Construction of an artificial system for ambrein biosynthesis and investigation of some biological activities of ambrein

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Ambergris, a sperm whale metabolite, has long been used as a fragrance and traditional medication, but it is now rarely available. The odor components of ambergris result from the photooxidative degradation of the major component, ambrein. The pharmacological activities of ambergris have also been attributed to ambrein. However, efficient production of ambrein and odor compounds has not been achieved. Here, we constructed a system for the synthesis of ambrein and odor components. First, we created a new triterpene synthase, “ambrein synthase,” for mass production of ambrein by redesigning a bacterial enzyme. The ambrein yields were approximately 20 times greater than those reported previously. Next, an efficient photooxidative conversion system from ambrein to a range of volatiles of ambergris was established. The yield of volatiles was 8–15%. Finally, two biological activities, promotion of osteoclast differentiation and prevention of amyloid β-induced apoptosis, were discovered using the synthesized ambrein.

Ambergris, a metabolic product of the sperm whale (Physeter catodon or Physeter macrocephalus), accumulates as gut concretions with a probability of about 1%, and is one of the most valuable scents of animal origin1–3. Ambergris, which exhibits various medicinal properties, has also been used worldwide as a traditional medication for maladies such as migraines, the common cold, constipation, disease of the nervous system, and rheumatism4–6. In addition, it is often used as an aphrodisiac7. However, ambergris is almost inaccessible because sperm whales are now protected under the Convention on the International Trade of Endangered Species of Wild Fauna and Flora. On rare occasions, jetsam ambergris is found on beaches around the world, and traded at a high price8. The odor components of ambergris result from the photooxidative degradation of the major component, ambrein (1)2,3,9–11, and the pharmacological activity is also believed to be due to 1 (Fig. 1)5,6. To date, efficient conversion to both 1 and odor components (2–5, Fig. 1) has never been achieved. The reported yields of chemical synthesis of 1 and conversion of 1 to odor components are 1.3–3.8% and approximately 1%, respectively11–14. In addition, since the biosynthetic pathway of 1 in sperm whales remains unclear, it is not possible to utilize the biosynthetic enzyme that produces 1.

Successful artificial enzymatic synthesis of 1 was reported in 2013 using onoceroid synthase, BmeTC, from Bacillus megaterium, which sequentially cyclizes both termini of squalene (6; Fig. 2a)12. Presumably, BmeTC first cyclizes one side end of 6, converts it into a bicycle 7, and then incorporates 7 into the same active site as a substrate and cyclizes the remaining terminal to produce onoceroids 8 and 9 (Fig. 2a). BmeTC catalyzes this two-step reaction (acycle [6] → bicycle [7] → bicycle-bicycle [8 and 9]; Fig. 2a)15. BmeTC can successfully form 1 using an abnormal monocyclic product (10), synthesized by a squalene-hopene cyclase variant (SHCD377C), as a substrate (Fig. 2b)15. Hence, enzymatic synthesis of 1 from inexpensive 6 was achieved via the route “acycle 6 → monocycle 10 → bicycle-monocycle 1” (Fig. 2b)15. Furthermore, the BmeTC variant BmeTCD373C, which is an equivalent point mutation as SHCD377C, could reportedly synthesize 1 from 6 both in vitro and in vivo (Fig. 2c)16–18.

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acquiring a “new route” that forms a monocycle 10, in addition to the wild-type route (WT route) that forms bicycle 7, BmeTCD373C synthesizes 1 via the two pathways, 6 → 7 → 1 (WT route → new route) and 6 → 10 → 1 (new route → WT route) (Fig. 2c). In a bioreactor, the production of 1 by BmeTCD373C in yeast Pichia pastoris reached a maximum titer of 105 mg L−1 of culture medium17, which was higher than that produced using other host systems or with the two enzyme system involving SHCD377C and BmeTCWT (Supplementary Table 1)17–19.

Since the production efficiency of 1 and odor components was still too low for industrialization, we sought to construct an artificial synthesis system that is dramatically more efficient than the one used currently. First, we created an “ambrein (1) synthase” that produces a higher yield of 1 than that of the final products (8 and 9) produced by wild-type BmeTC. Next, we established an efficient photooxidative conversion system to transform 1 to volatile components of ambergris. Finally, the synthesized 1 was analyzed for its bioactivities that have not yet been explored, by using cell culture assays. In this study, we focused on the effects of 1 on bone cell differentiation and against amyloid β neurotoxicity, since ambergris has been used in traditional medicine to treat.

