	69.9	74.2
6	46.2	45.3	47.7	48.0	48.1	46.8	46.8	46.9
7	166.6	166.5	167.9	168.7	168.7	167.8	168.7	168.6
8	52.5	52.5	52.5	52.5	52.4	52.4	52.5	52.4
1′	35.3	37.5	36.8	37.4	37.4	38.5	39.2	38.0
2′	36.5	136.9	37.4	37.7	35.8	139.1	139.4	139.4
3′	140.2	130.9	141.8	142.1	142.1	132.1	132.3	132.2
4′	128.7	131.9	129.6	129.6	129.6	132.6	132.5	132.5
5′	128.7	121.4	129.4	129.4	129.4	121.9	121.7	121.6
6′	126.6	131.9	127.3	127.2	127.2	132.6	132.5	132.5
7′	128.7	130.9	129.4	129.4	129.4	132.1	132.3	132.2
8′	128.7		129.6	129.6	129.6			

[a]Spectra measured in $CDCl_3$. [b]Spectra measured in methanol-d_4.

While optimizing the reaction conditions to prepare **7**, varying ratios of **1**, pericosine A, and Et_3N were tested. It was observed that, in the presence of excess Et_3N, pericosine A was converted to an epoxide, pericoxide (**9**),[7] as well as the methoxy analogue (−)-pericosine C (**10**)[8] (Figures 3A−D and S6A−D). Considering the conversion of pericosine A to epoxide **9** under basic conditions, the question arose whether pericosine A reacted directly with **1** or, instead, was being converted first to reactive species **9**. To address this problem, the hydroxy groups in pericosine A were protected, yielding triacetylated derivative **11**. This compound proved readily able to react with **1** in the presence of Et_3N to form **12** (Figure 3I). Notably, **12** was determined to bear the same absolute configuration as **7a** (Figure S5D,G). These results indicated that pericosine A could serve as a direct precursor to **7a** (i.e., pericosine A did not require activation to its epoxide form for the reaction to occur). Additionally, we tested whether **9** might be involved in the formation of *ent*-**7a**, but that test revealed **7a** was only produced when **9** and **1** were reacted in the absence of Et_3N (Figure 3G). Furthermore, we noted that, in reactions carried out with either excess pericosine A or **9**, no *ent*-**7a** was detected. It was also determined that incubation of **7** with Et_3N did not contribute to its racemization (Figure S6E). Taken in combination, the accumulated evidence suggested that *ent*-**7a** was not produced through a reaction with **9**, but instead, its production was based on the amount of **1** used in the reactions. Subsequently, we observed that racemization of **7a** occurred when it was exposed to **1** and that no basic catalyst was required for this process to proceed (Figure 3H). Thus, the overall reaction mechanism was proposed to occur as depicted in Figure 3J. To summarize, under basic conditions, pericosine A was partially converted to epoxide analogue **9**, and both pericosine A and **9** are believed to have competed to react with **1** (*syn*-S_N2' for pericosine A and *anti*-S_N2' for **9**). This was followed by racemization of product **7a** when excess **1** was present. A similar mechanistic process was determined to occur when pericosine A was reacted with thioacetate **2** (Figure S7). These observations agree with our previous computational studies,[7] which supported the stereoselective S_N2' substitution-type reaction mechanisms for pericosine A and **9**. Although our accumulated chemical and spectroscopic evidence support this general pathway, at this time, we cannot fully rule out the potential contribution that a Michael-addition-like pathway may play in this process.

To further investigate and optimize the reactivity of pericosines with thiols and thioesters, two chlorinated pericosine analogues, **19** and **20**, were synthesized from (−)-shikimic acid as described[8−13] with some procedural modifications (Scheme 1). Both **19** and **20** readily reacted with **1** and **2** in 50% MeOH in the presence of Et_3N (Figure S8B). Although the reaction rates of thiols with **19** and **20** were comparable to what had been observed for pericosine A, **19** and **20** appeared to be more water-soluble than pericosine A (i.e., solutions containing pericosine A appeared slightly cloudy, whereas solutions of **19** and **20** were clear). This proposition was also supported by the observed increased relative polarity of **19** and **20** as compared to pericosine A as estimated by LCMS analysis (Figure S8A). Since **19** and **20** showed similar substrate reactivities (Figure S8B), the more hydrophilic analogue **19** (Figure S8A) was selected for reactivity studies. Like pericosine A, **19** was converted to epoxide analogue **21** and the methoxy analogue **22** in 50% MeOH in the presence of Et_3N (Scheme 1 and Figures S12A−C). The reaction of **19** with **1** yielded three thiol-substituted products, **23**−**25**, and the ratios of those compounds varied on the basis of the amount of Et_3N (2 to 10 equiv) used in each reaction (Scheme 1 and Figure S8B). A similar reactivity profile was observed for the reaction between **19** and **2** resulting in the formation of three substituted products **26**−**28** (Scheme 1 and Figure S8B). The absolute configurations of **21**−**28** were determined by comparing their $^3J_{H,H}$ coupling constants and ^{13}C NMR data with those of selected model compounds (Figures S9−S11). The results of those tests (Figure S12D) suggested that both pericosines reacted with thiol **1** via the same S_N2' mechanisms (*syn*-S_N2' for **19** and *anti*-S_N2' for **21**) that had been identified for pericosine A and **9** (Figure S6E). Despite the similarities, two minor thiol-substituted products, **23** and **25**, were consistently detected under all tested conditions even when excess **19** or **20** was reacted with **1** (Scheme 1 and Figure S12D). We speculate that these products were formed via dual mechanisms: isomerization of the major product **24** due to excess **1** as well as through direct substitution of **19** and **21** by **1** through alternative S_N2' and/or S_N2 mechanisms (Figure S12).

