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Practical synthesis of blepharismone, a mating inducing pheromone of *Blepharisma japonicum*

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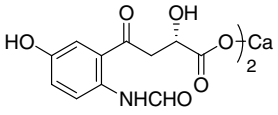
Abstract—Both enantiomers of blepharismone, a mating inducing pheromone produced by type II cells of *Blepharisma japonicum*, were synthesized via the Stille cross-coupling reaction of [4-(*tert*-butyl-dimethyl-silanyloxy)-2-trimethylstannanyl-phenyl]-carbamic acid *tert*-butyl ester with an acid chloride derived from (*S*)- and (*R*)-malic acid as a key reaction. The mating inducing activity of synthetic (*S*)-blepharismone was as effective as that of the natural one. The enantiomer (*R*)-blepharismone showed no mating inducing activity.

1. Introduction

In the ancestral ciliate *Blepharisma japonicum*, conjugation occurs between mating type I and II cells under a nutrient-deficient condition by secreting gamones (mating inducing pheromones) in the medium.[1] Blepharismone[†] **1** was isolated as gamone 2 from mating type II cells, which induces conjugation at 1 ng/ml. Further, the structure of **1** was determined as a calcium salt of 4-(2-formylamino-5-hydroxy-phenyl)-2(*S*)-hydroxy-4-oxo-butyric acid.[2] On the other hand, the amino acid sequence of blepharismone, a glycoprotein isolated as gamone 1 from mating type I cells of *B. japonicum*,[3] was recently determined by Harumoto and Sugiura;[4] further, it was shown that the expression of blepharismone was regulated developmentally and environmentally.[5] The syntheses of racemic **1** were reported by Tokoroyama et al.[6] and Jaenicke et al.,[7] albeit in low yield. The enantiomer of **1** has also been synthesized, and it was reported that it had no mating inducing activity.[8] In our effort to clarify the molecular mechanisms that regulate the conjugation of *B. japonicum*, we required molecular probes for elucidating the receptor of the gamones. In this paper, we describe the practical syntheses of enantiomerically pure **1** and its enantiomer using the Stille cross-coupling reaction as a key reaction.



Blepharismone **1**

2. Results and discussion

2.1. Synthesis of both enantiomers of blepharismone

Blepharismone **1** is a tri-substituted benzene derivative, which was fully functionalized and presumably synthesized biogenetically from tryptophan by hydroxylation at the C5 position of the indole ring, oxidative cleavage of the indole, and replacement of the amino group by a hydroxyl group. Compound **1** bears a structural resemblance to kynurenine, which was successfully synthesized by Salituro et al.[9] using the Stille coupling method;[10] their success has prompted us to apply their method for the synthesis of **1**. [4-(*tert*-Butyl-dimethyl-silanyloxy)-phenyl]-carbamic acid *tert*-butyl ester (**2**) was prepared from *p*-aminophenol according to the procedure reported by Isobe et al.[11] While the hydroxyl group of *p*-aminophenol was initially protected by a methoxy group, deprotection of derivative **6b** could not be achieved without a dehydration reaction of the side chain (vide infra); therefore, the hydroxyl group was protected by a *tert*-butyldimethylsilyl (TBDMS) group. The directed metallation of **2** using *tert*-butyllithium[12] proceeds regioselectively[11] to afford **3** by the addition of

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† Although gamone 2 was originally called blepharismin, the name was changed to blepharismone to avoid confusion since the red pigment of *Blepharisma*, zoopurpurin, was renamed as blepharismin.[1c]