Results

Screening for variants suitable for the synthesis of 1. Mutation analysis of BmeTC and SHC suggested that the D373 mutation of BmeTC was important for the synthesis of 1. However, none of the studies have analyzed D373 variants other than BmeTCD373C. In this study, an Escherichia coli cell-free system expressing the D373 variants (Supplementary Fig. 1) substituted with 11 amino acids (C, A, F, G, H, L, M, N, Q, S and W) was used to confirm their ability to convert 6 to 1. The results indicated that only the C mutant produced 1 (12.5% yield) (Fig. 3). This revealed that cysteine at position D373 is critical to form the monocyclic part of 1. The results indicated that only the C mutant produced 1 (12.5% yield) (Fig. 3). This revealed that cysteine at position D373 is critical to form the monocyclic part of 1.

Based on this working hypothesis, 6 residues (Y167, Y255, Y257, N302, L596, and F600) that were presumed to be located near the bicyclic or monocyclic structure during the second reaction were selected based on the modeling structure of BmeTC (Fig. 4) and replaced by a smaller Ala. Enzymatic reactions of 6 Ala mutants were performed using a cell-free system (Supplementary Fig. 1). Y167A, Y257A, and N302A variants showed a higher level of activity on substrates 6 and 7 to produce 8 and 9, compared with BmeTCWT (Fig. 5a,b and Supplementary Figs. 2 and 3). Whereas the product of BmeTCY167A containing a novel tricyclic compound was previously reported20, the enzyme activity was first revealed in this study.

On the other hand, the yield of 1 synthesized from 10 by the L596A variant was 8 times that synthesized by BmeTCWT (Fig. 5c and Supplementary Fig. 4). Thus, it may be useful to replace BmeTCWT with BmeTCL596A during the second step of the two-enzyme system consisting of SHCD377C and BmeTCWT (Fig. 2b). However, as the single enzyme system of BmeTCD373C has been shown to be more advantageous for producing of 1 in yeast (Supplementary Table 1)17,18, the present study further improved upon BmeTCD373C.

Construction of the system for artificial biosynthesis of 1. In order to improve its reactivity with monocyclic 10 and bicyclic substrate 7, 4 mutations (Y167A, Y257A, N302A, and L596A) were introduced into BmeTCD373C, following which the reactivity of these variants (Y167A/D373C, Y257A/D373C, N302A/D373C, and D373C/L596A) with 3 substrates (6, 7, and 10) was accurately analyzed using the purified enzyme (Fig. 6, Supplementary Figs. 5 and 6). The results indicated that of the double mutants, only BmeTCY167A/D373C produced...
from 6, in which the yield of 1 (21.5%) was improved approximately tenfold over that of BmeTC\textsuperscript{D373C} (2.2%) (Fig. 6a). If BmeTC\textsuperscript{Y167A/D373C} produces 1 in \textit{P. pastoris} with the same efficacy as BmeTC\textsuperscript{D373C}, an end yield of approximately 1 g L\textsuperscript{-1} culture medium can be expected.