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

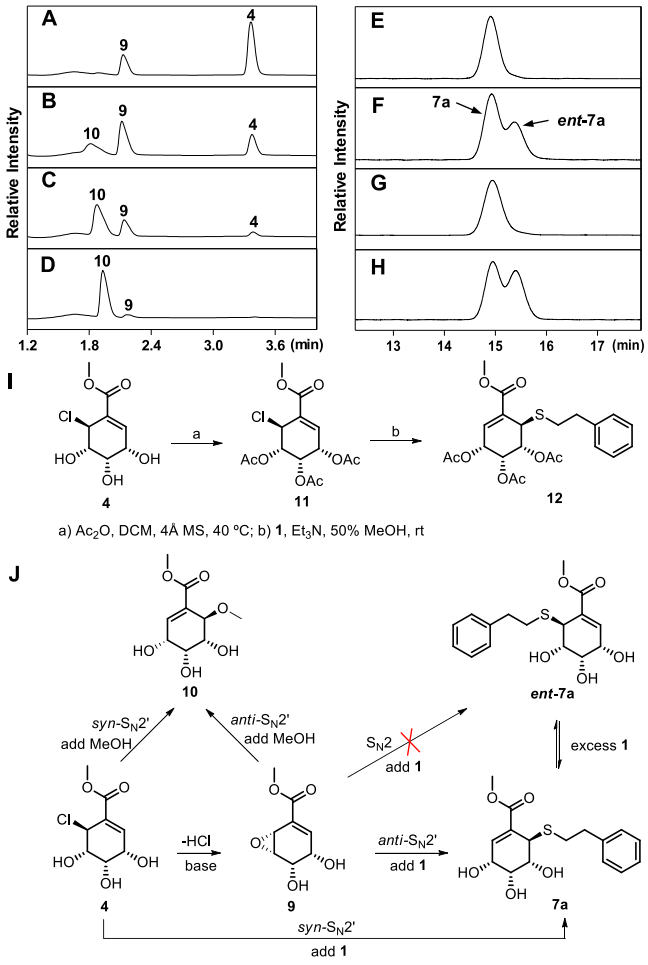


Figure 3. Investigating the mechanism of Et_3N-catalyzed substitution involving **1** and pericosine A (**4**). (A–D) Time-dependent conversion of pericosine A. Pericosine A (1 equiv) was mixed with Et_3N (2 equiv) in 50% MeOH, and the reactant mixture was monitored by LCMS at several time points [(A) 1 min; (B) 15 min; (C) 30 min; (D) 100 min]. (E–H) Chiral-HPLC analysis of **7** derived from different conditions. The reactants/reagents [initial ratios: (E) pericosine A (1 equiv)/**1** (1 equiv); (F) pericosine A (1 equiv)/**1** (1.5 equiv)/Et_3N (2 equiv); (G) **9** (1 equiv)/**1** (1 equiv); (H) **7a** (1 equiv)/**1** (1 equiv)] were incubated in 50% MeOH overnight prior to HPLC analysis. HPLC conditions: Lux cellulose-2 analytical column, 30% MeCN elution, monitored at 200 nm. (I) Synthesis of the fully acetylated pericosine analogue (**11**) and its reaction with **1**. (J) Proposed mechanism for the Et_3N-catalyzed substitution involving pericosine A with **1** or MeOH.

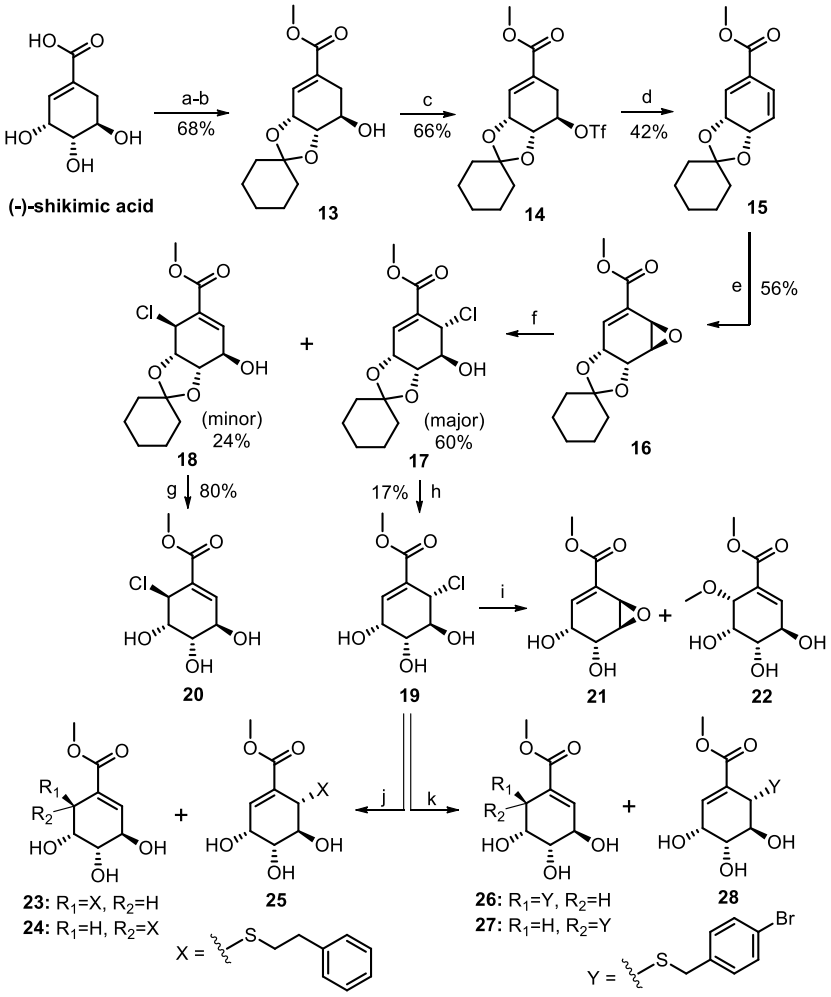
Encouraged by the robust reactivity of pericosine A and analogues **19** and **20**, we set about identifying reagents that would be potentially safer for topical applications on humans and other animals. First, we sought to replace Et_3N with a more suitable base because its presence was essential for the successful conversion of thioacetates into reactive thiols. To begin, 21 inorganic and organic bases (2 equiv) were tested for their catalytic capabilities in reactions between pericosine A (1 equiv) and **2** (1 equiv) under 100% aqueous conditions. Such reaction conditions had been noted by us as unfavorable for supporting Et_3N-facilitated catalysis (Figure S14A). From that initial set, 6 bases including K_2CO_3 and 5 types of polyamines (Figure S13) were found to have varying degrees of catalytic capabilities. One of the most active of those agents was the naturally occurring polyamine spermine (**29**) (Figures 4A and S1), which facilitated the complete consumption of **2** and pericosine A in H_2O (Figure S14B). This compound is a ubiquitous component of eukaryotic cells and has been reported to be incorporated into some cosmetics as an antioxidant agent.[14,15] While experiments with **29** were underway, parallel efforts to uncover additional alternative reaction conditions that were even more favorable and safer revealed a noteworthy surprise; propylene glycol (PG) was determined to be a highly effective cosolvent (20–50% PG) that facilitated the rapid solubilization of the hydrophobic thiols and thioacetates but did not impede the catalytic properties of **29** (Figure S14C). Importantly, PG is widely used in skin care products as a humectant, making it a useful addition to the suite of optimized reaction conditions and reagents.

