Regular Article

Synthesis and antifungal activity of the proposed structure of a volatile compound isolated from the edible mushroom *Hypsizygus marmoreus*

Nguyen Thi Ngoc Anh,[1] Daisuke Miyaji,[2] Kumiko Osaki-Oka,[2] Tatsuo Saito,[3] Atsushi Ishihara[2] and Arata Yajima[3],*

[1] Graduate School of Agriculture, Tokyo University of Agriculture, 1–1–1 Sakuragaoka, Setagaya-ku, Tokyo 156–8502, Japan
[2] Faculty of Agriculture, Tottori University, 4–101 Koyama-Minami, Tottori 680–8553, Japan
[3] Department of Chemistry for Life Sciences and Agriculture, Faculty of Life Sciences, Tokyo University of Agriculture, 1–1–1 Sakuragaoka, Setagaya-ku, Tokyo 156–8502, Japan

Ⓢ *Supplementary material*

We synthesized the proposed structure of an antifungal compound detected in the culture broth of the edible mushroom *Hypsizygus marmoreus*. Using the Evans aldol and Abiko–Masamune aldol reactions as the key steps, we synthesized all of the stereoisomers of the compound with high stereoselectivity. The GC retention times and the fragmentation patterns in the mass spectra of the synthesized isomers did not match those of the natural product. Therefore, this result may imply that it is necessary to reisolate the natural product and reconsider its structure. All of the synthesized isomers were found to exhibit antifungal activity against the phytopathogenic fungus *Alternaria brassicicola*. Due to their simple structures, the obtained isomers could be lead compounds for new pesticides.
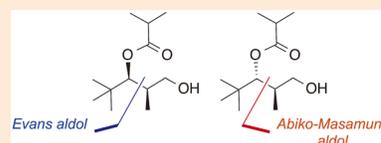
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Introduction

The increase in the world population has led to an urgent need for increased food production and efficient agriculture, resulting in the continued growth of the global fungicide market for food crops. This is because plant diseases caused by fungi, bacteria, and other pathogenic microorganisms can lead to reduced yields of agricultural products. The crops for which fungicides are mainly used include fruit crops, wheat, soybeans, grapes, and rice. In addition to the direct positive impact, pest control by fungicides has secondary positive effects due to high fertilization and early cultivation. On the other hand, it is clear that the evaluation of the benefits of fungicide use on humans and the natural environment varies depending on one's position. The adverse effects of pesticides on the cultivation of these crops have already become apparent. The impact of pesticide use on human society and the environment is becoming impossible to ignore. In modern pesticide development, in addition to pest and weed control efficacy, emphasis has been placed on safety for humans and animals and consideration for pollution of the natural environment, and excellent pesticides have been developed.[1] From this perspective, it would be advantageous if ingredients obtained from readily available foods for which safety can be guaranteed could be used as pesticides.

In 2015, Oka and coworkers discovered that 1-hydroxy-2,4,4-trimethylpentan-3-yl isobutyrate (HTI, **1**) from the mycelia and culture filtrates of the edible mushroom *Hypsizygus marmoreus* (TUFC 11906) had antifungal activity against some phytopathogenic fungi, including *Alternaria brassicicola* (Fig. 1).[2] *A. brassicicola* causes black spot disease (also known as dark leaf spot) on almost all Brassica species and essential cultivated crops such as cabbage, broccoli, and canola. This disease is of global economic importance and can reduce crop yields. Black spot, like

other diseases caused by *Alternaria* species, appears as necrotic lesions on the leaves.[3,4] The compound was reported to significantly inhibit *A. brassicicola* mycelial growth and conidial germination by 60% and 100%, respectively, indicating that it could be an effective alternative to pesticides for reducing plant diseases. Furthermore, Oka *et al.* noted that this volatile compound could be used as a new control agent to protect crops in the field and storage.[2] The proposed structure of HTI (**1**) is shown in Fig. 1 based on the GC-MS analysis of the partially purified fraction of the extract of the culture broth of *H. marmoreus*. It was proposed that HTI has a *tert*-butyl group, which is unique for a natural product.[5] Because the NMR spectra of natural HTI have not been obtained, no information about the relative and absolute configuration of HTI is available. In addition, since a quantitative supply by chemical synthesis is necessary for research for practical applications of HTI, we were interested in the asymmetric synthesis of HTI as part of our research on the control of plant pathogens.[6] This paper describes the asymmetric synthesis of the four stereoisomers of HTI and the inhibitory activities of the synthesized isomers against *A. brassicicola*.