The conversion of 6 and 7 by BmeTC\textsuperscript{Y167A/D373C} to yield 1 (21.5 and 62.0%, respectively) were 4.7 and 7.2 times greater than the conversion of 6 and 7 by BmeTC\textsuperscript{WT} to 8 and 9 (4.6 and 8.6%, respectively) by BmeTC\textsuperscript{WT} (Fig. 6a,b). This indicated that the double mutant enzyme displayed activity beyond its original function to form onoceroids (8 and 9). Since the activity of previously constructed BmeTC\textsuperscript{D373C} on substrates 6 and 7 (producing 2.2 and 0% of 1, respectively) was lower than that of BmeTC\textsuperscript{WT} (Fig. 6a,b), we named the novel enzyme created in this study, BmeTC\textsuperscript{Y167A/D373C}, as “ambrein synthase.”
BmeTCY167A/D373C reacted best with 7 rather than 6 (Fig. 6a,b), indicating that the reaction with 6 was slower in the first step (6 → 7) than in the second step (7 → 1) (Fig. 2c); thus, the first step would be rate limiting. In addition, BmeTCY167A/D373C did not react with 10 (Fig. 6c), suggesting that 1 was specifically synthesized from 7 when 6 was used as a substrate, and that BmeTCY167A/D373C mainly catalyzed the reaction 6 → 7 → 1 (Fig. 2c). Hence, to accelerate the reaction in the first step of 6 → 7 → 1 and reduce the overall quantity of by-product 10, 6 was converted to 1 by adding BmeTCWT to the reaction solution of BmeTCY167A/D373C (Fig. 2d). The yield of 1 was evaluated via a system, in which the total amount of enzymes BmeTCWT and BmeTCY167A/D373C was the same as that of a single enzyme. The yield of 1 (46.0%) obtained via the new system, comprising BmeTCWT and BmeTCY167A/D373C, was approximately twice that obtained via BmeTCY167A/D373C alone (21.5%) and approximately 20 times that of BmeTCW/D373C (2.2%) (Fig. 6a). Since the 2 enzymes can be co-expressed in the yeast P. pastoris \(^{17}\), the new system should be applicable in vivo (calculated titer: approximately 2 g 1/L culture medium in the bioreactor).

**Conversion of synthetic 1 into volatile components.** Enzymatically synthesized 1 was converted to volatiles by \( \text{O}_2 \) and these volatiles were compared with the volatiles present in ambergris. Ethanol tinctures of two ambergris samples (Supplementary Fig. 7) mainly contain 4 known compounds (2–5) and 6 other unknown compounds (12–17) (Fig. 7a,b). The ratio of volatile components was slightly different between the two ambergris samples obtained by us (Fig. 7b), suggesting that the slight difference between their odors could be due to differences in the oxidizing conditions of 1 in the environment. The conversion of 1 to volatile compounds was conducted via UV or visible light treatment using 3 photosensitizers (rose Bengal: RB, 5,10,15,20-tetraphenyl-
porphine: TPP, and methylene blue: MB). Volatile components similar to that of ambergris (2–5 and 12–17) were detected (Fig. 7b and Supplementary Figs. 8–12).

The yield of volatile components (ca. 1%) and the residual rate of 1 (ca. 68%) obtained from synthetic 1 via UV treatment were similar to those of ambergris (ca. 1% and ca. 68%, respectively) (Fig. 7c,d), and also similar to previously reported results obtained by the conversion of 1 to volatile components via visible light using a photosensitizer 5,10,15,20-tetraphenyl-21H, 23H-porphine copper (II) (yield: ca. 1% and residual rate: ca. 50%)11. In the current study, visible light treatment using 3 photosensitizers (RB, TPP and MB) resulted in higher yields of volatile components (ca. 8–15%) and a lower retention of 1 (ca. 0–13%) (Fig. 7c,d). The formation rates of volatiles from UV and RB treated 1 were more similar to those of ambergris, while the TPP and MB treated samples had a much higher proportion of 14 (12.2%) and 3 (25.5%), respectively, than the ethanol tinctures of two ambergris samples (1.2–2.1% and 1.2–4.2%) (Fig. 7b). After wetting samples with filter papers and evaporating the solvent, we compared the scents and found that the scents associated with UV and RB treatments of 1 were similar to those obtained from the ethanol tinctures of two ambergris, whereas the scents associated with TPP and MB treatments were different. Notably, the yield of valuable fragrance compound 3, which is used as a substitute for ambergris samples1–4, was 6–21 times higher in MB treated 1 than that obtained from the ethanol tinctures of two ambergris (Fig. 7b). The artificial synthetic system for the major component 1 and odor components of ambergris achieved efficient enzymatic conversion of 6 to 1 as well as efficient conversion of 1 to volatile components by 1O2.