While ascertaining the mechanistic role that **29** played in substitution reactions between pericosines and thiols/thioesters, an analysis of the reaction products revealed that some significant changes had occurred. For example, it was observed that, in reactions between pericosine A and **2** in the presence of **29**, **29** itself was rapidly reacting with pericosine A and epoxide **9** to form the new substitution product **30** (Figures 4, S1, and S14E–H). The reaction between **29** (1 equiv) and [^{13}C]-labeled pericosine A (1 equiv) revealed **30** was formed via an S_N2' mechanism (Figure 4A), and the absolute configuration of C-6 was determined to be *R* on the basis of the large $^3J_{H5,H6}$ value (9.4 Hz) observed in the ^{1}H NMR spectrum of the TFA salt of **30** (**TFA-30**) (Figure S15). Interestingly, unlike the reactions of **2** with pericosine A that were catalyzed by Et_3N (Figure S7E), catalysis involving **29** uniformly led to 1:1 racemates of **8** regardless of the reaction conditions employed (Figure S16E–H). Thus, we hypothesized that racemization of **8** was not only induced by excess **2′** (Figure S7F) but also facilitated by the presence of **29**. The reactions between **30** and **2** (Figures S1 and S16A,B) and between **8** and **29** (Figures S1 and S16C,D) revealed that **8** and **30**/*ent*-**30** were likely to undergo a virtually continuous cycle of interconversion via a *syn*-S_N2' pathway resulting in the racemization of both structures (Figure 4B). Furthermore, catalysis by **29** facilitated the 1,4-addition of **2′** (Figure S1) to the alkene group of **8** forming **31** (Figures 4B, S14, and S16), which had previously only appeared as a minor product in the reaction of excess **2** with pericosine A in the presence of Et_3N (Figure S7D). However, the formation of **31** was completely suppressed when excess pericosine A was used in the reaction (Figure S14D), indicating the 1,4-addition of **2′** is a much slower process. Finally, catalysis by **29** led to the virtually instantaneous (immediately after the mixing of reagents) and complete conversion of thiol **1** (Figure 4C) to racemic **7** (Figure 4D). Thus, reaction conditions employing spermine in 50% aqueous PG exhibited enhanced rapidity for the consumption of thiols compared to the system using Et_3N and 50% aqueous MeOH (Figures 4C and S6A).

On the basis of our success identifying an efficacious set of reaction conditions and reagents expected to be safe for dermal use, we proceeded to test our processes on the complex cocktail of odoriferous sulfur-containing metabolites directly harvested from skunk anal glands. Using a prior inventory of the thiols and thioesters found in skunk glands,[3] we tested pericosine A and analogues **19** and **20** in both the Et_3N/MeOH and aqueous spermine/PG systems. The results of those reactions were analyzed by LCMS, which revealed a multitude of thioether substitution products had formed [for

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX–XXX

Scheme 1. Synthesis of the Pericosine A Analogues 19 and 20 and Base-Catalyzed Nucleophilic Substitutions (Thiol 1 and/or Thioacetate 2) of 19[a]



[a](a) CSA, MeOH; (b) cyclohexanone, CSA; (c) Tf_2O, pyr., DMAP (cat.); (d) CsOAc, DMF; (e) oxone, $NaHCO_3$, 1,1-trifluoroacetone/H_2O; (f) HCl in Et_2O; (g) MeOH, acetyl chloride (cat.), (h) incomplete deprotection by MeOH/acetyl chloride (cat.) (thus, reprotected by CSA in acetone and then complete deprotection by MeOH/acetyl chloride (cat.)); (i) Et_3N, 50% MeOH; (j) **1**, Et_3N, 50% MeOH; (k) **2**, Et_3N, 50% MeOH.

example, reaction products **32**–**36** (Figure S1) were detected as having been formed from pericosine A, Figures S17A–G]. After overnight reactions, the spermine/PG-based reaction system exhibited comparable levels of consumption of the skunk-gland-derived sulfur-containing metabolites as had been observed for the Et_3N/MeOH system (Figures S17B,C). While formal odor identification tests were not performed, the effectiveness of the reactions at neutralizing the odor of skunk-gland secretions was readily apparent to the research team: whereas even single-digit microliter volumes of the skunk scent were capable of creating an overwhelmingly foul aroma (even when briefly removed from the chemical fume hood), samples of skunk secretions that had been properly treated with pericosine A, **19**, and **20** could be handled on the benchtop for extended periods without emitting any trace of unpleasant odor.

Tests were performed to assess the safety of pericosine A alone and under the optimized reaction conditions we developed. We were initially taken aback by a report suggesting pericosine A exhibited notable *in vitro* cytotoxicity against the murine P388 lymphocytic leukemia cell line (ED_{50} value of 0.45 μM).[16] However, the same group also reported that pericosine A only showed weak antiproliferative activities against 20 human cancer cell lines with an average GI_{50} value of 15.1 μM. Moreover, our *in vitro* antiproliferative/cytotoxicity tests also showed that pericosine A only weakly inhibited the growth of the Ect 1 human normal cervical cell line (IC_{50} value of 18.3 μM) and the NIH/3T3 normal mouse fibroblast cell line (IC_{50} value of 8.4 μM). Thus, we determined it was prudent to test pericosine A for its potential to cause skin and eye damage or irritation. To test the dermal and ocular toxicity of pericosines, we evaluated the test substances using the EpiDerm skin irritation and EpiOcular eye irritation models, respectively (Figure 5).[18,19] Pericosine A did not exhibit toxic or irritating effects at concentrations of 0.5 and 1 mM (Figure 5A), nor did it induce the expression of the cytokine interleukin-1α (IL-1α) at 0.5 mM (Figure 5B). Furthermore, pericosine A (0.5 and 1 mM) was determined to be nonirritating in the EpiOcular eye irritation assay, and it did not reduce the viability of the EpiOcular tissues as compared to the recommended control (Figure 5C). When the EpiOcular tissues were exposed to pericosine A (1 mM) for

