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Casuarine Stereoisomers from Achiral Substrates: Chemoenzymatic Synthesis and Inhibitory Properties

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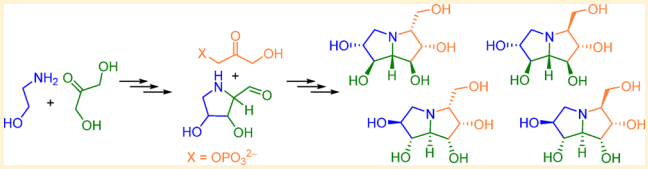
Supporting Information

ABSTRACT: A straightforward chemoenzymatic synthesis of four uncovered casuarine stereoisomers is described. The strategy consists of L-fuculose-1-phosphate aldolase F131A-variant-catalyzed aldol addition of dihydroxyacetone phosphate to aldehyde derivatives of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and its enantiomer (LAB) and subsequent one-pot catalytic deprotection−reductive amination. DAB and LAB were obtained from dihydroxyacetone and aminoethanol using D-fructose-6-phosphate aldolase and L-rhamnulose-1-phosphate aldolase catalysts, respectively. The new *ent*-3-*epi*-casuarine is a strong inhibitor of α-D-glucosidase from rice and of rat intestinal sucrase.



Casuarine (1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine, **1**; Figure 1) is a pyrrolizidine-type sugar mimic

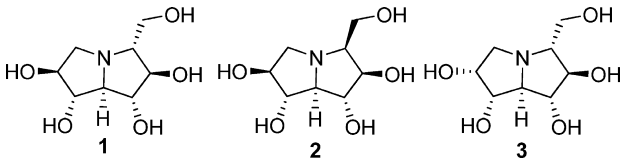


Figure 1. Naturally occurring casuarine (**1**), 3-*epi*-casuarine (**2**), and 6-*epi*-casuarine (**3**).

that was first isolated from *Casuarina equisetifolia* (Casuarinaceae) and *Eugenia jambolana* (Myrtaceae).[1−3] Bark extracts of *C. equisetifolia* have been claimed to be useful for treatment of diarrhea and colic and have been prescribed for the treatment of cancer in Western Samoa. Infusions prepared from *E. jambolana* and *Eugenia uniflora* were used in natural Paraguayan and Indian medicine as antidiarrheics, diuretics, antirheumatics, antifebriles, and antidiabetics.[4−7] Casuarine possesses six adjacent stereogenic centers, and therefore, 64 stereoisomers are possible. Two diastereomers were isolated from natural sources, namely, 3-*epi*-casuarine (**2**) from the shrub *Myrtus communis* L. (Myrtle)[8] and 6-*epi*-casuarine (**3**) from *E. uniflora* (Figure 1).[4]

Casuarine (**1**) is a potent and specific inhibitor of α-glucosidase from rice (IC_{50} = 1.2 μM), amyloglucosidase from *Aspergillus niger* (IC_{50} = 0.7 μM), rat intestinal maltase (IC_{50} = 0.7 μM), and isomaltase (IC_{50} = 3.9 μM).[9] It has recently been reported that casuarine inhibits human maltase-glucoamylase (MGAM)[10] more strongly than acarbose, a tetrasaccharide analogue currently on the market as an antidiabetic drug (Glucobay, Precose).[11]

Because of these potentially useful biological activities, synthetic efforts have been devoted to the preparation of casuarines and their stereoisomers.[3,8,12−18] The presence of six stereogenic centers is a challenge for their synthesis, which usually involves elaborated chiral starting materials and cumbersome protection−deprotection schemes that lead to moderate stereoselection and global yields.[15,16] Therefore, it is regarded as significant to devise new routes that provide concise access to casuarine stereoisomers.