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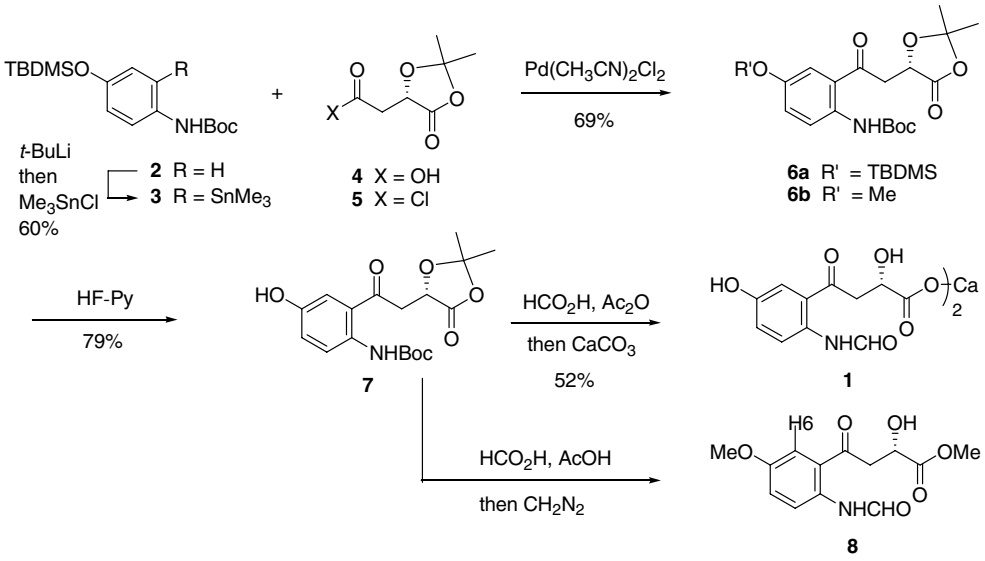

Scheme 1.

trimethyltin chloride. The crucial cross-coupling reaction of **3** and acid chloride **5** derived from (*S*)-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetic acid (**4**),[13] which was prepared from (*S*)-(+)-malic acid, was carried out using 10 mol % of $PdCl_2(CH_3CN)_2$ in toluene to afford **6a** in 63% yield. It should be mentioned that the use of acid chloride **5**, which was free from acid and prepared by adding a small amount of dimethylformamide in oxalyl chloride,[14] was crucial. Otherwise, the coupling yield of **6a** decreased dramatically. The use of palladium catalyst $Pd_2(dba)_3$ instead of $PdCl_2(CH_3CN)_2$ did not improve the yield. The removal of the TBDMS group by tetrabutylammonium fluoride was unsuccessful[15] because a dehydration reaction occurred on the side chain of the β-alkoxy carbonyl functionality to afford a 4-keto-2-butenoic acid derivative. Instead, removal was accomplished by the treatment of **6a** with HF·pyridine[16] to afford **7** in 79% yield. Removal of the acetonide and *N*-Boc groups, and formylation of the resulting amino group were carried out in one step by the treatment of **7** with a 1:5 mixture of acetic anhydride and formic acid. Triturating the resulting polar product in a saturated aq calcium carbonate solution yielded crystalline (*S*)-**1** in 52% isolated yield (Scheme 1); this was identical to natural blepharismone in all respects. For biological evaluation, (*R*)-blepharismone ((*R*)-**1**), an enantiomer of natural blepharismone, was also synthesized from (*R*)-malic acid by using the same method described above.

2.2. Enantiomeric purity and biological activity of synthetic (*S*)- and (*R*)-blepharismones

In order to determine the enantiomeric purity of synthetic (*S*)- and (*R*)-**1**, we tried to prepare a Mosher ester of **1**;[17] however, a dehydration reaction on the side chain occurred, and we could not obtain the ester. Hence, dimethyl derivative **8** was synthesized by treating with diazomethane, the crude intermediate after deprotection and formylation of **7** with acetic anhydride in formic acid. We could determine the enantiomeric purity using ^{1}H NMR measurement of **8** in the presence of a chiral shift reagent [(+)-Eu(hfc)$_3$]. We discovered that the ^{1}H NMR signals of H6 on the aromatic ring of (*S*)- and (*R*)-**8** in the presence of 1 equiv of (+)-Eu(hfc)$_3$ were separated at the baseline level (Table 1); further, none of the enantiomers were detected in each sample. Hence, it was concluded that the synthetic (*S*)- and (*R*)-**1** were enantiomeric pure.