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

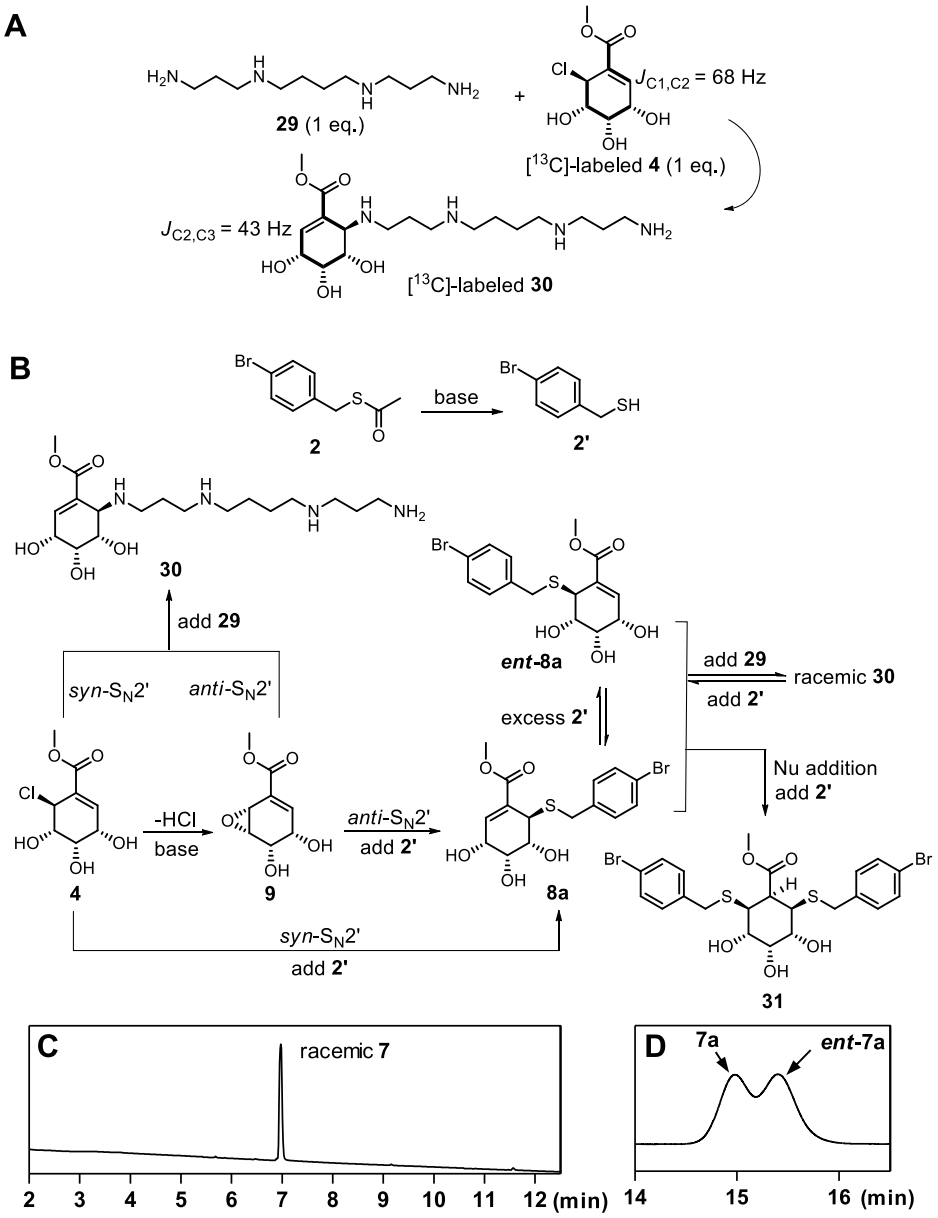


Figure 4. (A) The conjugation of [^{13}C]-labeled pericosine A (**4**) (accomplished by feeding [U−$^{13}C_6$]-D-glucose to the producing fungus) with spermine (**29**). (B) The proposed mechanism of the reaction between **2** and pericosine A with **29** as the catalyst. (C) LCMS analysis (UV 210 nm) of the reaction between pericosine A (1 equiv) and **1** (1 equiv) in 50% propylene glycol (PG) with spermine (2 equiv) as the catalyst. The reaction mixture was injected into LCMS immediately upon mixing of the reagents. Product **7** was analyzed by chiral HPLC (UV 210 nm) (D).

an extended period (24 h), it was found to meet the classification of an ultramild chemical agent with an ET-50 value of >24 h (Figure 5D). Additionally, the safety of pericosine A remained unchanged in both the EpiDerm and EpiOcular tests when 50% PG was used as the test vehicle as well as when mixtures of **29** (0.5 mM) and pericosine A (0.5 mM) were used as the test sample (Figure 5A−D). Further testing was performed to assess the potential mutagenic properties of pericosine A. Pericosine A showed no mutagenic properties in the Ames II mutagenicity assay,[17] even at a concentration as high has 1 mg/mL (Table S3). Taken in combination, these data suggested that pericosine-based odor neutralization strategies might be safe and effective for dermal uses on humans and other animals. Moreover, pericosine A appeared to have no immediately detectable problematic properties as it would relate to its accidental introduction to the eye.

In summary, our studies provide a scientific rationale for using pericosines as a new natural-product-based odor neutralization strategy. Studies with pericosine A and its analogues (**19** and **20**) have revealed an unusual suite of electrophilic properties incorporated into this family of fungal metabolites, which render them safe yet highly reactive (even under ultramild aqueous conditions) toward a variety of noxious sulfur-containing chemicals. Our team is continuing to probe the potential applications of pericosines as well as pursue studies aimed at improving routes toward the production of these shikimate-based metabolites. For example, in addition to the synthetic efforts reported in the manuscript, we have engaged in the process of subjecting the source fungus (*Tolypocladium* sp. MEA-2) to chemical mutagenesis and

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

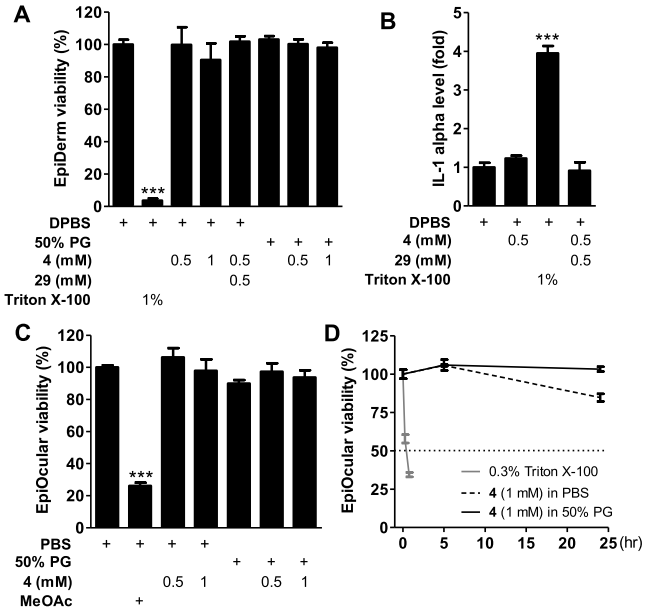


Figure 5. Safety tests of pericosine A (**4**) (A, B) using the *in vitro* EpiDerm skin irritation test. The EpiDerm tissues were exposed to the test chemicals for 60 min and then incubated in fresh medium for 24 h. The medium was collected for analysis of the cytokine interleukin-1α (IL-1α) (B), and the viability of the tissues was evaluated using the MTT assay. (C) For the EpiOcular eye irritation test, tissues were incubated with the test chemicals for 30 min, and cell viability was evaluated using the MTT assay. Viability: ≥60% is considered nonirritating while viability levels <60% are interpreted as irritating. (D) EpiOcular sub-draize ultra-mildness test. The ET-50 is defined as the time of exposure needed for a test substance to reduce the tissue viability by 50% relative to control tissues. For all tests: $n = 3$; ***, $p < 0.001$; 50% PG; 50% propylene glycol aqueous solution.

culture medium optimization, which has already led to a 15-fold increase in the yield of pericosine A (156 vs 10 mg/L, data not shown). The encouraging results obtained for pericosine A and its analogues have revealed a promising suite of commercial applications utilizing this robust odor neutralization technology. Further studies examining the activities of additional synthetic pericosines are anticipated to provide inspiration for the creation of a new generation of nontoxic materials engineered to counteract a range of noxious nucleophilic substances.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation data were obtained on a Rudolph Research Autopol III automatic polarimeter. NMR data were obtained on Varian VNMR spectrometers (400 and 500 MHz for ^{1}H, 100 and 125 MHz for ^{13}C) with broad band and triple resonance probes at 25 ± 0.5 °C. HRESIMS (high-resolution electrospray ionization mass spectrometry) data were collected on an Agilent 6538 high-mass-resolution QTOF mass spectrometer. LCESIMS (liquid chromatography-electrospray ionization mass spectrometry) data were obtained on a Shimadzu LC−MS 2020 system (ESI quadrupole) coupled to a photodiode array detector with a Phenomenex Kintex column (2.6 μm C_{18} column, 100 Å, 75 × 3.0 mm). Preparative HPLC separations were performed on a Shimadzu system using a SCL-10A VP controller and a Gemini 5 μm C_{18} column (110 Å, 250 × 21.2 mm) with a flow rate of 10 mL/min. Semipreparative HPLC separations were performed on a Waters 1525 system using a 2998 PDA detector and HPLC columns including a Gemini 5 μm C_{18} column (110 Å, 250 × 10.0 mm), a Kinetex 5 μm biphenyl column (110 Å, 250 × 10.0 mm), and a Kinetex 5 μm F5 column (110 Å, 250 × 10.0 mm) with a flow rate of 4 mL/min. Chiral analytical HPLC analyses were performed on a Waters 1525 system using Lux 5 μm cellulose-2 and Lux 5 μm cellulose-3 columns (110 Å, 250 × 4.6 mm) with a flow rate of 1 mL/min. All solvents were of ACS grade or better.