During our ongoing project on the chemoenzymatic synthesis of iminocyclitols, we developed a procedure for the preparation of polyhydroxylated pyrrolizidines of the hyacinthacine and alexine types that may successfully compete with those already described.[15,19−21] Our methodology is based on aldol addition reactions of dihydroxyacetone phosphate (DHAP) to *N*-Cbz-pyrrolidinecarbaldehyde derivatives catalyzed by L-rhamnulose-1-phosphate aldolase (RhuA) and L-fuculose-1-phosphate aldolase F131A variant ($FucA^{F131A}$), both from *Escherichia coli*.[22−24] Inspired by the potential versatility of this approach, we herein explore the chemoenzymatic synthesis of highly polyhydroxylated pyrrolizidines of the casuarine type using multistep aldol addition reactions catalyzed by D-fructose-6-phosphate aldolase (FSA) and the DHAP-dependent aldolases FucA and RhuA.

As shown in the retrosynthetic analysis (Scheme 1), we envisaged that casuarines (i.e., 1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidines) could be derived by two asymmetric aldol additions of dihydroxyacetone (DHA) and reductive amination reactions starting from a conveniently protected aminoethanal (e.g., *N*-Cbz-aminoethanal).

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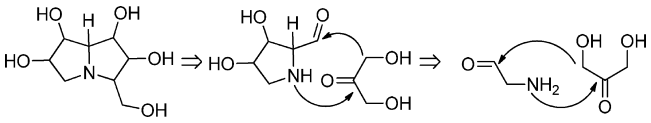
Scheme 1. Retrosynthetic Analysis of Casuarine-Type Iminocyclitols: Enzymatic Aldol Addition and Catalytic Reductive Amination



The aldol additions of DHA to *N*-Cbz-aminoethanal catalyzed by FSA and RhuA to furnish 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and its enantiomer (LAB), respectively, and their transformation into aldehydes **4** and *ent*-**4** (Scheme 2) was described in a previous work.[25] Hence, the aldol additions of DHAP to **4** and *ent*-**4** were investigated here using wild-type FucA, the FucA^{F131A} variant, and wild-type RhuA from *E. coli* (Scheme 2).

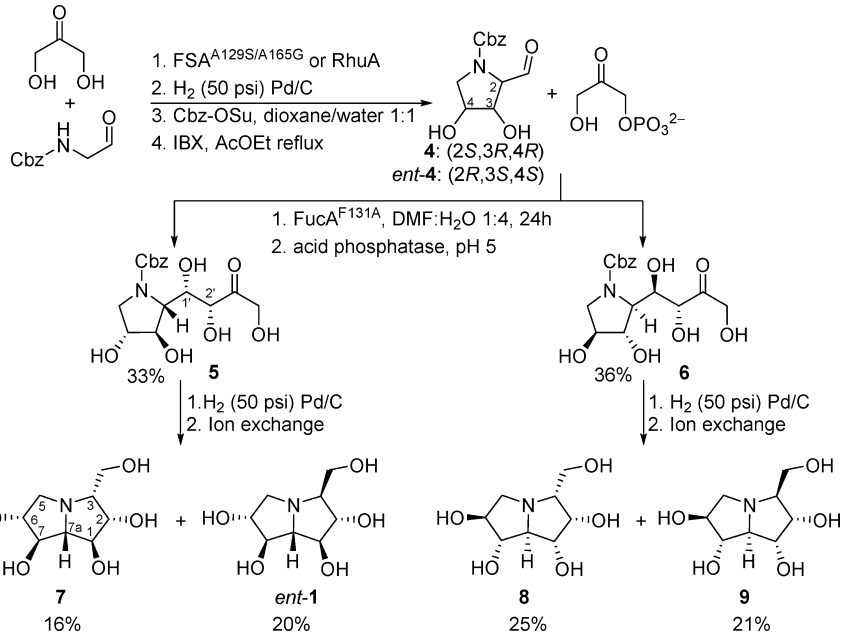
The biocatalyst screening assays (i.e., 1.2 mmol scale) revealed that only FucA^{F131A} tolerated both **4** and *ent*-**4** with 90 and 70% conversion to **5** and **6**, respectively. This is consistent with the results obtained in previous works, confirming the significant ability of FucA^{F131A} to accept constrained substrate acceptors.[23,24] Substitution of alanine for phenylalanine at position 131 in wild-type FucA creates the necessary space to accommodate bulky aldehyde acceptors.[23,24] Also noticeable is the fact that RhuA, which accepts a broad structural variation of substrates,[22,26] did not tolerate **4** and *ent*-**4**. It is likely that the sterically constrained structures of **4** and *ent*-**4** direct the hydroxyl groups toward hydrophobic areas of the enzyme active site, disfavoring the binding.