Materials and methods

1.   Synthesis of the four stereoisomers of HTI

1.1.   (S)-4-Benzyl-3-((2S,3S)-3-hydroxy-2,4,4-trimethylpentanoyl)oxazolidin-2-one (**3**)

To a stirred solution of (S)-**2** (500 mg, 2.14 mmol) in CH_2Cl_2 (16 mL), Et_3N (598 μL, 4.29 mmol) and dibutylboryl trifluoromethanesulfonate (*n*-Bu_2BOTf, 1.0 M in CH_2Cl_2, 4.29 mL, 4.29 mmol) were slowly added at 0°C under a nitrogen atmosphere. The solution was stirred for 2 hr at 0°C. After cooling to −78°C, pivalaldehyde (349 μL, 3.22 mmol) in CH_2Cl_2 (2 mL) was added to the solution over a period of 5 min. After stirring for 2 hr at the same temperature, the solution was stirred overnight at 0°C. The reaction was quenched by the addition of MeOH (20 mL) and phosphate buffer (pH=7, 6.2 mL), and a solution of MeOH (14 mL) and aq. H_2O_2 (30%, 7 mL) was added to the mixture. The mixture was stirred for 4.5 hr at room temperature (rt). The layers were then separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (hexane:ethyl acetate=97:1 to 60:40) to give **3**[7] (550 mg, 80%) as a colorless solid. $[α]_D^{20}$=+38.8 (*c* 1.00, $CHCl_3$), (Lit 7: $[α]_D^{20}$=+36.9 (*c* 1.0, $CHCl_3$)); IR (ATR): v_{max} (cm^{-1})=3523, 1766, 1675; ^1H NMR (400 MHz, $CDCl_3$): δ 7.36–7.20 (m, 5H), 4.68 (m, 1H), 4.25–4.17 (m, 2H), 4.09, (dq, J=6.9, 3.2 Hz, 1H), 3.67 (d, J=3.2 Hz, 1H), 3.26 (dd, J=13.3, 3.2 Hz, 1H), 2.77 (dd, J=13.3, 9.7 Hz, 1H), 2.5–1.6 (br, 1H), 1.30 (d, J=6.9 Hz, 3H), 0.97 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.3, 152.8, 135.0, 129.4 (×2), 129.0 (×2), 127.4, 77.7, 66.1, 55.1, 38.7, 37.8, 35.6, 26.8 (×3), 12.7; HRMS (ESI) *m/z* calcd. for $C_{18}H_{25}NNaO_4^+$ [M+Na]$^+$ 342.1676, found 342.1667.

1.2.   (2R,3S)-2,4,4-Trimethylpentane-1,3-diol (**4a**)

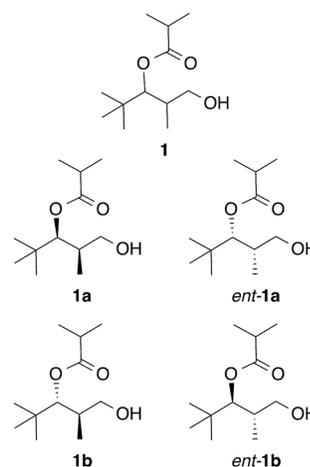To a stirred solution of **3** (525 mg, 1.65 mmol) in THF (25 mL)

Fig. 1.   Structures of 1-hydroxy-2,4,4-trimethylpentan-3-yl isobutyrate (HTI, **1**)

and MeOH (200 μL, 4.93 mmol), $LiBH_4$ (4 M in THF, 617 μL, 2.47 mmol) was added at 0°C under a nitrogen atmosphere; the mixture was stirred for 2 hr at 0°C and then overnight at rt. The reaction was quenched by the addition of aq. NH_4Cl and stirred for 1.5 hr at rt. The layers were separated, and the aqueous layer was extracted with Et_2O. The combined organic layers were washed with brine and dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (hexane:ethyl acetate=98:2 to 30:70) to give **4a** (218 mg, 91%) as a colorless oil. The enantiomeric purities of the products were determined by GC analyses. Column: Chirasil-Dex CB (25 m×0.25 mm, 0.25 μm thickness); carrier gas: He (172 kPa), 100°C (5 min) to 200°C (4.0°C/min), t_R **4a**=14.4 (100%), t_R *ent*-**4a**=14.2 (0%), t_R **4b**=13.9 (0%), t_R *ent*-**4b**=13.6 (0%). $[α]_D^{20}$=−23.9 (*c* 1.00, $CHCl_3$): IR (ATR): v_{max} (cm^{-1})=3273; ^1H NMR (400 MHz, $CDCl_3$): δ 3.66 (dd, J=10.2, 4.2 Hz, 1H), 3.62 (dd, J=10.2, 5.2 Hz, 1H), 3.51 (d, J=1.3 Hz, 1H), 1.94 (m, 1H), 1.75 (br, 2H), 1.01 (d, J=7.0 Hz, 3H), 0.95 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 80.7, 69.8, 35.6, 35.5, 26.7 (×3), 10.7; HRMS (ESI) *m/z* calcd. for $C_8H_{18}NaO_2^+$ [M+Na]$^+$ 169.1199, found 169.1200.