Reactivity Tests of the Commercial "De-Skunking" Products. Six "de-skunking" products were purchased from Amazon.com, including Skunk-Off Liquid Soaker, Mold Monster Stink Slayer, Clean +Green Deskunk Coat Cleaner, Nature's Miracle Skunk Odor Remover, SynergyLabs De-Skunk Odor Destroyer, and Skout's Honor Skunk Odor Eliminator (referred to as Product A). For each product, 50 μL was mixed with 2-phenylethanethiol (**1**, 3 μL from 100 mM DMSO stock solution). The reactant mixtures were kept static overnight (~16 h) at room temperature. Reaction products were mixed with MeOH (50 μL) and analyzed by LCMS.

Conversion of 2-Phenylethanethiol (1) by Commercial Product A. An aliquot of 1 mL of Product A was added to 20 μL of 2-phenylethanethiol (**1**). The mixture was stirred for 3 h at room temperature. The solvent was evaporated *in vacuo*, and the residue was subjected to semipreparative HPLC purification using a Kinetex 5 μm F5 column (isocratic, 40% MeCN in 10% TFA) to afford compound **3** (38 mg).

*Compound **3**:* white solid; ^{1}H NMR (400 MHz, methanol-d_4): δ 7.62 (d, J = 8.3 Hz, 4H), 7.26 (d, J = 8.3 Hz, 4H), 7.23 (m, 3H), 7.09 (d, J = 8.3 Hz, 2H), 3.52 (t, J = 7.4 Hz, 2H), 2.88 (t, J = 7.4 Hz, 2H), 2.40 (s, 6H); ^{13}C NMR (100 MHz, methanol-d_4): δ 144.8, 140.5, 138.5, 130.6 (4C), 129.9 (2C), 129.6 (2C), 128.1, 127.8 (4C), 57.0, 30.5, 21.5 (2C); HRESIMS m/z 499.0800, $[M + Na]^+$ (calcd for $C_{22}H_{24}N_2O_4S_3Na$, 499.0790).

General Methods for Reactivity Tests of Pericosine A (4) and Analogues. Reactions were performed in Eppendorf tubes with 50 μL of the test solvent [e.g., H_2O, 50% MeOH, and 50% propylene glycol (PG)]. Reagents were then added to the test solvent: pericosine A or analogues (1 equiv, 3 μL from 100 mM DMSO stock solution), sulfur-containing compounds [e.g., 2-phenylethanethiol (**1**), 4-bromo-α-toluene thioacetate (**2**), dibenzyl sulfide (**5**), and dibenzyl disulfide (**6**)] (1 equiv, 3 μL from 100 mM DMSO stock solution), and/or bases (e.g., Et_3N, spermine) (2 equiv, 6 μL from 100 mM DMSO stock solution). The reactant mixtures were then kept static overnight (~16 h, default incubation time unless otherwise specified) at room temperature. Test mixtures were combined with MeOH (50 μL) and analyzed by LCMS to measure reagent consumption as well as product formation.

Reaction of Pericosine A (4) with 2-Phenylethanethiol (1) Catalyzed by Et_3N. Pericosine A (**4**, 10 mg, 1 equiv) was mixed with 2-phenylethanethiol (**1**, 6 μL, 1.5 equiv) and Et_3N (12.5 μL, 2 equiv) in 50% MeOH (2 mL). The reactant mixture was stirred at room temperature for 3 h, and the solvent was removed *in vacuo*. The residue was purified by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 50% MeCN) to yield compound **7** (10 mg, scalemic mixture).

Scalemic-(3R,4R*,5R*,6R*)-methyl 3,4,5-Trihydroxy-6-(phenethylthio)cyclohex-1-enecarboxylate (Scalemic-**7**):* white solid; $[\alpha]^{20}_D$ −93 (c 0.15, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS m/z 347.0932, $[M + Na]^+$ (calcd for $C_{16}H_{20}O_5SNa$, 347.0924).

Pericosine A (**4**, 10 mg, 1 equiv) was mixed with 2-phenylethanethiol (**1**, 4 μL, 1 equiv) without Et_3N. After stirring for 16 h, the standard purification procedures were applied to generate the pure 3*R*,4*R*,5*R*,6*R* isomer of **7** (5.2 mg).

*(3R,4R,5R,6R)-Methyl 3,4,5-Trihydroxy-6-(phenethylthio)-cyclohex-1-enecarboxylate (**7a**):* $[\alpha]^{20}_D$ −145 (c 0.1, MeOH).

Reaction of Pericosine A (4) with 4-Bromo-α-toluene Thioacetate (2) Catalyzed by Et_3N. Pericosine A (**4**, 10 mg, 1 equiv) was mixed with 4-bromo-α-toluene thioacetate (**2**, 11.3 μL, 1.5 equiv) and Et_3N (62.5 μL, 10 equiv) in 50% MeOH (2 mL). The reactant mixture was stirred at room temperature overnight and before the solvent was removed *in vacuo*. The residue was purified by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 50% MeCN) to yield compound **8** (3.7 mg, racemic mixture).

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 3. ^{1}H and ^{13}C NMR Data of 19−22[a]

	19		20		21		22	
no.	^{13}C	^{1}H (*J*)	^{13}C	^{1}H (*J*)	^{13}C	^{1}H (*J*)	^{13}C	^{1}H (*J*)
1	133.0		129.8		128.6		132.1	
2	139.5	6.78, dd (4.3, 1.2)	144.7	6.87, d (2.5)	146.2	4.84, ddd (2.0, 1.9, 1.8)	141.0	6.59, dd (2.6, 1.6)
3	66.7	4.35, dd (4.3, 4.1)	69.9	4.38, dd (8.5, 2.5)	67.5	4.19, dd (5.4, 1.9)	70.1	4.40, ddd (7.1, 2.6, 2.6)
4	71.7	3.62, dd (8.2, 4.1)	71.1	3.97, dd (8.5, 2.6)	66.4	4.37, m	75.6	3.52, dd (7.1, 2.1)
5	74.5	4.13, dd (8.2, 5.4)	75.5	4.14, dd (3.0, 2.6)	53.7	3.56, dd (4.0, 3.0)	69.8	4.23, dd (4.0, 2.1)
6	58.5	4.58, ddd (5.4, 1.1, 1.0)	55.8	4.76, dd (3.0, 0.9)	47.3	3.91, dd (4.0, 2.0)	77.9	4.20, m
7	167.2		166.8		166.8		168.6	
8	52.6	3.78, s	52.7	3.78, s	52.5	3.81, s	52.4	3.50, s
9							58.7	3.76, s

[a]In methanol-d_4.