Biological activity of 1. Ambergris was previously used as a traditional medicine for various maladies47. However, the biological activities of natural 1, the main component of ambergris (ex. ambergris samples 1 and 2 contained 1 at ca. 68%; Fig. 7d) have not been assessed extensively due to its scarcity. To date, only its aphrodisiac, antinociceptive, and elastase release inhibitory activities are known46,21. Since the enzymatic synthesis in the current study enabled sufficient production of synthetic 1, its two biological activities were analyzed. First, we analyzed the effect of 1 on the differentiation of bone cells, osteoblasts and osteoclasts. Extracellular calcium deposited by mature osteoblasts was stained with alizarin red S after cells were incubated with or without 10 μM 1. However, a significant effect of 1 on the osteoblastic activity was not detected (Supplementary Fig. 13). In contrast, 1 enhanced osteoclastic differentiation at a concentration of 10 μM (Fig. 8). The results indicated that
significantly increased the number of mature osteoclasts in a concentration-dependent manner (Fig. 8). The effect of 50 μM 1 was similar to that of 5 μM kenpaullone22, which is a strong activator of osteoclastic differentiation. This result suggested that 1 may be a promising drug candidate for osteopetrosis, caused by defective osteoclast function.

Next, we analyzed the protective effect of 1 against amyloid β (Aβ)-mediated neurotoxicity. Alzheimer’s disease (AD), the most common type of dementia, is an age-related progressive neurodegenerative disorder characterized by depositions of amyloid β (Aβ), the primary component of senile plaques23. Aβ peptides elicit neurotoxicity, leading to neuronal loss and cognitive deficits. We examined the effect of 1 on Aβ-induced apoptotic cell death in human neuroblastoma SK-N-SH cells, in which Aβ 1–42 was used to induce cell death. Exposure of SK-N-SH cells to 1 µM Aβ 1-42 for 24 h led to a markedly increased percentage of early apoptotic cells (Annexin V+/7-AAD−), as well as late apoptotic and dead cells (Annexin V+/7-AAD+, Fig. 9). Aβ1-42-induced apoptosis was significantly inhibited by pretreatment with 1 at concentrations of 1–20 µM for 24 h prior to Aβ1-42 exposure (Fig. 9). These results implied that 1 possesses the potential to prevent Aβ neurotoxicity. Presently, we are investigating whether 1 modulates apoptosis signaling pathways and comparing the efficacy of 1 and other Alzheimer’s drug candidates targeting Aβ neurotoxicity.

Discussion
The present study redesigned BmeTC to create a new enzyme named “ambrein (1) synthase” (BmeTCY167A/D373C), which displays activity beyond its wild-type function (production of 8 and 9). We also constructed an efficient in vitro artificial biosynthetic pathway, which can be used for mass production of 1 in vivo. The new two-enzyme system (Fig. 2d) gives approximately 20 times more yield of 1 than the most efficient system currently known (BmeTCD373C)17 (Fig. 6a) and is expected to produce 2 g 1/L culture medium in yeast P. pastoris. Recently, it was hypothesized that 1 is biosynthesized via the pathway 6 → 7 → 1 in sperm whales24. In addition, 2 enzymes are utilized to convert symmetric compounds to asymmetric fern onoceroids and carotenoids25,26, via a strategy similar to the one we finally adopted. It is interesting that the pathways adopted by nature are similar to the artificial pathways (Fig. 2d) we have developed.
Although earlier studies on the photooxidation of 1 were aimed at mimicking the production of volatile components of natural ambergris and isolating the volatiles, none of the studies aimed to achieve the efficient
conversion of 1 to volatiles. In this study, we were able to obtain a yield of 8–15% (Fig. 7c), which was higher than the yields obtained previously for different purposes9–11 and the content in the natural ambergris analyzed by us (Fig. 7c). The synthetic system of volatiles constructed by us could change odor depending on the type of photosensitizers used (Fig. 7b). In the future, a variety of odors may be created by examining various reaction conditions including use of different photosensitizers. In addition, although unknown volatile compounds 12–17 were detected in this study (Fig. 7a and Supplementary Fig. 12), their structures could not be determined. New odor compounds may be identified in the future if a large amount of 1 is photooxidized. Further, we identified two biological activities of 1: promotion of osteoclast differentiation and prevention of Aβ neurotoxicity (Figs. 8 and 9). However, it remains unclear how this compound performs these activities. Identification of an intracellular target molecule of 1 may allow the discovery of therapeutic agents for osteopetrosis and Alzheimer’s disease in the future.