1.3.   (2R,3S)-1-((tert-*Butyldimethylsilyl*)oxy)-2,4,4-trimethylpentan-3-ol (**5a**)

To a stirred solution of **4a** (292 mg, 1.99 mmol) in CH_2Cl_2 (10 mL), Et_3N (834 μL, 5.98 mmol), 4-dimethylaminopyridine (DMAP, 731 mg, 5.98 mmol), and *tert*-butylchlorodimethylsilane (TBSCl, 451 mg, 2.99 mmol) were added at 0°C. After stirring for 1 hr at 0°C, the reaction was quenched with water, and the aqueous layer was extracted with Et_2O. The combined organic layer was washed with brine, dried with Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexane:EtOAc=98:2 to 60:40) to afford **5a** (517 mg, quant.) as a colorless oil. $[α]_D^{20}$=−8.3 (*c* 1.0, $CHCl_3$); IR (ATR): v_{max} (cm^{-1})=3516; ^1H NMR (400 MHz, $CDCl_3$): δ 3.66 (dd, J=9.1, 3.6 Hz, 1H), 3.58 (dd, J=9.1, 4.5 Hz, 1H), 3.48 (d, J=1.0 Hz, 1H), 1.86 (m, 1H), 1.54 (br, 1H), 0.97 (d, J=6.9 Hz,

3H), 0.93 (s, 9H), 0.89 (s, 9H), 0.06 (s, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 81.0, 70.7, 35.3, 35.2, 26.9 ($\times$3), 25.9 ($\times$3), 18.2, 11.1, -5.5, -5.6; HRMS (ESI) m/z calcd. for C$_{14}$H$_{32}$NaO$_2$Si$^+$ [M+Na]$^+$ 283.2064, found 283.2065.

1.4. (2R,3S)-1-((tert-Butyldimethylsilyl)oxy)-2,4,4-trimethylpentan-3-yl isobutyrate (6a)

To a stirred solution of **5a** (260 mg, 998 μmol) in CH$_2$Cl$_2$ (5 mL), Et$_3$N (417 μL, 2.99 mmol), DMAP (366 mg, 2.99 mmol), and isobutyryl chloride (209 μL, 2.00 mmol) were added at 0°C. After stirring for 2 hr at room temperature, the mixture was cooled to 0°C. The reaction was quenched with water, and the aqueous layer was extracted with Et$_2$O. The combined organic layer was washed with brine, dried with Na$_2$SO$_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexane : EtOAc = 98 : 2 to 60 : 40) to afford **6a** (297 mg, 90%) as a colorless oil. $[\alpha]_D^{20}$ = +5.5 (c 1.0, CHCl$_3$); IR (ATR): υ_{max} (cm^{-1}) = 1733; ^{1}H NMR (400 MHz, CDCl$_3$): δ 4.67 (d, J=2.3 Hz, 1H), 3.44 (dd, J=9.4, 7.5 Hz, 1H), 3.31 (dd, J=9.4, 7.1 Hz, 1H), 2.58 (m, 1H), 2.02 (m, 1H), 1.19 (d, J=6.9 Hz, 6H), 0.92 (s, 9H), 0.89 (s, 9H), 0.85 (d, J=6.9 Hz, 3H), 0.04 (s, 3H), 0.03 (s, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 176.6, 78.8, 67.4, 36.3, 35.4, 34.5, 26.5 ($\times$3), 25.9 ($\times$3), 19.3, 19.2, 18.2, 12.1, -5.43, -5.44; HRMS (ESI) m/z calcd. for C$_{18}$H$_{38}$NaO$_3$Si$^+$ [M+Na]$^+$ 353.2482, found 353.2451.