Rac-(3R,4R*,5R*,6R*)-methyl 6-((4-Bromobenzyl)thio)-3,4,5-trihydroxycyclohex-1-enecarboxylate (Racemic-**8**):* white solid; $[\alpha]^{20}_{D}$ 0 (c 0.35, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 410.9881, $[M + Na]^+$ (calcd for $C_{15}H_{17}O_5SBrNa$, 410.9782).

Transformation of Pericosine A (4) in 50% MeOH Catalyzed by Et_3N. Pericosine A (**4**, 15 mg, 1 equiv) was mixed with Et_3N (18.8 μL, 2 equiv) in 50% MeOH (12 mL). The reactant mixture was stirred at room temperature for 1 h before the solvent was removed *in vacuo*. The residue was purified by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 10% MeCN) to yield compounds **9** (3.5 mg)[7] and **10** (9.4 mg).[8]

Acetylation of Pericosine A (4). Pericosine A (**4**, 10 mg) was mixed with Ac_2O (1 mL) in DCM (1 mL) followed by the addition of 4 Å molecular sieves (100 mg). The reactant mixture was stirred at 40 °C overnight, and the solvent was removed *in vacuo*. The residue was purified by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 40% MeCN) to yield compound **11** (8.1 mg).

*(1S,2S,3S,6S)-6-Chloro-5-(methoxycarbonyl)cyclohex-4-ene-1,2,3-triyl Triacetate (**11**):* white solid; $[\alpha]^{20}_{D}$ 115 (c 0.29, MeOH); ^{1}H NMR (500 MHz, methanol-d_4): δ 6.80 (d, *J* = 3.4 Hz, 1H), 5.84 (m, 1H), 5.60 (dd, *J* = 2.3, 4.5 Hz, 1H), 5.43 (dd, *J* = 2.3, 5.9 Hz, 1H), 4.98 (d, *J* = 5.9 Hz, 1H), 3.82 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H); ^{13}C NMR (125 MHz, methanol-d_4): δ 171.5, 171.3, 171.2, 165.9, 137.4, 133.3, 73.7, 68.5, 66.7, 54.0, 53.0, 20.5 (2C), 20.4; HRESIMS *m/z* 371.0504, $[M + Na]^+$ (calcd for $C_{14}H_{17}O_8ClNa$, 371.0504).

Reaction of Compound 11 with 2-Phenylethanethiol (1) Catalyzed by Et_3N. Compound **11** (16 mg, 1 equiv) was mixed with 2-phenylethanethiol (**1**, 6 μL, 1.5 equiv) and Et_3N (12.8 μL, 2 equiv) in 50% MeOH (2 mL). The reactant mixture was stirred at room temperature overnight before the solvent was removed *in vacuo*. The residue was purified by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 70% MeCN) to yield compound **12** (13.5 mg).

*(1R,2R,3R,6R)-5-(Methoxycarbonyl)-6-(phenethylthio)cyclohex-4-ene-1,2,3-triyl Triacetate (**12**):* white solid; $[\alpha]^{20}_{D}$ −98 (c 0.68, MeOH); ^{1}H NMR (500 MHz, methanol-d_4): δ 7.21−7.28 (m, 4H), 7.17 (m, 1H), 6.66 (H-2, dd, *J* = 1.0, 4.2 Hz, 1H), 5.71 (H-3, t, *J* = 5.0 Hz, 1H), 5.57 (H-4, dd, *J* = 2.4, 5.0 Hz, 1H), 5.34 (H-5, dd, *J* = 2.4, 4.2 Hz, 1H), 3.96 (H-6, d, *J* = 4.2 Hz, 1H), 3.79 (CH_3-8, s, 3H), 2.86−3.06 (m, 4H), 2.06 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H); ^{13}C NMR (125 MHz, methanol-d_4): δ 172.0, 171.7, 171.5, 167.1, 141.6 (C-2), 134.2, 134.1, 129.7 (2C), 129.5 (2C), 127.4, 72.5 (C-5), 67.9 (C-4), 65.3 (C-3), 52.9 (CH_3-8), 44.3 (C-6), 37.2, 35.5, 20.8, 20.6 (2C); HRESIMS *m/z* 473.1255, $[M + Na]^+$ (calcd for $C_{22}H_{26}O_8SNa$, 473.1241).

Transformation of Compound 19 in 50% MeOH Catalyzed by Et_3N. Compound **19** (15 mg, 1 equiv) was mixed with Et_3N (18.8 μL, 2 equiv) in 50% MeOH (12 mL). The reactant mixture was stirred at room temperature for 1 h, and the solvent was removed *in vacuo*. The reaction products were purified from the residue by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 10% MeCN) to yield compounds **21** (4.5 mg) and **22** (3.8 mg).

*(1R,4R,5R,6S)-Methyl 4,5-dihydroxy-7-oxabicyclo[4.1.0]hept-2-ene-2-carboxylate (**21**):* colorless solid; $[\alpha]^{20}_{D}$ −54 (c 0.3, MeOH); for ^{1}H and ^{13}C NMR data, see Table 3; HRESIMS *m/z* 185.0459, $[M - H]^-$ (calcd for $C_8H_9O_5$, 185.0455).

*(3R,4S,5S,6R)-Methyl 3,4,5-Trihydroxy-6-methoxycyclohex-1-enecarboxylate (**22**):* colorless solid; $[\alpha]^{20}_{D}$ −58 (c 0.25, MeOH); for ^{1}H and ^{13}C NMR data, see Table 3; HRESIMS *m/z* 241.0694, $[M + H]^+$ (calcd for $C_9H_{14}O_6Na$, 241.0683).

Reaction of Compound 19 with 2-Phenylethanethiol (1) Catalyzed by Et_3N. Compound **19** (20 mg, 1 equiv) was mixed with 2-phenylethanethiol (**1**, 8 μL, 1 equiv) and Et_3N (125 μL, 10 equiv) in 50% MeOH (8 mL). The reactant mixture was stirred overnight at room temperature, and the solvent was removed *in vacuo*. The products were purified from the residue by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 30% MeCN) to yield compounds **23** (3.4 mg), **24** (9.5 mg), and **25** (9.0 mg).

*(3R,4S,5S,6S)-Methyl 3,4,5-Trihydroxy-6-(phenethylthio)-cyclohex-1-enecarboxylate (**23**):* white solid; $[\alpha]^{20}_{D}$ 17 (c 0.23, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 347.0928, $[M + Na]^+$ (calcd for $C_{16}H_{20}O_5SNa$, 347.0924).