This study differed from the conventional biosynthesis studies that have aimed to reconstruct natural biosynthetic pathways. It was a challenge to synthesize a rare natural product (1) whose biosynthetic pathway remains unclear, with an artificial biosynthetic route using an enzyme created in the laboratory. Many new natural products have been discovered by genome mining. However, if the biosynthetic enzyme is of a new type or if the natural producer of the product is unknown, genome mining cannot be performed. Therefore, it will be important in the future to synthesize desired compounds by artificially creating new biosynthetic enzymes. In addition, the system constructed in this study can be synthesize 1 analogues and fragrance analogues by redesigning enzymes and using substrate analogues, and will lead to the creation of compounds with numerous odors and biological activities beyond those found in nature in the future.

Methods

General. E. coli JM109 (Takara, Shiga, Japan) was used for sequencing analysis, and E. coli BL21(DE3) (Takara), pColdTF (Takara), and pColdI (Takara) were used to express BmeTCX genes. NMR spectra were recorded using a Bruker DPX 400 spectrometer (Billerica, MA, USA) at 400 MHz for protons (1H) and 100 MHz for carbon (13C). GC–MS was performed on a JMS-T100GCV spectrometer (JEOL, Tokyo, Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 µm; J&W Scientific, Inc., Folsom, CA, USA), using the EI mode operated at 70 eV. GC analyses were performed using a Shimadzu GC-2014 chromatograph equipped with a flame ionization detector and using a DB-1 capillary column (30 m × 0.25 mm × 0.25 µm; J&W Scientific, Inc.). GC and GC–MS conditions for the BmeTCX products were as follows: injection temperature = 300 °C, column temperature = 220–300 °C (1 °C min−1). GC and GC–MS conditions for the volatile compounds were as follows: injection temperature = 200 °C, column temperature = 40–300 °C (5 °C min−1) for GC and 30–300 °C (5 °C min−1) for GC–MS. Compound 6 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Two ambergris samples (NSMT M55020 and NSMT M55019; Supplementary Fig. 7) stored in the National Museum of Nature and Science (Japan) for more than 30 years were used for the analysis of volatile components.

Isolation and structural analysis of 1, 7, 10, and 11 synthesized from substrate 6 by BmeTCX. Compound 11, biosynthesized in the yeast P. pastoris, was identified previously by MS analysis37. However, no NMR data were available for 11. Therefore, isolation and structural analysis of 11 was performed in the present study. Compounds 1, 7, and 10 were isolated for use as substrates for enzymatic reactions and material for conversion into volatile components. As a typical example, the method used to isolate 1, 7, 10, and 11 produced by BmeTCX373C/L596A is described below. Compounds 1, 7, and 10 were synthesized and isolated via a method similar to BmeTCD373C, mainly using BmeTCY167A/D373C, BmeTCW7 and BmeTCX373C, respectively.
The expression and preparation of the cell-free extract was basically the same as in Ref. 20 as described below. *E. coli* BL21(DE3) harboring pColdTF-BmeTC<sup>D373C/L596A</sup> was grown at 37 °C in LB medium (1 L) with 100 μg mL<sup>-1</sup> ampicillin<sup>20</sup>. Expression of the recombinant protein was induced by adding 0.1 mM IPTG when OD<sub>600</sub> reached ~ 0.6<sup>20</sup>. Further cultivation of BL21(DE3) recombinants was performed for 24 h at 15°C<sup>20</sup>.

*E. coli* cells expressing recombinant BmeTCD<sup>D373C/L596A</sup> were harvested by centrifugation and resuspended in buffer A (15 mL/5 g) containing 50 mM Tris–HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM EDTA, 0.1% ascorbic acid and 0.1% Tween-80<sup>20</sup>. Cells were disrupted by sonication with UP200s (Hielscher Ultrasonics GmbH) at 4–10 °C for 15 min<sup>20</sup>. The resulting suspension was centrifuged at 12,300 × g for 20 min (twice)<sup>20</sup>. The pellet was discarded, and the resulting supernatant was used as the cell-free extract.