Table 1. ^{1}H NMR chemical shifts of H6 in compounds (*S*)- and (*R*)-**8**

Conditions	Chemical shift (ppm) of H6
(*S*)- and (*R*)-**8** without (+)-Eu(hfc)$_3$	7.33
(*S*)-**8** with 1 equiv of (+)-Eu(hfc)$_3$	8.03
(*R*)-**8** with 1 equiv of (+)-Eu(hfc)$_3$	8.23

The mating inducing activity of synthetic (*S*)-blepharismone (**1**) was as effective as that of the natural one (1 ng/l unit activity)[‡] for the type I cell of *B. japonicum*. Synthetic (*R*)-blepharismone, the enantiomer of **1**, showed no mating inducing activity at a concentration of up to 500 μg/mL.

3. Conclusion

We have synthesized enantiomerically pure (*S*)-blepharismone (**1**), a mating inducing pheromone produced by type II cell, of *B. japonicum*, via the Stille cross-coupling reaction of [4-(*tert*-butyl-dimethyl-silanyloxy)-2-trimethylstannanyl-phenyl]-carbamic acid *tert*-butyl ester (**3**) and acid chloride **5** derived from (*S*)-malic acid as a key reaction. Enantiomer (*R*)-**1** was also prepared by an identical method. The enantiomeric purities of synthetic blepharismones were unam-

[‡] The mating inducing activity of blepharismone in the conjugation of *B. japonicum* can be defined as follows: 1 ng/l unit activity denotes that a concentration of 1 ng of blepharismone in 1 mL of the culture medium containing 1000 cells of *B. japonicum* causes one conjugation pair in the medium.[2]

biguously determined by ^{1}H NMR analysis of the dimethyl derivative of **1** in the presence of a chiral shift reagent. The mating inducing activity of synthetic (*S*)-**1** was found to be as potent as that of natural **1**, while enantiomer (*R*)-**1** was found to have no mating inducing activity at all. The intermediates in our synthesis of **1** could be applied to the synthesis of molecular probes for elucidating the gamone receptor of *B. japonicum*, and the results will be reported in due course.

4. Experimental

4.1. General

^{1}H and ^{13}C NMR spectra were recorded, respectively, at 400 and 100 MHz (JEOL JNM LA400) or 300 and 75 MHz (JEOL JNM LA300). Chemical shifts are reported in ppm relative to Me_4Si and $CDCl_3$ with $CHCl_3$ as the internal reference (7.26 ppm for ^{1}H NMR and 77.0 ppm for ^{13}C NMR). Coupling constants are reported in hertz (Hz) and determined directly from ^{1}H NMR spectra. Spectral splitting patterns were designated as s (singlet), d (doublet), t (triplet), m (multiplet), and br (broad). Mass spectra were obtained on JEOL JMS-700T or JEOL JMS-AX500 spectrometers. Infrared spectra were taken on a Jasco A-100 spectrometer or a Hitachi 270-30 spectrophotometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Elemental analyses were carried out at Analytical Center of Osaka City University.

All air- and moisture-sensitive reactions were carried out in flame-dried, argon-flushed, two-necked flask sealed with rubber septum, and dry solvents and reagents were introduced with a syringe. THF and Et_2O were freshly distilled from sodium benzophenone ketyl. CH_2Cl_2, $CHCl_3$, and toluene were distilled from P_2O_5 and stored over 4-Å molecular sieves. Pyridine was dried over KOH and stored over 4-Å molecular sieves. TLC was performed on Merck precoated silica gel plates (#5715), and the TLC spots were visualized under 254-nm UV light and/or by charring after dropping the plate into vanillin solution in 5% sulfuric acid/methanol. Purification of products was accomplished via Merck silica gel (#7734 and #9385) flash chromatography. Hexane and ethyl acetate were distilled and used in column chromatography.

