Designed Hapten Aimed at Anti-ciguatoxin Monoclonal Antibody: Synthesis, Immunization and Discrimination of the C2 Configuration

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Abstract: The ABC-ring fragment of ciguatoxin was synthesized as a C2 epimeric mixture (1:1). The mixture, which was designed as a possible hapten for preparing an anti-ciguatoxin monoclonal antibody was conjugated with a carrier protein (KLH) and then used for immunization of mice. The three monoclonal antibodies (mAbs) obtained have shown an appreciable reactivity to the hapten. Each mAb discriminated configuration of the C2 hydroxy group of the ABC-ring fragment. The mAb which showed reactivity to the 2*S*-fragment was cross-reacted with ciguatoxin .

Key words: ciguatoxin, anti-ciguatoxin antibody, synthetic hapten, artificial antigen, immunization.

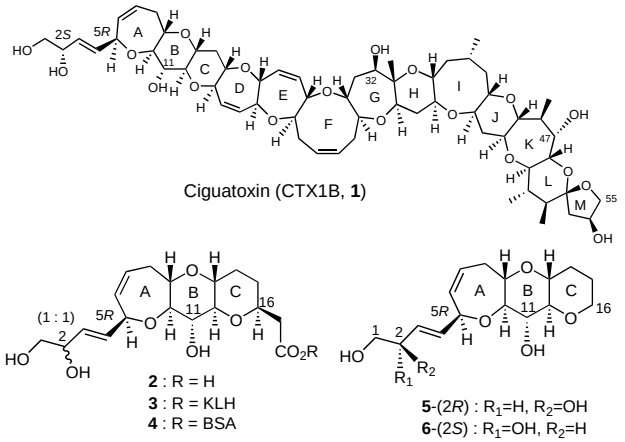


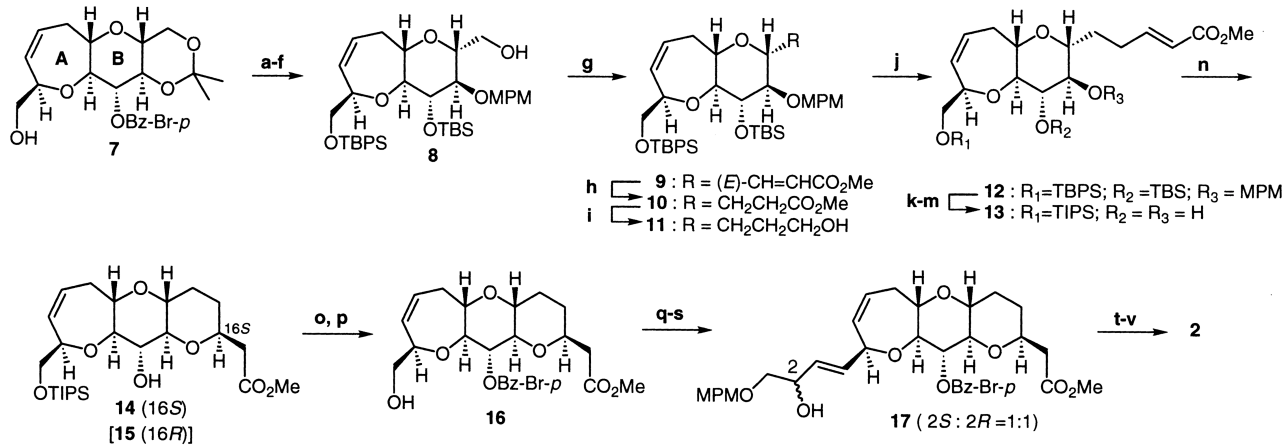
Figure 1. Structures of Ciguatoxin **(1)**, hapten **2**, hapten-protein conjugates **3** and **4**, and ABC-ring fragments **5** and **6**

Over twenty thousand people in the tropics and subtropics suffer annually from ciguatera, a widespread human food poisoning that causes gastrointestinal, neurological and cardiovascular disorders.[2] Ciguatoxin (CTX1B, **1**),[3,4] the most potent ciguatoxin, which is isolated from the moray eel, is regarded as the principal causative toxin of ciguatera. Considerable attention has been directed to the development of accurate methods for detecting ciguatoxins.[5] Although Hokama et al. prepared a monoclonal antibody (mAb) using CTX1B (**1**)[6] itself, the antibody showed a high cross-reactivity to okadaic acid. The LM-ring moiety was believed to be an antigenic determinant.[6b] Due to the extremely low accessibility of **1**, immunization with a protein conjugate composed of a structurally defined synthetic hapten should prove useful for the preparation of the antibody.[7] We focused on the ABC-ring fragment **2** (Figure 1) as a haptenic group with a carboxylic acid linker. Synthesis of the artificial antigen, immunization and reactivities of mAbs are described herein.