Adducts **5** and **6** were treated with acid phosphatase and purified by HPLC in ca. 30−36% isolated yield. The reductive aminations of **5** and **6** with H_2 and Pd/C catalyst rendered four casuarine stereoisomers. As ascertained in previous synthetic reports, the attack of the FucA^{F131A}-bound nucleophile to the aldehyde always occurs from the *Re* face, rendering the (*R*)-C2′ configuration (Scheme 2).[23,24,27−31] Moreover, the specific absolute configurations introduced by the stereogenic centers of the aldehydes allow the unequivocal determination of the stereochemistries of the molecules. Thus, *ent*-3-*epi*-casuarine (**7**), *ent*-casuarine (*ent*-**1**), 2-*epi*-casuarine (**8**), and 2,3-*epi*-casuarine (**9**) were obtained.

From the configurations of the stereocenters of the casuarine stereoisomers thus obtained, it can be inferred that the aldol addition of DHAP to **4** and *ent*-**4** was highly stereoselective. The stereochemical outcome depended on the aldehyde: **4** gave the *syn*-(1′*S*,2′*R*)-configured aldol adduct, while *ent*-**4** gave the *anti*-(1′*R*,2′*R*)-configured adduct, typical of L-fuculose-1-phosphate, the natural FucA substrate (Scheme 2). This stereochemical outcome appears to be general, as judged for the examples reported in previous works.[23,24] On the other hand, the reductive amination was not stereoselective, and mixtures of epimers at C3 were obtained. The reactions were scaled up to 5.2−6.0 mmol under the screening assay conditions, except for the enzyme content (i.e., 10 mg of protein/mL for 1.2 mmol scale vs 4 mg/mL for 5.2−6 mmol scale). Each pair of diastereoisomers, **7** + *ent*-**1** and **8** + **9**, was separated by ion-exchange chromatography. Purification procedures of the aldol adduct and the final compounds were not optimized.

Intriguingly, in the screening experiments conducted at the 1.2 mmol scale, the aldol addition reaction of DHAP to **4** followed by dephosphorylation and reductive amination furnished three casuarine stereoisomers: **7**, *ent*-**1**, and a minor one (~8%) identified as (1*R*,2*S*,3*R*,6*R*,7*R*,7a*S*)-1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine (*ent*-1,3-*epi*-casuarine) (compound **10**; see the Supporting Information), which was not recovered from the purification. This compound arose from the aldol adduct epimer of **5** at C1′ [i.e., (*R*)-C1′] as a result of the attack of the DHAP-FucA^{F131A} complex to the *Re* face of **4**. However, *ent*-1,3-*epi*-casuarine was not detected in the NMR spectra of the reaction performed under the 5.2−6 mmol scale conditions. It is likely that the aldol adduct precursor of *ent*-1,3-*epi*-casuarine was a thermodynamically controlled product that could appear as a consequence of the higher enzyme concentration used in the 1.2 mmol scale reactions.

Scheme 2. Chemoenzymatic Synthesis of Four Stereoisomers of Casuarine



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Compounds **7**, *ent*-**1**, **8**, and **9** were assayed as inhibitors against a panel of commercial glycosidases and rat intestinal disaccharide hydrolases. The compounds were found to be inhibitors of α-D-glucosidase from baker's yeast and rice and α-rhamnosidase from *Penicillium decumbens* (Table 1).

Table 1. Activities (IC_{50}, μM) of the Synthesized Casuarine Stereoisomers against Commercial Glycosidases;[a] For Comparison, the Activities of DAB and LAB Iminosugars Are Included

product	α-glucosidase (baker's yeast)	α-glucosidase (rice)	α-rhamnosidase (*P. decumbens*)
7	250 ± 71	7.9 ± 5.2	680 ± 193
ent-**1**	297 ± 44	261 ± 47	94 ± 32
8	320 ± 19	51 ± 8	–[b]
9	530 ± 138	111 ± 3	–[b]
DAB[c]	0.32 ± 0.03	61 ± 7	–[b]
LAB[d]	1.9 ± 0.2	0.05 ± 0.2	56 ± 5