To isolate product 11 formed by BmeTC<sup>D373C/L596A</sup>, 6 (7.5 mg) was emulsified with Tween 80 (150 mg) in buffer A (75 mL), and incubated with the cell-free extract (300 mL) at 30 °C for 112 h. Subsequently, 15% KOH/MeOH solution (450 mL) was added to the reaction mixture and lipophilic products were extracted with n-hexane (400 mL × 3) and concentrated. The gas chromatogram of n-hexane extract is shown (Supplementary Fig. 14). The n-hexane extract (5.29 g) was partially purified using silica gel (250 g) column chromatography with n-hexane and n-hexane/EtOAc (100:20). The fraction (Fra. A: 5.0 mg) eluted with n-hexane contained substrates 6, 8, 10, and 11, whereas the fraction (Fra. B: 188.0 mg) eluted with n-hexane/EtOAc (100:20) contained 1, 7, and 9. Pure 11 (oil; 1.5 mg) and 10 (oil; 0.1 mg) were obtained by SiO<sub>2</sub> HPLC (Inertsil 100A, 7.6 × 250 mm; GL Science) with n-hexane from Fra. A, and pure 1 (oil; 2.1 mg) and 7 (oil; 0.6 mg) were obtained by SiO<sub>2</sub> HPLC (Inertsil 100A, 7.6 × 250 mm; GL Science) with n-hexane/THF (100:2) from Fra. B.

Figure 9. Protective effect of 1 on Aβ<sub>1-42</sub>-induced apoptosis in SK-N-SH cells. Cells were pretreated with different concentrations of 1 (1, 2, 5, 10 and 20 μM) for 24 h before being exposed to 1 μM Aβ<sub>1-42</sub> for 24 h. Apoptotic cells were analyzed via flow cytometry using an Annexin V/7-AAD staining assay. (a) Dot plots of representative experiments. The number in the lower right quadrant signifies the percentage of early apoptotic cells (Annexin V<sup>+</sup>/7-AAD<sup>−</sup>), and the upper right quadrant signifies the percentage of late apoptotic and dead (Annexin V<sup>+</sup>/7-AAD<sup>+</sup>) cells. (b) Percentages of early apoptotic cells (Annexin V<sup>+</sup>/7-AAD<sup>−</sup>) and late apoptotic and dead cells (Annexin V<sup>+</sup>/7-AAD<sup>+</sup>). Data are expressed as mean ± SEM; (n = 3). * and † refer to the comparison of Aβ<sub>1-42</sub> alone group versus control group in Annexin V<sup>−</sup>/7-AAD<sup>−</sup> and Annexin V<sup>−</sup>/7-AAD<sup>+</sup> cells, respectively; * or † = P < 0.01. # and ‡ refer to the comparison of 1 pretreatment groups versus Aβ<sub>1-42</sub> alone group in Annexin V<sup>−</sup>/7-AAD<sup>−</sup> and Annexin V<sup>−</sup>/7-AAD<sup>+</sup> cells, respectively; # or ‡ = P < 0.01.
The structure of compound 11 was determined using MS (Supplementary Fig. 15) and NMR (Supplementary Figs. 16–21). HR-ESI-MS detected m/z 410.3904 [M]+ (calculated 410.3913 for C23H26O). The structures and purity (> 99%) of 1, 7, and 10 were confirmed by 1H NMR to be consistent with those of previous reports35,37,38.

**Analysis of BmeTCX products using a cell-free system.** Construction of pColdTF-BmeTCX WT and pColdTF-BmeTCX D373C has been previously reported36,37. Site-directed mutagenesis of pColdTF-BmeTCX WT and pColdTF-BmeTCX D373C was performed using the Quick Change Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) and primers which are listed in Supplementary Table 2. The pColdTF-BmeTCX WT was synthetized by Genewiz (Morrisville, NC, USA) using codon-optimized sequences for *E. coli*. *E. coli* BL21 (DE3) harboring pColdTF-BmeTCX (X: WT, D373C/A/F/G/H/L/M/N/Q/S/W, Y167A, Y255A, Y257A, N302A, L596A and F600A) was grown at 37 °C in LB medium (1 L) with 100 µg mL−1 ampicillin. The expression and preparation of the cell-free extracts was basically the same as in Ref. 20 as described below. Expression of the recombinant protein was induced by adding 1.0 mM IPTG when OD600 reached ~0.6. BL21 (DE3) recombinants were further cultured for 24 h at 15°C. *E. coli* cells expressing recombinant BmeTCX were harvested by centrifugation and resuspended in buffer A (3 mL/g cells) containing 50 mM Tris–HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM EDTA, 0.1% ascorbic acid and 0.1% Tween-80. Cells were disrupted via sonication using an UP200s (Hielscher Ultrasonics GmbH) at 4–10 °C for 15 min. The resulting suspension was centrifuged at 12,300 x g for 20 min (twice)5. The pellet was discarded, and the resulting supernatant was used as cell-free extracts. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gel confirmed that all cell-free extracts contained approximately the same amount of BmeTCX (Supplementary Fig. 1).