Synthesis of the haptenic group **2** is shown in Scheme 1.[8,9] The AB-ring fragment **7**, which is readily available from D-glucose[8] was subjected to the protecting group manipulation to yield the primary alcohol **8**. Swern oxidation of **8** followed by Wittig reaction yielded the α,β-unsaturated ester **9**. 1,4-Reduction of **9** with L-Selectride® in the presence of *tert*-butyl alcohol produced the ester **10**,[10] followed by reduction with DIBAL which yielded the alcohol **11**. Oxidation of **11** and subsequent Wittig reaction afforded the α,β-unsaturated ester **12**. Removal of silyl groups of **12** with TBAF followed by selective protection of the resulting primary alcohol with TIPS group and deprotection of MPM group produced the diol **13**. The diol **13** was treated with K_2CO_3 at room temperature for 2.5 hours and then at 50 °C for 4 hours to give the *trans*/*syn*-fused (7,6,6)-tricyclic ethers, **14** and **15** in 72% yield and a 6:1 ratio, respectively, via hetero-conjugate addition and epimerization processes.[11,12] *p*-Bromobenzoylation of **14** followed by deprotection of TIPS groups yielded **16**. Construction of the A-ring side chain from **16** was achieved by applying the reported protocol[8] to furnish a 1:1 epimeric mixture of the allylic alcohol **17**. Stepwise removal of the protecting groups[8] followed by hydrolysis of the methyl ester produced the haptenic group **2**.

The haptenic group **2** was conjugated with keyhole limpet hemocyanine (KLH) and bovine serum albumin (BSA) through their lysine residue using carbodiimide and *N*-hydroxysuccinimide.[13] The resulting hapten-protein conjugates **3** and **4** were purified by dialysis. The haptenic density in the conjugates was estimated by 2,4,6-trinitrophenylation to be 137/246 and 19/30 for KLH and BSA, respectively.[14]

The artificial antigen **3** was emulsified in RIBI adjuvant and was injected in five Balb/c mice intraperitoneally at three week intervals. After four injections, the spleen was exiled from the mice and the cells were fused with myelo-



Reagents and conditions: (a) TBPSCl/imidazole/DMF, 84%; (b) PPTs/MeOH, 98%; (c) *p*-$MeOC_6H_4CH(OMe)_2$/PPTs/CH_2Cl_2, 96%; (d) K_2CO_3/MeOH, 84%; (e) TBSCl/imidazole, 87%; (f) DIBAL/CH_2Cl_2, –30 °C, 99%; (g) $(COCl)_2$/DMSO/CH_2Cl_2, –78 °C, then Et_3N, –78° to 0 °C, then Ph_3PCHCO_2Me, 0 °C to r.t., 90%; (h) L-Selectride/*t*-BuOH/THF, –78 °C, 89%; (i) DIBAL/Et_2O, –78 °C, 88%; (j) SO_3•Py/DMSO/CH_2Cl_2, 0 °C to r.t., then Ph_3PCHCO_2Me, 93%; (k) TBAF/THF, 63%; (l) TIPSCl/Et_3N/DMAP/CH_2Cl_2, 74%; (m) DDQ/H_2O/CH_2Cl_2, 89%; (n) K_2CO_3/MeOH, r.t., 2.5 h, then 50 °C, 4 h, 72%; (o) *p*-BrBzCl/Et_3N/DMAP/CH_2Cl_2, 95%; (p) HF/MeCN, 99%; (q) Dess–Martin periodinane/CH_2Cl_2, r.t.; (r) $Ph_3PCHCOCH_2OMPM$/toluene, r.t.; (s) $NaBH_4$/$CeCl_3$•7 H_2O, –78 °C, 83% (3 steps); (t) *p*-BrBzCl/Et_3N/DMAP/CH_2Cl_2, 84%; (u) DDQ/CH_2Cl_2, 85%; (v) LiOH/H_2O/*t*-BuOH, >90%