4.2. [4-(*tert*-Butyl-dimethyl-silanyloxy)-2-trimethylstannanyl-phenyl]-carbamic acid *tert*-butyl ester 3

To a solution of [4-(*tert*-butyl-dimethyl-silanyloxy)-phenyl]-carbamic acid *tert*-butyl ester **2** (3.62 g, 11.2 mmol) in anhydrous THF (40 mL) was added *t*-BuLi (1.35 M pentane solution, 20 mL, 27.0 mmol) at −78 °C under an argon atmosphere. The mixture was stirred for 15 min at −78 °C, and 2.5 h at −20 °C. After dropwise addition of trimethyltin chloride (1.0 M THF solution, 11.2 mL, 11.2 mmol) at −20 °C, the mixture was stirred for an additional 20 min. The resulting mixture was treated with H_2O and extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel chromatography (Et_2O/hexane, 1:14) to afford 3.25 g of **3** (60%) as a slightly yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.18 (s, 6H), 0.31 (s, 9H), 0.98 (s, 9H), 1.49 (s, 9H), 6.11 (br s, 1H), 6.76 (dd, 1H, $J = 8.2$, 3.1 Hz), 6.85 (d, 1H, $J = 3.1$ Hz), 7.25 (d, 1H, $J = 8.2$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ −8.7, −4.4, 18.2, 25.7, 26.3, 28.4, 80.0, 120.2, 120.6, 127.2, 136.3, 153.0, 154.4; IR (neat) ν_{max} 3300, 1715, 1520, 1150 cm^{-1}; MS (EI) *m*/*z* 487 (M^+, 0.8), 472 (11), 416 (100), 398 (36), 372 (17), 342 (6), 284 (5), 267 (21), 223 (25), 210 (31), 192 (13), 166 (64), 135 (6), 106 (5), 73 (41), 57 (46). HRDEIMS *m*/*z*; $[M-H]^+$ calcd for $C_{20}H_{36}NO_3SiSn$, 486.1486. Found: 486.1491.

4.3. (*S*)-{4-(*tert*-Butyl-dimethyl-silanyloxy)-2-[2-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-phenyl}-carbamic acid *tert*-butyl ester (*S*)-6a

To a solution of (*S*)-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetic acid (*S*)-**4**[13] (1.19 g, 6.84 mmol) in CH_2Cl_2 (15 mL) in the presence of a catalytic amount of DMF was added $(COCl)_2$ (0.59 mL 16.9 mmol) at room temperature under an argon atmosphere, and the mixture was stirred for 30 min. The solvent was removed under reduce pressure to afford acid chloride (*S*)-**5**. The acid chloride was dissolved in anhydrous toluene (10 mL), and to a solution were added $PdCl_2(CH_3CN)_2$ (187 mg, 0.720 mmol) and a solution of **3** (3.25 g, 6.69 mmol) in anhydrous toluene (30 mL) and the mixture was stirred for 4 h at room temperature. Palladium was removed by filtration, and the filtrate was concentrated under reduced pressure. The resulting crude product was purified by silica gel chromatography (Et_2O/hexane = 1:12–1:5) to give 2.21 g of (*S*)-**6a** (69%) as a pale yellow oil. $[\alpha]_D^{28} = +4.61$ (*c* 3.93, CH_3OH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.20 (s, 6H), 0.99 (s, 9H), 1.51 (s, 9H), 1.62 (s, 3H), 1.65 (s, 3H), 3.39 (dd, 1H, $J = 18.0$, 7.3 Hz), 3.61 (dd, 1H, $J = 18.0$, 3.1 Hz), 4.94 (dd, 1H, $J = 7.3$, 3.1 Hz), 7.06 (dd, 1H, $J = 9.2$, 3.1 Hz), 7.24 (d, 1H, $J = 3.1$ Hz), 8.36 (d, 1H, $J = 9.2$ Hz), 10.44 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.3, 18.2, 25.5, 25.6, 26.9, 28.3, 41.4, 70.0, 80.4, 111.3, 115.0, 120.9, 121.5, 127.5, 136.9, 149.3, 153.2, 173.0, 197.8. IR (Nujol): ν_{max} 3220, 1785, 1715, 1655, 1515, 1180, 1150, 1120, 1045, 940, 840 cm^{-1}. MS (EI) *m*/*z* 479 (M^+, 12), 423 (13), 379 (100), 348 (5), 321 (20), 293 (23), 276 (13), 264 (57), 250 (13), 220 (12), 192 (49), 166 (6), 146 (6), 129 (22), 106 (4), 85 (6), 73 (36), 57 (30). HRDEIMS *m*/*z*; $[M]^+$ calcd for $C_{24}H_{37}NO_7Si$, 479.2339. Found: 479.2334.