1.5. (2R,3S)-1-Hydroxy-2,4,4-trimethylpentan-3-yl isobutyrate (HTI, 1a)

To a stirred solution of **6a** (140 mg, 424 μmol) in THF (2 mL), hydrogen fluoride pyridine complex (*ca.* 70% HF, 110 μL) was added at 0°C. After stirring for 5 hr at 0°C, the reaction was quenched with water, and the aqueous layer was extracted with Et$_2$O. The combined organic layer was washed with water and brine, dried with Na$_2$SO$_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexane : EtOAc = 98 : 2 to 60 : 40) to afford **1a** (88.5 mg, 97%) as a colorless oil. $[\alpha]_D^{20}$ = +8.7 (c 1.0, CHCl$_3$); IR (ATR): υ_{max} (cm^{-1}) = 3448, 2969, 2874, 1731, 1711, 1469, 1387, 1366; ^{1}H NMR (400 MHz, CDCl$_3$): δ 4.71 (d, J=1.9 Hz, 1H), 3.42 (dd, J=11.4, 5.1 Hz, 1H), 3.21 (dd, J=11.4, 8.3 Hz, 1H), 2.75–2.86 (br, 1H), 2.64 (sep, J=6.9 Hz, 1H), 2.15–2.05 (m, 1H), 1.22 (d, J=7.0 Hz, 6H), 0.97 (s, 9H), 0.85 (d, J=7.0 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 178.4, 78.9, 66.2, 35.9, 35.2, 34.5, 26.8 ($\times$3), 19.24, 19.19, 11.3; HRMS (ESI) m/z calcd. for C$_{12}$H$_{24}$NaO$_3^+$ [M+Na]$^+$ 239.1618, found 239.1620.

Ent-**1a**, **1b**, and *ent*-**1b** were synthesized in a manner similar to that described for the synthesis of **1a** (see Supplementary Material).

1.6. GC-MS analysis of the synthetic compound

GC-MS analyses were performed according to the reported procedure.[2] A Shimadzu GCMS-QP2010 Plus equipped with a DB-1 column (30 m×0.25 mm, film: 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) was used. The injection volume was 1 μL, and the temperature of the injector port was 250°C. The injections were performed in the split mode at a ratio of 1/50. The carrier gas was helium, and the initial column head pressure

was 69.4 kPa. The oven temperature was set at 40°C for 4 min, linearly increased to 280°C at 10°C/min, and kept at 280°C for 2 min. All mass spectra were acquired in the electron impact (EI) mode. Ionization was kept off during the first 5 min to avoid solvent overloading. The measurable mass weight range was from m/z 50 to 500.

*2. Evaluation of the antifungal activity of the synthesized compounds*

We assayed the antifungal activity of the synthesized compounds as reported previously.[2] For the conidial germination inhibition test, conidia of *A. brassicicola* (O-264) were suspended in distilled water (DW), and the concentration was adjusted to 5×10^5 conidia/mL using a hemocytometer. One milliliter of each sample (5 mg/mL in *n*-hexane) was pipetted onto Whatman filter paper No. 2 (6 cm) and dried for 30 sec. As a control, *n*-hexane (1 mL) was pipetted. The filter paper was attached to the bottom of a petri dish, and the petri dish was inverted. On the bottom of the inverted petri dish, a glass slide drop of three drops (50 μL each) of conidial suspensions was placed on moistened papers. The dish was sealed with Parafilm and incubated at 25°C in the dark. After incubation for 24 hr, conidial germination was stopped with a lactophenol cotton blue solution and observed under a microscope (Eclipse 80i; Nikon, Tokyo, Japan). The percentage of conidial germination was calculated by counting the germinated conidia in 100 conidia, and the inhibition rate of conidial germination was calculated as compared to a control.

Results and discussion

1. Synthesis of the four stereoisomers of HTI

To synthesize the four stereoisomers of HTI, we considered it appropriate to use *syn*- and *anti*-selective asymmetric aldol reactions as the key steps. Scheme 1 summarizes our synthesis of the four stereoisomers of HTI. First, the *syn*-selective Evans asymmetric aldol reaction[8] of pivalaldehyde with (S)-**2** in accordance with the literature[7] gives **3**. After the separation of the undesired minor stereoisomers, **3** was reduced with LiBH$_4$ to give diol **4a**. The primary hydroxy group of obtained **4a** was protected as TBS ether (**5a**), and the remaining secondary hydroxy group was acylated with isobutyl chloride to give **6a**. In the final step of the synthesis, the removal of the TBS group was necessary. We examined the conditions in several ways: under the first reaction conditions using TBAF, some intramolecular acyl rearrangement proceeded as a side reaction, so that the reaction was then carried out using HF·pyridine, and we succeeded in obtaining a high yield of the desired (2S,3R)-**1a**. The overall yield of **1a** was 63.6% from commercially available (S)-**2**. The corresponding enantiomer (*ent*-**1a**) was synthesized from (R)-**2** in the same manner as was the (2S,3R)-isomer (Scheme S1).