Scheme 1

ma cells (P3X63-Ag8.653). Three hybridomas, which were designated as 4H2, 6F12, and 6H7, were obtained after the subcloning.[15]

The mAbs were tritrated with hapten-BSA conjugate **4** by ELISA, and dose-response curves were obtained as shown in Figure 2. The cross-reactivities of the mAbs to the carrier proteins, KLH and BSA, were not detectable (data not shown). The ABC-ring moiety is most likely to act as an antigenic determinant in the mAbs (4H2, 6F12 and 6H7).

To evaluate the relative affinity of these three mAbs to the haptenic group, competitive inhibition with the epimeric mixture (**2**) was then investigated (Figure 3). Satisfactory inhibition of the binding of 6H7 and 6F12 to the conjugate **4** by **2** was observed in more than 90 and 70%, respectively, while weak inhibition of 4H2 binding was seen. The order of reactivity of mAbs to **2** was estimated to be 6H7 > 6F12 > 4H2.

The mAbs to the synthetic ABC-ring fragment were thus prepared using the hapten-KLH conjugate **3** as an artificial antigen. In order to evaluate the binding selectivity of these mAbs to the C2 stereoisomers, the epimers, (2*R*,5*R*)-**5** and (2*S*,5*R*)-**6**, were then synthesized from the tetraol **18**,[8] separated and used as competitive inhibitors (Scheme 2). Two-carbon elongation from **18** followed by manipulation of the protecting groups and tosylation yielded **21**. Ring closure of the tosylate **21**[9a] upon treatment with TBAF produced the BC ring fragment **22** in