*(3R,4S,5S,6R)-Methyl 3,4,5-Trihydroxy-6-(phenethylthio)-cyclohex-1-enecarboxylate (**24**):* white solid; $[\alpha]^{20}_{D}$ −137 (c 0.63, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 347.0930, $[M + Na]^+$ (calcd for $C_{16}H_{20}O_5SNa$, 347.0924).

*(3R,4R,5S,6S)-Methyl 3,4,5-Trihydroxy-6-(phenethylthio)-cyclohex-1-enecarboxylate (**25**):* white solid; $[\alpha]^{20}_{D}$ 24 (c 0.60, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 347.0928, $[M + Na]^+$ (calcd for $C_{16}H_{20}O_5SNa$, 347.0924).

Reaction of 19 with 4-Bromo-α-toluene Thioacetate (2) Catalyzed by Et_3N. Compound **19** (20 mg, 1 equiv) was mixed with 4-bromo-α-toluene thioacetate (**2**, 15 μL, 1 equiv) and Et_3N (125 μL, 10 equiv) in 50% MeOH (8 mL). The reactant mixture was stirred at room temperature overnight, and the solvent was removed *in vacuo*. The compounds were purified from the residue by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 35% MeCN) to yield compounds **26** (2.8 mg), **27** (6.8 mg), and **28** (7.0 mg).

*(3R,4S,5S,6S)-Methyl 6-((4-Bromobenzyl)thio)-3,4,5-trihydroxy-cyclohex-1-enecarboxylate (**26**):* white solid; $[\alpha]^{20}_{D}$ 38 (c 0.19, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 410.9878, $[M + Na]^+$ (calcd for $C_{15}H_{17}BrO_5SNa$, 410.9872).

*(3R,4S,5S,6R)-Methyl 6-((4-bromobenzyl)thio)-3,4,5-trihydroxy-cyclohex-1-enecarboxylate (**27**):* white solid; $[\alpha]^{20}_{D}$ −181 (c 0.45, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 410.9885, $[M + Na]^+$ (calcd for $C_{15}H_{17}BrO_5SNa$, 410.9872).

*(3R,4R,5S,6S)-Methyl 6-((4-bromobenzyl)thio)-3,4,5-trihydroxy-cyclohex-1-enecarboxylate (**28**):* white solid; $[\alpha]^{20}_{D}$ 80 (c 0.47, MeOH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 410.9885, $[M + Na]^+$ (calcd for $C_{15}H_{17}BrO_5SNa$, 410.9872).

Reaction of Pericosine A (4) with Spermine (29). Pericosine A (**4**, 4.4 mg, 1 equiv) was mixed with spermine (**29**, 4 μL, 1 equiv) in 50% MeOH (1 mL). The reactant mixture was stirred for 1 h at room temperature before the solvent was removed *in vacuo* to yield compound **30** (8.2 mg). Compound **30** (8.2 mg) was dissolved in 50% MeOH (1 mL) followed by the addition of TFA (7.6 μL), and

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 4. ^{1}H and ^{13}C NMR Data of 30 and 31

	30[a]		TFA-30[a]		[^{13}C]-30[a]	31[b]		31[c]
no.	^{13}C	^{1}H (J)	^{13}C	^{1}H (J)	$J_{C,C}$ (Hz)	^{13}C	^{1}H (J)	^{1}H (J)
1	132.0		124.1		44, 73	50.7	2.90, br s	3.15, t (4.6)
2	142.7	6.77, br s	149.8	7.04, ddd (1.8, 1.8, 1.9)	43	46.1	2.89, br d (11.0)	2.94, dd (4.6, 11.1)
3	68.6	4.31, br s	69.0	4.45, m	43, 38	69.1	4.02, br dd (2.9, 11.0)	3.80, m
4	72.3	4.04, br s	73.9	4.10, m	37, 38	71.5	4.28, br t (2.9)	3.80, m
5	72.2	3.87, br d (6.8)	70.6	4.05, dd (2.0, 9.4)	37	69.1	4.02, br dd (2.9, 11.0)	3.80, m
6	58.6	3.70, br d (6.8)	60.0	4.18, ddd (2.6, 2.6, 9.4)	44	46.1	2.89, br d (11.0)	2.94, dd (4.6, 11.1)
7	168.6		167.3		73	171.5		
8	52.6	3.77, s	53.3	3.85, s		51.9	3.72, s	3.60, s
1′/1″						36.1	3.69, d (13.4)	3.76, d (13.0)
							3.74, d (13.4)	3.82, d (13.0)
2′/2″	45.5	2.74, m	45.1	3.41, m		136.7		
				3.25, m				
3′/3″	28.3	1.74, m	24.4	2.20, m		130.8	7.16, d (8.0)	7.24, d (8.4)
4′/4″	49.2	2.90, m	45.9	3.15, m		131.9	7.46, d (8.0)	7.50, d (8.4)
5′/5″						121.5		
6′/6″	49.2	2.81, m	48.6	3.08, m		131.9	7.46, d (8.0)	7.50, d (8.4)
7′/7″	26.8	1.68, m	24.2	1.82, m		130.8	7.16, d (8.0)	7.24, d (8.4)
8′	27.6	1.61, m	24.2	1.82, m				
9′	49.8	2.68, m	48.6	3.08, m				
11′	47.9	2.74, m	45.8	3.12, m				
12′	30.7	1.76, m	25.4	2.09, m				
13′	40.3	2.82, m	37.8	3.06, m				

[a]Measured in methanol-d_4. [b]Measured in $CDCl_3$. [c]Measured in DMSO-d_6.

the mixture was stirred for 5 min. The solvents and unreacted TFA were removed *in vacuo* to yield the TFA salt of **30**, **TFA-30** (15.2 mg).

(3R,4R,5S,6R)-Methyl 6-((3-((4-((3-Aminopropyl)amino)butyl)-amino)propyl)amino)-3,4,5-trihydroxycyclohex-1-enecarboxylate (30): colorless solid; $[\alpha]^{20}_{D}$ −14 (c 1.0, MeOH); for ^{1}H and ^{13}C NMR data, see Table 4; HRESIMS m/z 389.2762, $[M + H]^+$ (calcd for $C_{18}H_{37}N_4O_5$, 389.2758).

TFA-30. $[\alpha]^{20}_{D}$ −24 (c 1.0, MeOH); for ^{1}H and ^{13}C NMR data, see Table 4.

Production of [^{13}C]-Labeled 30. To produce the [^{13}C]-labeled **30**, [^{13}C]-labeled pericosine A (3.0 mg, 1 equiv, accomplished by feeding [U-$^{13}C_6$]-D-glucose to the source fungus)[7] was reacted with spermine (**30**, 3.0 μL, 1 equiv) under the same conditions as previously described. The $J_{C,C}$ coupling constants of the [^{13}C]-labeled **30** (6.6 mg) are listed in Table 4.