To analyze BmeTCX products, the substrate (6, 7, or 10; 0.1 mg) was emulsified with Tween 80 (20 mg) in buffer A (1 mL), and incubated with the cell-free extracts containing BmeTCX (4 mL) at 37 °C for 64 h. After 15% KOH/MeOH solution (6 mL) was added to the reaction mixture, the lipophilic products were extracted with n-hexane (10 mL x 3) and concentrated. Next, the n-hexane extract containing the products and residual substrate was analyzed by GC and GC–MS. Standard deviations were calculated from the results of 3 replicates.

**Analysis of BmeTCX products using purified enzymes.** In order to analyze the correct enzyme activity, BmeTCX (X: WT, D373C/A/F/G/H/L/M/N/Q/S/W, Y167A, Y255A, Y257A, N302A and D373C/L596A) was expressed without the fused TF-tag. The BmeTCX WT gene was excised from the Ndel and Xhol sites of pColdTF-BmeTCX WT, and introduced into the same site of pColdI to construct pColdI-BmeTCX WT. Next, pColdI-BmeTCX WT was transformed into BL21 (DE3) cells and the expression and purification of BmeTCX was basically the same as in Ref. 29 as described below. After culturing in the case of pColdTF-BmeTCX WT, cells expressing the recombinant protein were harvested by centrifugation and disrupted by sonication in buffer B (200 mM Tris–HCl (pH 7.9) and 300 mM NaCl) (30 mL/L cultured cells) containing 10 mM imidazole and 0.1% Tween 80 at 4 °C. The homogenate was centrifuged at 18,270 x g for 20 min to prepare the supernatant containing soluble Histagged fusion protein, which was loaded into a Ni–NTA agarose column (0.2 mL; Qiagen, Hilden, Germany), followed by washing with 10 mL of buffer B containing 10 mM imidazole and then by 12 mL of buffer B containing 50 mM imidazole and 0.1% Tween 80. The purified protein was eluted with 3 mL buffer C [50 mM Tris–HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM EDTA, 300 mM NaCl and 0.1% Tween-80] by gel-filtration chromatography using a Sephadex G-10 column (GE Healthcare, Pittsburgh, PA, USA). The expression and purification of BmeTCX WT were analyzed via 10% SDS-PAGE (Supplementary Fig. 5).

The reaction mixture used to analyze the BmeTCX products in buffer C (total volume: 1 mL) contained 8.2 mM (50 µg) substrate (6, 7 or 10) emulsified with 1 mg Tween 80 and 1.4 µM (50 µg) purified BmeTCX. Reactions were performed at 37 °C for 64 h. As shown in the representative example in Supplementary Fig. 22, the time-dependent activities of BmeTCX were linear at 64 h. After 15% KOH/MeOH solution (6 mL) was added to the reaction mixture, the lipophilic products were extracted using n-hexane (2 mL x 3) and the products and residual substrate was analyzed by GC and GC–MS. Standard deviations were calculated from the results of 3 replicates.

**Conversion of 1 into volatile components.** Two ambergris tinctures were prepared with 1 mg ambergris/200 µL (95% EtOH). UV treatment of 1 was performed by irradiating a sample [1 mg 1/200 µL (95% EtOH)] in a glass vial with a UV lamp (15 W) at 26 °C for 6 weeks. Visible light treatment of 1 was carried out by irradiating a sample [1 mg 1/200 µL (RB and MB: 95% EtOH; TPP: dichloromethane)] in a glass vial with LED visible light lamp (60 W) at 26 °C for 4 h, followed by adding the photosensitizer (RB and MB: 100 µM; TPP: 50 µM) every hour and stirring. The samples were directly injected for analysis of volatile compounds by GC and GC–MS. The compounds 2–5 were identified by comparing the EI-MS spectra of 2–5 with those of the NIST library (Supplementary Figs. 8–11). Quantification of volatile compounds by GC was performed by comparison with the peak area of authentic 3 (Kao, Tokyo, Japan). Standard deviations were calculated from the results of 3 replicates.