Having succeeded in synthesizing both enantiomers of the *syn*-isomers of HTI, we next attempted to synthesize the *anti*-isomers (Scheme 1). The *anti*-selective Abiko–Masamune asymmetric aldol reaction[9] of pivalaldehyde with (1S,2R)-**7** gave a
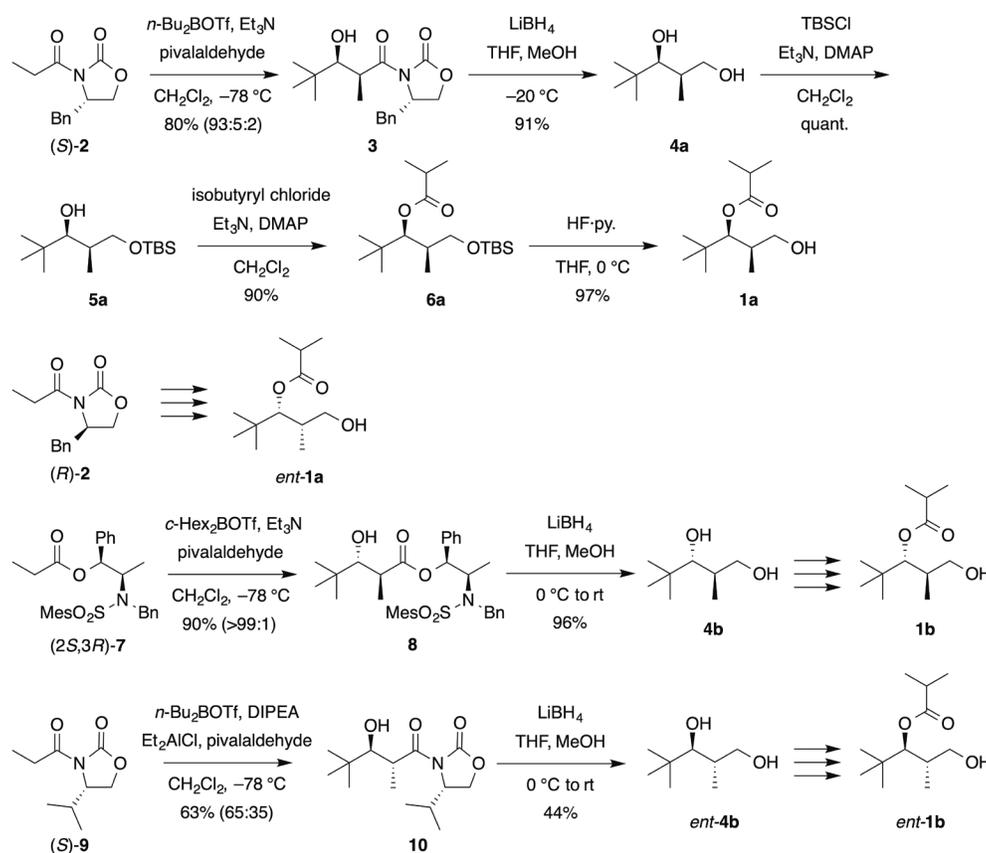
Scheme 1.   Synthesis of the four stereoisomers of HTI

high yield of **8**[10)] and had selectivity. Then, the chiral auxiliary group of **8** was reductively removed to afford *anti*-diol **4b**, and the resulting **4b** was converted into (2*R*,3*R*)-**1b** in a manner similar to the synthesis of *syn*-isomers (Scheme S1). The overall yield of (2*R*,3*R*)-**1b** was 72.5% from commercially available (1*S*,2*R*)-**10**. Although we successfully synthesized (2*R*,3*R*)-**1b**, we could not synthesize the corresponding enantiomer (*ent*-**1b**) by the same method because the starting material, *ent*-**10**, was not available. Therefore, we selected the *anti*-selective Evans aldol reaction[11)] for the synthesis of *ent*-**1b**. The Evans aldol reaction of (*S*)-**9** with pivalaldehyde in the presence of Et$_2$AlCl gave **10** in a 63% isolated yield. Although the stereoselectivity was not satisfactory (*anti*:*syn*=65:35), the resulting isomers were easily separated by chromatography. Then, the chiral auxiliary group of **10** was reductively removed to afford *ent*-**4b**, and *ent*-**4b** was converted into *ent*-**1b** (Scheme S1). The overall yield of *ent*-**1b** was 26.1% from commercially available (*S*)-**9**. The ¹H and ¹³C NMR spectra of all synthesized isomers supported their structures well. As mentioned above, none of the NMR data of natural HTI (**1**) have been reported, so it was impossible to compare the NMR data of natural HTI to the data for the synthesized isomers. Unfortunately, when we performed GC-MS analysis of the synthesized compounds, none of the isomers matched the data of the natural products.[2)] In other words, the GC retention times for both *syn*-isomer (t_R=15.67) and *anti*-