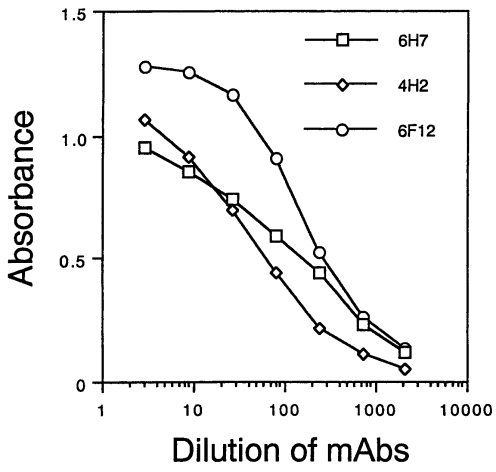


Figure 2. Titration curves of mAbs by hapten-BSA conjugate **4**

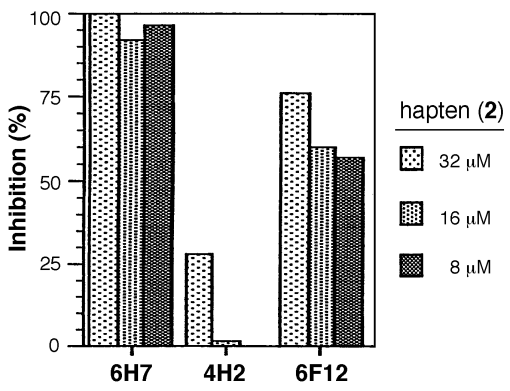
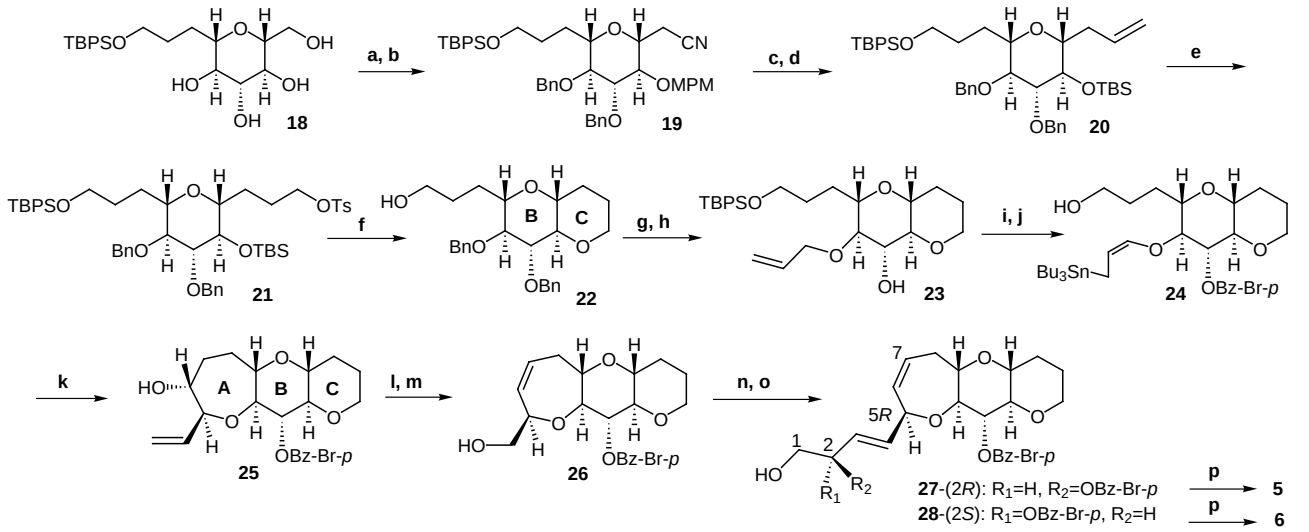


Figure 3. Competitive inhibition of the binding of mAbs to hapten-BSA conjugate **4** by **2**



Reagents and conditions: (a) *p*-$MeOC_6H_4CH(OMe)_2$/PPTs/CH_2Cl_2, r.t., then BnBr/NaH/THF/DMF (2:1), 0 °C to r.t., 90% (2 steps); (b) DIBAL/CH_2Cl_2, –20 °C, then TsCl/Py/DMAP/CH_2Cl_2, r.t., NaCN/18-crown-6/DMF, 80 °C, 73% (3 steps); (c) DDQ/H_2O/CH_2Cl_2, r.t., TBSOTf/2,6-lutidine/CH_2Cl_2, 0 °C to r.t., 66% (2 steps); (d) DIBAL/CH_2Cl_2, –78 °C, $(TMS)_2NNa$/Ph_3PMeBr/THF, 0 °C, 90% (2 steps); (e) 9-BBN/THF, sonication, then NaOH/H_2O, then TsCl/Py/, r.t., 85% (2 steps); (f) TBAF/THF, r. t., 91%; (g) TBPSCl/imidazole/DMF, r.t., then H_2/$Pd(OH)_2$/EtOH/EtOAc (1:1), 81% (2 steps); (h) PivCl/Py/DMAP, 0 °C, then Pd $(dba)_2$ (10 mol%)/dppb (40 mol%)/CH_2 = $CHCH_2CO_2Me$/THF, 65 °C, then DIBAL/CH_2Cl_2, –78 °C, 87% (3 steps); (i) BuLi/HMPA/THF, –78 °C, then Bu_3SnCl, 29% (**23** 60% recovered); (j) *p*-BrBzCl/Et_3N/DMAP/CH_2Cl_2, r.t.; then TBAF/THF, r.t., 93% (2 steps); (k) SO_3•Py/DMSO/Et_3N/CH_2Cl_2, 0 °C to r.t.; then BF_3•OEt_2/CH_2Cl_2, –78 °C, 84% (2 steps); (l) O_3, CH_2Cl_2/MeOH (3:1), –78 °C, then $NaBH_4$, –78 to 0 °C, then TBSCl/Et_3N/DMAP/CH_2Cl_2, r. t. 82% (2 steps); (m) MsCl/Et_3N/CH_2Cl_2, 0 °C to r.t., then DBU/toluene, 100 °C, TBAF/THF, r.t. 63% (3 steps); (n) Dess–Martin periodinane/CH_2Cl_2, r.t., then $Ph_3PCHCOCH_2OMPM$/benzene, r.t., then $NaBH_4$/$CeCl_3$•$7H_2O$/MeOH, –78 °C, 34% (3 steps); (o) *p*BrBzCl/Et_3N/DMAP/CH_2Cl_2, r.t., then DDQ/H_2O/CH_2Cl_2, r.t., 85%; (p) K_2CO_3/MeOH, r.t., 92% for **5** and 72% for **6**