[a]Data are means of triplicate experiments ± standard error of the mean (SE). [b]No inhibition. [c]DAB = 1,4-dideoxy-1,4-imino-D-arabinitol. [d]LAB is the enantiomer of DAB.

ent-3-*epi*-Casuarine (**7**) was a strong and selective inhibitor of α-D-glucosidase from rice. Moreover, **7** was also a strong inhibitor of rat intestinal sucrase (IC_{50} = 3.5 ± 0.6 μM) and moderate against rat intestinal maltase (IC_{50} = 39 ± 13 μM). The rest of compounds showed only moderate to weak inhibitory activities. *ent*-Casuarine (*ent*-**1**) was a moderate inhibitor of L-rhamnosidase but with lower inhibitory potency against α-glucosidase from rice.[9] Compounds **7**, *ent*-**1**, **8**, and **9** were inactive against β-D-glucosidase from sweet almonds, β-D-galactosidase bovine liver, α-D-mannosidase from jack beans, α-L-fucosidase from bovine kidney, and in front of rat intestinal lactase and threalase.

In summary, we have developed a concise chemoenzymatic method for the synthesis of four uncovered casuarine stereosiomers. The method consists of two key enzymatic aldol addition reactions to control four stereogenic centers, two of them being controlled by catalytic reductive amination. This approach did not require protection of the hydroxyl groups, minimizing the intermediate chemical transformations and the purification steps. The reductive amination was not stereoselective and thus generated two epimers, which also conferred structural diversity. The new compound *ent*-3-*epi*-casuarine (**7**) was found to be a strong and selective inhibitor of α-D-glucosidase from rice and of rat intestinal sucrase.

EXPERIMENTAL SECTION

Methods. *N*-Cbz-(2*S*,3*R*,4*R*)- and -(2*R*,3*S*,4*S*)-3,4-dihydroxypyrrolidine-2-carbaldehyde (**4** and *ent*-**4**, respectively) were prepared as previously described by our group.[25] After the oxidation step with IBX, the organic phase containing the aldehyde was washed with 5% (w/v) aqueous $NaHCO_3$, and the ethyl acetate was removed under reduced pressure.

Synthesis of (1*S*,2*S*,3*R*,6*R*,7*R*,7a*S*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (*ent*-3-*epi*-Casuarine, 7) and (1*S*,2*S*,3*S*,6*R*,7*R*,7a*S*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (*ent*-Casuarine, *ent*-1). *Enzymatic Aldol Addition.* Reactions (100 mL total volume) were carried out in 250 mL Erlenmeyer flasks. *N*-Cbz-(2*S*,3*R*,4*R*)-3,4-dihydroxypyrrolidine-2-carbaldehyde (**4**) (1.6 g, 6 mmol) was dissolved in DMF (20 mL) and cooled to 0 °C. A freshly prepared solution of DHAP at pH 6.9 (62 mL of a 97.7 mM solution, 6 mmol), KCl (2.5 mL of a 200 mM solution), and triethanolamine (TEA) buffer (5 mL of a 1 M, pH 6.7 solution) were added under vigorous agitation. Finally, $FucA^{F131A}$ (400 mg, 4 mg/mL of reaction mixture) was added, and the solution was mixed again. The reaction mixture was placed on a horizontal shaking bath (100 rpm) at a constant temperature of 4 °C. The reactions were monitored by HPLC until the peak of the aldol adduct was constant with time (24 h, 90% conversion). The enzymatic reactions were stopped by addition of MeOH (100 mL), and the precipitated enzyme was removed by centrifugation (3000 rpm, 15 min). Then the methanol was evaporated, and the aqueous solution was washed with ethyl acetate (4 × 100 mL) to remove the unreacted **4**. The aqueous layer was collected and the pH adjusted to 5 with HCl (0.5 M), and the remaining ethyl acetate dissolved in the aqueous layer was removed under reduced pressure.