4.4. (*R*)-{4-(*tert*-Butyl-dimethyl-silanyloxy)-2-[2-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-phenyl}-carbamic acid *tert*-butyl ester (*R*)-6a

Using the same procedure as above, (*R*)-**4**[13] (398 mg, 2.29 mmol) and **3** (1.10 g, 2.27 mmol) gave 644 mg of (*R*)-**6a** (59%) as a pale yellow oil. $[\alpha]_D^{24} = -4.73$ (*c* 4.14, CH_3OH). HRDEIMS *m*/*z*; $[M]^+$ calcd for $C_{24}H_{37}NO_7Si$, 479.2339. Found: 479.2320.

4.5. (*S*)-{2-[2-(2,2-Dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-4-hydroxy-phenyl}-carbamic acid *tert*-butyl ester (*S*)-7

To a solution of (*S*)-**6a** (962 mg, 2.01 mmol) in anhydrous THF (10 mL) was added HF·pyridine (2 mL) at 0 °C under

an argon atmosphere, and the mixture was stirred for 1 h. The cooling bath was removed and stirred for additional 1 h. To the reaction mixture was added saturated aq $NaHCO_3$ solution, and extracted with AcOEt. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the resulting residue was purified by silica gel chromatography (AcOEt/hexane = 1:3). The crystalline product was further recrystallized from Et_2O/hexane to give 580 mg of (*S*)-**7** (79%) as pale yellow needles; mp 147–148 °C. $[\alpha]_D^{25} = +6.55$ (*c* 3.97, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 1.51 (s, 9H), 1.62 (s, 3H), 1.65 (s, 3H), 3.39 (dd, 1H, $J = 18.0$, 7.3 Hz), 3.61 (dd, 1H, $J = 18.0$, 3.1 Hz), 4.92 (dd, 1H, $J = 7.3$, 3.1 Hz), 5.70 (br s, 1H), 7.04 (dd, 1H, $J = 9.2$, 2.4 Hz), 7.25 (d, 1H, $J = 2.4$ Hz), 8.30 (d, 1H, $J = 9.2$ Hz), 10.36 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.9, 26.8, 28.3, 41.4, 70.0, 80.6, 111.6, 115.0, 121.3, 121.6, 123.1, 135.4, 149.7, 153.4, 173.4, 197.8. 1R (Nujol) ν_{max} 3440, 3300, 1770, 1725, 1655, 1510, 1290, 1180, 1150, 1130, 830 cm^{-1}. MS (EI) *m*/*z* 365 (M^+, 16), 354 (2), 309 (17), 291 (13), 265 (94), 234 (12), 220 (4), 207 (57), 179 (24), 162 (100), 151 (28), 136 (39), 108 (20), 91 (6), 85 (12), 71 (7), 57 (73). HRDEIMS *m*/*z*; $[M]^+$ calcd for $C_{18}H_{23}NO_7$, 365.1475. Found: 365.1463. Elemental Anal. Calcd: C, 59.17; H, 6.35; N, 3.83. Found: C, 59.10; H, 6.40; N, 3.95.

4.6. (*R*)-{2-[2-(2,2-Dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-4-hydroxy-phenyl}-carbamic acid *tert*-butyl ester (*R*)-7

Using the same procedure as above, (*R*)-**6a** (644 mg, 1.43 mmol) gave 432 mg of (*R*)-**7** (83%) as pale yellow needles; mp 147–149 °C. $[\alpha]_D^{26} = -6.26$ (*c* 3.92, CH_3OH). HRDEIMS *m*/*z*; $[M]^+$ calcd for $C_{18}H_{23}NO_7$, 365.1475. Found: 365.1462. Elemental Anal. Calcd: C, 59.17; H, 6.35; N, 3.83. Found: C, 59.16; H, 6.38; N, 3.91.