Scheme 2

91% yield. To construct A-ring, **22** was converted to the allylic tin compound **24**. Oxidation of **24** followed by treatment with BF_3•OEt_2 yielded the ABC-ring fragment **25** in 84% yield. Construction of the C1-C7 functional groups from **25** was achieved according to the reported protocol to yield a 1:1 mixture of **27** and **28**. At this stage the epimers were separated. Methanolysis of **27** and **28** produced **5** and **6**, respectively. The C2 configuration of **5** and **6** was determined by the CD spectra of the corresponding tris-*p*-bromobenzoates.[16,17]

Binding of the mAb 6H7 to the conjugate **4** was inhibited by (2*R*)-**5** more effectively than by (2*S*)-**6** (Figure 4). In contrast, 4H2 and 6F12 were selectively bound to (2*S*)-**6**, but not to (2*R*)-**5**. Thus, each mAb distinguished the C2 configuration of the epimeric ABC-ring fragments, though the affinities to **5** and **6** were not high. As CTX1B (**1**) has a (2*S*)-configuration,[4] competitive inhibition of the binding of 6F12 to the conjugate **4** by CTX1B was then examined. Figure 5 shows the observed weak but definite cross-reactivity of 6F12 with CTX1B (**1**).

In conclusion, the ABC-ring fragment **2** of CTX1B (**1**) was synthesized as a 1:1 C2 epimeric mixture. The mixture, which was designed as a haptenic group was conjugated with a carrier protein (KLH) and then used in immunization as an artificial antigen. The three mAbs obtained discriminated the configuration of the C2 hydroxy group of the ABC-ring fragment. The mAb which showed reactivity to the fragment (2*S*)-**6** also showed a cross-reactivity with CTX1B (**1**). Affinity maturation of the mAbs and further efforts to prepare the highly sensitive anti-ciguatoxin antibodies are currently underway in our laboratory.

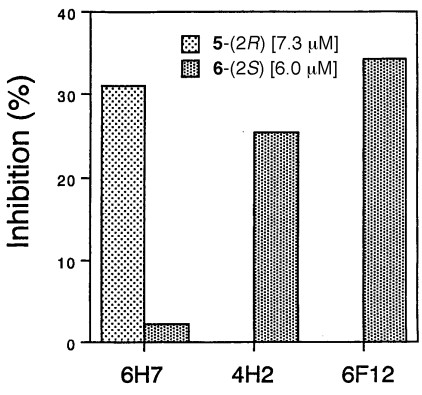


Figure 4. Competitiv inhibition of the binding of mAbs to hapten-BSA conjugate **4** by (2*R*)-**5** and (2*S*)-**6**

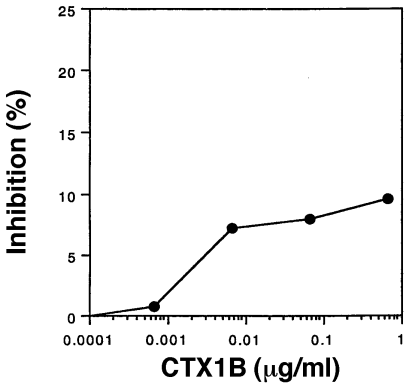


Figure 5. Competitive inhibition of the binding of mAb 6F12 to hapten-BSA conjugate **4** by CTX1B

Hapten 2

Colorless solid.

^{1}H NMR (600 MHz, CD_3OD/TMS): δ = 1.59 (1 H, m, H-15), 1.67 (1 H, m, H-14), 2.05 (1 H, m, H-15), 2.21 (1 H, m, H-14), 2.54 (1 H, br, H-17), 2.57 (1 H, m, H-8), 2.71 (1 H, br, H-17), 2.78 (1 H, m, H-8), 3.19 (1 H, t, *J* = 9 Hz, H-12), 3.27 (1 H, m, H-13), 3.41 (1 H, ddd, *J* = 9.7, 8.8, 3.8 Hz, H-9), 3.52 (1 H, m, H-10), 3.66 (1 H, dd, m, H-1), 3.69 (1 H, dd, m, H-1), 3.71 (1 H, m, H-11), 3.97 (1 H, br, H-16), 4.31 (1 H, m, H-2), 4.76 (1 H, m, H-5), 5.98 (2 H, m, H-6,7), 5.98 (1 H, m, H-3), 6.05 (1 H, m, H-4).