**Cell culture and treatments.** Murine pre-osteoblastic MC3T3-E1 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in Minimum Essential Medium Eagle-alpha modification (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin. For induction of osteoblastic

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differentiation, the cells were incubated in α-MEM complete medium supplemented with 10 mM β-glycerol phosphate, 50 μg/mL ascorbic acid, 10 nM dexamethasone, and 10 μM 1 or 0.1% DMSO as a control; the medium was exchanged with fresh medium every 3 d. Murine macrophage-like pre-osteoclastic RAW264.7 cells (ATCC, Manassas, VA, USA) were cultured in α-MEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. In order to induce osteoclastic differentiation, 100 ng/mL sRANKL (Oriental Yeast, Japan) was added to the medium with different concentrations of 1 or 0.1% DMSO as a control and incubated for 4 d with 5% CO₂ at 37 °C. Human neuroblastoma SK-N-SH cells were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and cultured in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate and 1% penicillin/streptomycin. SK-N-SH cells were seeded at a density of 1 × 10⁶ cells/mL, and following overnight incubation, treated with or without 1 (1–20 μM) for 24 h. Subsequently, culture supernatants containing 1 were removed, and the cells were exposed to 1 μM Aβ₁-₄₂ (Wako, Osaka, Japan) for an additional 24 h to induce Amyloid β (Aβ)-mediated neurotoxicity.

**Alizarin red S staining.** MC3T3-E1 cells cultured in the osteoblastic differentiation medium for 3 weeks and were subsequently fixed with 4% PFA for 30 min at 24 °C. Finally, cells were incubated for 45 min at 24 °C with 1% alizarin red S (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), and then washed by distilled water³⁰. TRAP staining. Matured osteoclasts were fixed with 10% glutaraldehyde for 15 min at 37 °C and subsequently incubated for 10 min at 37 °C in TRAP staining buffer containing 10 mg/mL naphthol AS-MX phosphate, 0.3 mg/mL Fast Red Violet LB Salt, 0.1 M sodium acetate, 0.3 M potassium tartrate, 0.1% Triton X-100, and 0.1 M acetic acid³¹. TRAP-positive osteoclasts with more than 3 nuclei were considered as mature osteoclasts and counted using a light microscope (Olympus IX73, Tokyo, Japan).

**Apoptosis assay by flow cytometry.** For the apoptosis assay, flow cytometric analysis was performed using FITC-Annexin V (Biolegend, San Diego, CA, USA) and 7-Amino-Actinomycin (7-AAD; Biolegend). Cells were pretreated with or without 1 for 24 h, followed by 24 h exposure to 1 μM Aβ₁-₄₂. Subsequently, cells were harvested, washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin, and resuspended in Annexin V binding buffer at 1 × 10⁶ cells/mL. Thereafter, cells were stained with FITC-Annexin V (5 μg/mL) and 7-AAD (0.5 μg/mL) for 15 min in the dark. Finally, cells were analyzed using FACSCalibur flow cytometry system (BD Bioscience, San Jose, CA, USA). A minimum of 10,000 events was collected and the percentage of apoptotic cells was calculated using the CellQuest software (BD Bioscience).

**Statistical analysis.** Statistical significance was determined by one-way analysis of variance followed by the Tukey–Kramer test for multiple comparisons at P < 0.01.

**Data availability**

Data supporting the findings of this study are available within the article and the Supplementary Information files, and from the corresponding author upon reasonable request.

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Author contributions
Yo.K., T.H., and T.S. designed the project and wrote the manuscript. Y.Y., K.O., M.I., K.C., D.U., and T.S. performed the analysis of enzymes. Yu.K., Y.T., T.K.Y., and T.S. conducted the analysis of volatiles. Yo.K. performed the experiments of bone cells. H.T. performed the experiments of apoptotic cells. Y.Y., Yu.K., K.O., M.I., K.C., D.U., Y.T., T.K.Y., Yo.K., T.H., and T.S. analyzed the data.

Competing interests
The authors declare no competing interests.

Additional information
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