isomer (t_R=15.86) were slightly different from those of the natural product (t_R=15.66), and the fragmentation patterns in the mass spectra were far from consistent [MS *m/z*; natural product: 173, 143, 98, 89, 71, 56; *syn*-isomer: 159, 141, 127, 113, 98, 89, 83, 57, 55; *anti*-isomer: 157, 141, 127, 113, 98, 89, 83, 71, 57, 55 (Figs. S1 and S2)]. A significant difference is that the characteristic peaks—*m/z*=173, 143, and 56—observed in the spectrum of the natural product were not observed in the spectra of the synthetic compounds. Thus, these results suggest that we may need to reconsider the reported structure determination of the original natural product.

2. Evaluation of the antifungal activity of the synthetic compounds

Next, we tested the antifungal activity of the synthesized isomers of HTI against *A. brassicicola* in accordance with the reported procedure.[2)] As shown in Table 1, all stereoisomers exhibited moderate to good inhibitory activity against conidial germination at 5 mg/mL. Although only slight differences between the isomers were observed, the (3*S*)-isomers (*ent*-**1a** and *ent*-**1b**) tended to show more potent activity. This result indicates that the three-dimensional spatial arrangement of the methyl and hydroxymethyl groups rather than the stereochemistry of the ester moiety has a strong influence on the antifungal activity, suggesting that there may be some target molecule such as

Table 1. Inhibitory activity of the four stereoisomers against conidial germination of *A. brassicicola* (O-264).
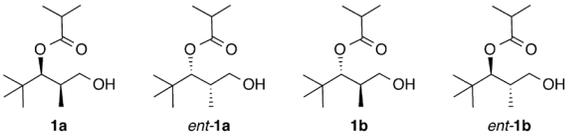
Compound	Inhibition rate (%)[a]
1a	49.0±6.6
ent-**1a**	75.3±1.2
1b	44.6±2.3
ent-**1b**	76.4±3.4

[a] The inhibition rate (%) was calculated by comparison with a control (5 mg/plate). Each value shows the average of three replications with a standard deviation (*n*=3).

protein in the mode of action of HTI. Because no pure natural product is available, and the precise IC_{50} value of the natural product has not been reported,[2] it is not possible to compare the biological activity of the synthetic product to that of the natural product. However, the antifungal activity of the synthetic products does not appear to be lower than that of the natural products.

Conclusion

In summary, we synthesized four stereoisomers of the proposed structure of an antifungal compound detected in the culture broth of the edible mushroom *H. marmoreus*. Using three different aldol reaction conditions, we synthesized all stereoisomers with high stereoselectivity. Regrettably, we were unable to determine the stereochemistry of the natural product because the GC-MS data of the natural product did not match the data of the synthetic product. Uncertainty in the structure determination is often observed in the case of compounds for which instrumental analysis data such as NMR are not sufficiently available because they can only be obtained in trace amounts from nature.[12,13] This result may suggest that for the structure determination of the antifungal compound of *H. marmoreus*, it is necessary to cultivate a large amount of *H. marmoreus* and then once again attempt to extract the natural products. Although the initial research objective—to determine the structure of the natural product—was not achieved, all synthesized isomers were found to exhibit antifungal activity against the phytopathogenic fungus *A. brassicicola*. The (3*S*)-isomers **1a** and *ent*-**1b** showed more potent activity than the (3*R*)-isomers *ent*-**1a** and **1b**, but since all isomers were active, it is clear that the racemic form can also be used as an antifungal agent. Because the synthesized compounds showed antimicrobial activity despite their simple structure, they may be leading compounds for new pesticides.

Electronic supplementary materials

The online version of this article contains supplementary materials which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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