IR (film): ν = 3348, 2928, 2860, 2366, 1727, 1578, 1439, 1094, 752, 615 cm^{-1}.

MALDI-TOFMS: m/z calcd for $C_{18}H_{26}O_8Na$ ($M+Na^+$) 393.1526, found 393.1517.

Hapten-Protein Conjugates 3 and 4

To a stirred mixture of **2** (4.0 mg, 10 μmol) and *N*-hydroxysuccinimide (23.0 mg, 200 μmol) in DMF (200 μL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (19.2 mg, 100 μmol). The mixture was stirred at r.t. for 5 h to give 200 μL of DMF solution of the crude activated ester (ca 10 μmol).

To a stirred solution of keyhole limpet hemocyanine (5 mg) in PBS aqueous solution (800 μL) was added the resultant crude activated ester (ca. 7 μmol) in DMF (140 μL) and the mixture was allowed to stand at r.t. for 3.5 h without stirring. The resultant mixture was dialyzed against PBS aqueous solution (1 L) at 4 °C to give hapten-KLH conjugate **3**.

To a stirred solution of bovine serum albumin (2 mg, 2 μmol) in PBS aqueous solution (500 μL) was added the resultant the crude activated ester (ca. 3 μmol) in DMF (60 μL) was added and the mixture was allowed to stand at r.t. for 3.5 h without stirring. The resultant mixture was dialyzed against PBS aqueous solution (1 liter) at 4 °C to give hapten-BSA conjugate **4**.

BC-Ring Fragment 22

A solution of **21** (142 mg, 0.152 mmol) in THF (5 mL) was treated with TBAF (608 μL of 1.0 M THF solution, 0.608 mmol) and the mixture was stirred at r.t. for 12 h. Concentration and flash column chromatography (silica gel, hexane/EtOAc, 2:1) gave **22** (56.9 mg, 91%) as a colorless solid; $[\alpha]_D^{28}$ –8.73 (*c* = 1.00, $CHCl_3$).

^{1}H NMR (200 MHz, $CDCl_3$/TMS): δ = 1.48 (2 H, m), 1.70 (4 H, m), 1.98 (1 H, m), 2.14 (1 H, m), 3.12 (2 H, m), 3.26 (1 H, t, *J* = 9.5 Hz), 3.29 (1 H, t, *J* = 9.5 Hz), 3.40 (1 H, m), 3.62 (3 H, m), 3.98 (1 H, m), 4.62 (1 H, d, *J* = 11.0 Hz), 4.75 (1 H, d, *J* = 11.0 Hz), 4.94 (1 H, d, *J* = 11.0 Hz), 4.99 (1 H, d, *J* = 11.0 Hz), 7.2–7.4 (10 H, m).

IR (film): ν = 3418, 3030, 2854, 1453, 1097, 1028, 736, 698 cm^{-1}.

ABC-Ring Moiety 25

Oxidation of the Primary Alcohol 24 to Aldehyde

A solution of the alcohol **24** (510 mg, 0.685 mmol) and Et_3N (955 μL, 6.85 mmol) in DMSO (1.5 mL) and CH_2Cl_2 (6 mL) was treated with SO_3•pyridine (435 mg, 2.74 mmol) at 0 °C and allowed to stand at r.t. for 40 min. The mixture was quenched with H_2O (2 mL) and extracted with EtOAc (200 mL). The separated organic layer was washed with H_2O, brine and dried (Na_2SO_4). The filtrate was concentrated and subjected to flash column chromatography (silica gel, hexane/EtOAc/Et_3N, 100:25:1) to give the corresponding aldehyde (473 mg, 93%).