4.7. (*S*)-4-(2-Formylamino-5-methoxy-phenyl)-2-hydroxy-4-oxo-butyric acid methyl ester (*S*)-8

To a suspension of (*S*)-**7** (80.3 mg, 0.22 mmol) in formic acid (1.5 mL) was added acetic anhydride (0.30 mL) at room temperature. The reaction mixture was stirred for 1.5 h and then concentrated in vacuo. The residue was treated with an excess of diazomethane in Et_2O (8 mL). The reaction mixture was concentrated under reduced pressure and the resulting crude product was purified by preparative silica gel TLC (AcOEt/hexane = 1:1) to give 47.3 mg of (*S*)-**8** (76%) as a pale yellow solid; mp 125–127 °C. $[\alpha]_D^{25} = +3.52$ (*c* 0.4, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 3.28 (br s, 1H), 3.49 (dd, 1H, $J = 17.7$, 6.1 Hz), 3.60 (dd, 1H, $J = 17.7$, 3.7 Hz), 3.83 (s, 3H), 3.84 (s, 3H), 4.65 (dd, 1H, $J = 6.1$, 3.7 Hz), 7.15 (dd, 1H, $J = 9.2$, 2.4 Hz), 7.37 (d, 1H, $J = 2.4$ Hz), 8.42 (d, 1H, $J = 1.8$ Hz), 8.68 (d, 1H, $J = 9.2$ Hz), 11.04 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 43.4, 52.9, 55.8, 66.8, 80.6, 116.0, 120.6, 122.7, 123.3, 133.4, 154.9, 159.5, 174.2, 201.0. IR ($CHCl_3$) ν_{max} 3550, 3300, 1735, 1685, 1660, 1280, 1250, 1100 cm^{-1}. HRFABMS *m*/*z*; $[M+H]^+$ calcd for $C_{13}H_{16}NO_6$, 282.0978. Found: 282.0984.

4.8. (*R*)-4-(2-Formylamino-5-methoxy-phenyl)-2-hydroxy-4-oxo-butyric acid methyl ester (*R*)-8

Using the same procedure as above, (*R*)-**7** (80.0 mg, 0.219 mmol) gave 46.2 mg of (*R*)-**8** (75%) as a pale yellow solid.

4.9. (*S*)-Blepharismone (*S*)-1

To a suspension of (*S*)-**7** (166 mg, 0.455 mmol) in formic acid (3 mL) was added acetic anhydride (0.6 mL) at room temperature. The reaction mixture was stirred for 1.5 h, and then concentrated in vacuo. To the residue were added saturated aq $CaCO_3$ (1 mL) and $CaCO_3$ (21.2 mg, 0.21 mmol) to give a precipitate, which was collected by filtration. Recrystallization from H_2O gave 64 mg of (*S*)-**1** (52%) as a yellow solid. $[\alpha]_D^{27} = -94.5$ (*c* 1.07, DMSO). ^1H NMR (400 MHz, DMSO-d_6) δ 3.17 (br s, 1H), 3.34 (s, 2H), 4.31 (br s, 1H), 6.04 (br s, 1H), 6.92 (d, 1H, $J = 8.8$, 2.4 Hz), 7.49 (s, 1H), 8.07 (d, 1H, $J = 8.8$ Hz), 8.29 (d, 1H, $J = 2.4$ Hz), 10.64 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 45.8, 69.3, 116.7, 120.1, 123.1, 127.5, 128.9, 153.4, 160.1, 177.6, 202.5. IR (Nujol) ν_{max} 3600–3000 (br), 1680, 1650, 1600, 1440 cm^{-1}.

4.10. (*R*)-Blepharismone (*R*)-1

Under the same procedure as above, (*R*)-**7** (151 mg, 0.414 mmol) gave 58 mg of (*R*)-**1** (54%). $[\alpha]_D^{26} = +94.7$ (*c* 1.08, DMSO), $[\alpha]_D^{25} = +17.9$ (*c* 0.50, H_2O), (lit.: $[\alpha]_{578\ nm}^{rt} = +24.3$ (H_2O)).[8]

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