Dephosphorylation. The aqueous solution was diluted with pH 5 citrate buffer (25 mL of a 1 M solution) and H_2O to a final volume of 500 mL. Acid phosphatase (572 units) was then added. The reaction was monitored by HPLC until no starting material was detected, typically 24 h. The crude reaction mixture was loaded onto a glass column (5 × 30 cm) packed with Amberlite XAD 16 (Rohm and Haas) stationary phase, previously equilibrated with H_2O. The column was washed with H_2O (2 × 600 mL) first, and the product was then eluted with 7:3 EtOH/H_2O (1750 mL, taking fractions of 250 mL). Fractions containing **5** were collected. EtOH was removed in vacuum, and the aqueous residue was lyophilized.

Removal of the Cbz Group and Reductive Amination. Pd/C (600 mg) was added to a solution of **5** (1.23 g) in 4:1 H_2O/MeOH (200 mL). The reaction mixture was shaken under H_2 (50 psi) overnight at rt, and then the catalyst was filtered off. The solvent was evaporated under reduced pressure, and the residue was lyophilized, furnishing a mixture of **7** and *ent*-**1** (400 mg, 33% yield). Compounds **7** and *ent*-**1** were separated and purified by ion-exchange chromatography (see below).

Synthesis of (1*R*,2*S*,3*R*,6*S*,7*S*,7a*R*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (2-*epi*-Casuarine, 8) and (1*R*,2*S*,3*S*,6*S*,7*S*,7a*R*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (2,3-*epi*-Casuarine, 9). The same procedure was followed starting with *N*-Cbz-(2*R*,3*S*,4*S*)-3,4-dihydroxypyrrolidine-2-carbaldehyde (*ent*-**4**) (1.4 g, 5.2 mmol). The enzymatic aldol addition provided 70% conversion after 24 h. The crude unphosphorylated aldol adduct **6** (1.1 g) gave a mixture of diasteromers **8** and **9** (380 mg, 36% yield).

Purification by Ion-Exchange Chromatography. The mixture of diasteromers **7** and *ent*-**1** (400 mg) was separated by ion-exchange chromatography on an FPLC system, and the fractions were analyzed by NMR. A glass column (450 mm × 25 mm) was packed with stationary phase (CM-Sepharose CL-6B in NH_4^+ form) into a final bed volume of 220 mL. The flow rate was 4 mL/min. The CM-Sepharose-NH_4^+ was washed initially with H_2O, and then **7** + *ent*-**1** (100 mg) dissolved in water adjusted to pH 7 with HCOOH (1 M) was loaded onto the column. Minor colored impurities were washed away with H_2O (440 mL, 2 bed volumes). The retained compounds **7** and *ent*-**1** were eluted with 320 and 240 mL, respectively, of aqueous 10 mM NH_4OH. The process was repeated (i.e., four runs) until all of the crude material was consumed. Pure fractions were pooled and lyophilized, affording **7** (65 mg, 16% from the mixture) and *ent*-**1** (80 mg, 20% from the mixture). The diasteromers **8** and **9** were separated and purified using the same procedure as above. The **8** + **9** mixture (380 mg) was loaded onto the column packed with CM-Shepharose-NH_4^+. After the column was washed with plain water, the retained compounds were eluted isocratically with aqueous 10 mM NH_4OH. The procedure was repeated (i.e., four runs) until all of the crude material was consumed, affording **8** (96 mg, 25% from the crude mixture) and **9** (80 mg, 21% from the crude mixture). The products were lyophilized at pH 5.

(1*S*,2*S*,3*R*,6*R*,7*R*,7a*S*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (*ent*-3-*epi*-Casuarine, 7). $[\alpha]_D^{22}$ −5.9 (*c* 0.85, MeOH) [lit.[15] of the enantiomer $[\alpha]_D^{25}$ +2 (*c* 0.04, H_2O); lit.[8] of the enantiomer $[\alpha]_D^{23}$ +5.7 (*c* 0.5, H_2O)]. ^1H NMR (400 MHz, D_2O): δ 4.51 (s, 1H, H-1), 4.38 (d, *J* = 2.3 Hz, 1H, H-2), 4.28 (m, 2H, H-7, H-6), 4.10 (dd, *J* = 7.1, 3.8 Hz, 2H, H-8, H-8′), 4.03 (m, 1H, H-3), 3.82

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(d, J = 8.1 Hz, 1H, H-7a), 3.72 (dd, J = 10.9, 6.2 Hz, 1H, H-5), 3.58 (t, J = 10.7 Hz, 1H, H-5′). ^{13}C NMR (101 MHz, D_2O): δ 76.7 (C-1), 76.7 (C-2), 75.9 (C-7), 75.7 (C-7a), 72.8 (C-6), 67.4 (C-3), 55.0 (C-8), 50.4 (C-5). HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for $C_8H_{16}NO_5^+$ 206.1022, found 206.1011.