Reaction of Pericosine A (4) with 4-Bromo-α-toluene Thioacetate (2) Catalyzed by Spermine (29). Pericosine A (**4**, 20 mg, 1 equiv) was mixed with 4-bromo-α-toluene thioacetate (**2**, 15 μL, 1 equiv) and spermine (20 μL, 2 equiv) in 50% propylene glycol (PG) (2 mL). The reactant mixture was stirred at room temperature overnight, and the solvent was removed *in vacuo*. Compounds were obtained from the organic residue by semipreparative HPLC using a Gemini 5 μm C_{18} column (isocratic, 65% MeCN) to yield compounds **8** (8.7 mg, racemic mixture) and **31** (12.5 mg).

(1R,2R,3R,4R,5S,6S)-Methyl 2,6-bis((4-Bromobenzyl)thio)-3,4,5-trihydroxycyclohexanecarboxylate (31): white solid; $[\alpha]^{20}_{D}$ −4 (c 0.4, MeOH); for ^{1}H and ^{13}C NMR data, see Table 4; HRESIMS m/z 612.9323, $[M + Na]^+$ (calcd for $C_{22}H_{24}O_5S_2Br_2Na$, 612.9324).

Computational Details. Conformational analyses were carried out using ComputeVOA v1.1. Geometry, frequency, ^{13}C NMR, ECD, and specific rotation calculations were applied at the DFT (density functional theory) and TD-DFT (time-dependent density functional theory) levels [B3LYP functional/6-31G(d,p) or 6-311+G(2d,p) basis set] with Gaussian '09 carried out in the gas phase. For each substance, subsets of the lowest energy conformers in the gas phase were obtained by selecting only those conformers with energies predicted to be within 2.0 kcal/mol of their respective lowest-energy conformers. The ^{13}C NMR data, ECD spectra, and specific rotation values of these conformers were summed after a Boltzmann statistical weighting. Single ECD spectra of the calculated conformers were determined by SpecDis 1.60 using a sigma value of 0.2−0.3 eV. After applying a proper scaling factor and a UV-shift correction, the Boltzmann-summed computed ECD spectra were compared with the experimentally obtained ECD data.

Biological Tests. The *in vitro* antiproliferative/cytotoxic properties of pericosine A were tested in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay against the Ect 1 human normal cervical cell line and the NIH/3T3 normal mouse fibroblast cell line.[20] The potential mutagenic activity of pericosine A was evaluated in the Ames II mutagenicity assay[17] performed by BioReliance Corporation. The dermal and ocular toxicities of pericosine A were evaluated using the EpiDerm skin irritation and EpiOcular eye irritation models[18,19] following the standard protocols provided by the manufacturer (MatTek Corporation).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.9b00415.

Synthesis of the pericosine A analogs **19** and **20**; crystal data and structure refinement for compound **3**; calculated carbon chemical shifts; results of the AMES II assay; structures of numbered compounds **1**−**36**, including examples of atom-numbering schemes for the different chemical classes (as a convenience to the reader); NMR spectra; LCMS analysis; absolute configuration and mechanism of formation; principal component analysis; ^{13}C chemical shifts; $^{3}J_{H,H}$ coupling constants; catalytic effects; catalyzed reactions; generation of the TFA salt of **30**; optimized geometries, relative energies, and Boltzmann populations; optimized conformers; ^{1}H and ^{13}C NMR data; $^{1}H-^{1}H$ COSY, HSQC, and HMBC data (PDF)

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX

AUTHOR INFORMATION

Corresponding Author

*Tel: (405) 325-6969. E-mail: rhcichewicz@ou.edu (R.H.C.).

ORCID

Lin Du: 0000-0001-8751-4482

Doug E. Frantz: 0000-0002-4509-4579

Robert H. Cichewicz: 0000-0003-0744-4117

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support from the University of Oklahoma, Office of Technology Development, Growth Fund, is appreciated. We are grateful for helpful recommendations provided by the University of Oklahoma, Center for the Creation of Economic Wealth, Technology Commercialization Program.

REFERENCES

(1) Anand, S.; Radhakrishna, S. *J. Asia Pac. Biodivers.* **2017**, *10*, 154−161.

(2) Nyhus, P. J. *Annu. Rev. Environ. Resour.* **2016**, *41*, 143−171.

(3) Wood, W. F. *Chem. Educ.* **1999**, *4*, 44−50.

(4) Aldrich, T. B. *J. Exp. Med.* **1896**, *1*, 323−340.

(5) Zaks, K. L.; Tan, E. O.; Thrall, M. A. *J. Am. Vet. Med. Assoc.* **2005**, *226*, 1516−1518.

(6) Shim, I.; Seo, G. B.; Oh, E.; Lee, M.; Kwon, J. T.; Sul, D.; Lee, B. W.; Yoon, B. I.; Kim, P.; Choi, K.; Kim, H. M. *J. Toxicol. Sci.* **2013**, *38*, 937−946.

(7) Du, L.; You, J.; Nicholas, K. M.; Cichewicz, R. H. *Angew. Chem., Int. Ed.* **2016**, *55*, 4220−4225.

(8) Usami, Y.; Ohsugi, M.; Mizuki, K.; Ichikawa, H.; Arimoto, M. *Org. Lett.* **2009**, *11*, 2699−2701.

(9) Zhang, E. S.; Xu, T. L.; Wang, D. J.; Huang, T. K.; Yuan, M.; Li, J.; Zou, Y. *RSC Adv.* **2014**, *4*, 10022−10027.

(10) Valsecchi, E.; Tacchi, A.; Prosperi, D.; Compostella, F.; Panza, L. *Synlett* **2004**, *2004*, 2529−2532.

(11) Mizuki, K.; Iwahashi, K.; Murata, N.; Ikeda, M.; Nakai, Y.; Yoneyama, H.; Harusawa, S.; Usami, Y. *Org. Lett.* **2014**, *16*, 3760−3763.

(12) Usami, Y.; Mizuki, K.; Ichikawa, H.; Arimoto, M. *Tetrahedron: Asymmetry* **2008**, *19*, 1461−1464.

(13) Usami, Y.; Mizuki, K. *J. Nat. Prod.* **2011**, *74*, 877−881.

(14) Lovaas, E. *Adv. Pharmacol.* **1996**, *38*, 119−149.

(15) Lupo, M. P. *Clin. Dermatol.* **2001**, *19*, 467−473.

(16) Yamada, T.; Iritani, M.; Ohishi, H.; Tanaka, K.; Minoura, K.; Doi, M.; Numata, A. *Org. Biomol. Chem.* **2007**, *5*, 3979−3986.

(17) Gee, P.; Maron, D. M.; Ames, B. N. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 11606−11610.

(18) Kandarova, H.; Hayden, P.; Klausner, M.; Kubilus, J.; Sheasgreen, J. *J. Vis. Exp.* **2009**, *29*, 1366.

(19) Kaluzhny, Y.; Kandarova, H.; Handa, Y.; DeLuca, J.; Truong, T.; Hunter, A.; Kearney, P.; d'Argembeau-Thornton, L.; Klausner, M. *ATLA, Altern. Lab. Anim.* **2015**, *43*, 101−127.

(20) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.

DOI: 10.1021/acs.jnatprod.9b00415
J. Nat. Prod. XXXX, XXX, XXX−XXX