(1*S*,2*S*,3*S*,6*R*,7*R*,7a*S*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (*ent*-Casuarine, *ent*-1). $[\alpha]_D^{22}$ −13.5 (c 1.0, H_2O) [lit.[16] of the enantiomer $[\alpha]_D^{23}$ +18.1 (c 1.0, H_2O); lit.[1] of the enantiomer $[\alpha]_D^{24}$ +16.9 (c 0.8, H_2O)]. ^{1}H NMR (400 MHz, D_2O) δ 4.52 (s, 1H, H-7), 4.50 (d, J = 2.1 Hz, 1H, H-6), 4.41 (t, J = 8.6 Hz, 1H, H-1), 4.01 (m, 2H, H-2, H-8), 3.89 (dd, J = 13.1, 5.3 Hz, 1H, H-8′), 3.76 (m, 2H, H-5, H-7a), 3.64 (ddd, J = 10.3, 5.2, 3.1 Hz, 1H, H-3), 3.55 (d, J = 13.1 Hz, 1H, H-5′). ^{13}C NMR (101 MHz, D_2O): δ 76.7 (C-6), 76.5 (C-7), 75.3 (C-1), 75.0 (C-7a), 73.2 (C-2), 71.0 (C-3), 58.6 (C-5), 56.8 (C-8). HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for $C_8H_{16}NO_5^+$ 206.1022, found 206.1011.

(1*R*,2*S*,3*R*,6*S*,7*S*,7a*R*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (2-*epi*-Casuarine, 8). $[\alpha]_D^{22}$ −20.8 (c 1.2, MeOH). ^{1}H NMR (400 MHz, D_2O): δ 4.37 (dd, J = 8.8, 4.0 Hz, 1H, H-1), 4.26 (m, 2H, H-2, H-6), 4.21 (t, J = 3.0 Hz, 1H, H-7), 3.85 (dd, J = 11.3, 7.2 Hz, 1H, H-8), 3.71 (dd, J = 11.2, 6.4 Hz, 1H, H-8′), 3.31 (m, 3H, H-3, H-5, H-7a), 2.94 (dd, J = 12.3, 3.7 Hz, 1H, H-5′). ^{13}C NMR (101 MHz, D_2O): δ 78.5 (C-7), 77.9 (C-6), 74.9 (C-1), 73.6 (C-7a), 73.0 (C-2), 70.3 (C-3), 59.7 (C-8), 57.9 (C-5). HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for $C_8H_{16}NO_5^+$ 206.1022, found 206.1007.

(1*R*,2*S*,3*S*,6*S*,7*S*,7a*R*)-1,2,6,7-Tetrahydroxy-3-hydroxymethylpyrrolizidine (2,3-*epi*-Casuarine, 9). $[\alpha]_D^{22}$ −12.8 (c 0.7, H_2O). ^{1}H NMR (400 MHz, D_2O): δ 4.23 (m, 1H, H-1), 4.16 (m, 2H, H-2, H-6), 3.92 (m, 3H, H-7, H-8, H-8′), 3.35 (m, 3H, H-3, H-5, H-7a), 3.00 (t, J = 9.4 Hz, 1H, H-5′). ^{13}C NMR (101 MHz, D_2O): δ 77.8 (C-7), 74.4 (C-6), 74.3 (C-7a), 73.0 (C-1), 70.2 (C-2), 64.5 (C-3), 58.2 (C-8), 50.0 (C-5). HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for $C_8H_{16}NO_5^+$ 206.1022, found 206.1007.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, experimental biological activities, and copies of ^{1}H and ^{13}C NMR, COSY, and HSQC spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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