^{1}H NMR (200 MHz, $CDCl_3$/TMS): δ = 0.60–0.90 (15 H, m), 1.15–1.65 (20 H, m), 1.81 (1 H, m), 2.16 (1 H, m), 2.57 (2 H, m), 3.09 (1 H, t, *J* = 9.5 Hz), 3.24 (2 H, m), 3.48 (2 H, m), 3.92 (1 H, m), 4.33 (1 H, td, *J* = 9.0, 6.0 Hz), 5.38 (1 H, t, *J* = 9.0 Hz), 5.77 (1 H, dt, *J* = 6.0, 1.5 Hz), 7.57 (2 H, m), 7.90 (2 H, m), 9.78 (1 H, t, *J* = 1.5 Hz).

Cyclization of Aldehyde to 25

To a stirred solution of the resultant aldehyde (473 mg, 0.637 mmol) in CH_2Cl_2 (30 mL) was added dropwise BF_3•OEt_2 (8.2 mL of 0.1 M CH_2Cl_2 solution, 0.82 mmol) over a period of 20 min at –78 °C. After 40 min, the mixture was quenched with aq satd $NaHCO_3$ solution (2 mL) and extracted with Et_2O and EtOAc. The organic layer was washed with brine and dried ($MgSO_4$). Filtration, concentration and flash column chromatography (silica gel, hexane/EtOAc, 2:1) afforded **25** (259 mg, 90%) as a colorless oil; $[\alpha]_D^{24}$+5.55 (*c* = 1.03, $CHCl_3$).

^{1}H NMR (200 MHz, $CDCl_3$/TMS): δ = 1.52 (1 H, m), 1.72 (2 H, m), 1.95 (4 H, m), 2.14 (1 H, m), 3.14 (1 H, t, *J* = 9.5 Hz), 3.28 (2 H, m), 3.42 (1 H, m), 3.52 (1 H, t, *J* = 9.0 Hz), 3.78 (2 H, m), 3.93 (1 H, m), 4.92 (1 H, dt, *J* = 11.0, 1.5 Hz), 5.08 (1 H, dt, *J* = 17.0, 1.5 Hz), 5.32 (1 H, t, *J* = 9.0 Hz), 5.72 (1 H, ddd, *J* = 17.0, 11.0, 5.0 Hz), 7.57 (2 H, m), 7.82 (2 H, m).

IR (film): ν = 3496, 2946, 2870, 1727, 1591, 1487, 1458, 1400, 1332, 1272, 1174, 1100, 1054, 1013, 946, 843, 754, 685, 623, 596 cm^{-1}.

MALDI-TOFMS: m/z calcd for $C_{21}H_{25}{}^{79}BrO_6Na$ ($M+Na^+$) 475.0733, found 475.0815.

Allylic *p*-Bromobenzoates 27, 28

A solution of **26** (70.6 mg, 0.161 mmol) in CH_2Cl_2 (4 mL) was treated with Dess–Martin periodinane (340 mg, 0.803 mmol) and the mixture was stirred at r.t. for 45 min. The mixture was quenched with aq sat. $Na_2S_2O_3$ solution (1 mL) at 0 °C, diluted with Et_2O (20 mL) and stirred at r.t.for 20 min. The organic layer was separated, washed with aq sat. $NaHCO_3$ solution, dried (Na_2SO_4) and filtered. The filtrate was concentrated in vacuo and treated immediately with a solution of $Ph_3PCHCOCH_2OMPM$ (731 mg, 1.61 mmol) in benzene (3 mL) at r.t. After stirring for 2 h, the mixture was concentrated and subjected to column chromatography on florisil (hexane/EtOAc, 2:1) to give a crude enone. To a stirred mixture of $CeCl_3$•$7H_2O$ (300 mg, 0.803 mmol) in MeOH (2 mL) was added a solution of the enone in MeOH (3 mL) at –78 °C. The mixture immediately treated with $NaBH_4$ (30 mg, 0.803 mmol) and was allowed to warm to –35 °C for 40 min. The mixture was quenched with aq sat. NH_4Cl (1 mL) and diluted with EtOAc. The organic layer was concentrated, diluted with EtOAc, washed with H_2O, aq sat. $NaHCO_3$ and dried ($MgSO_4$). Filtration, concentration and flash column chromatography (silica gel, hexane/EtOAc, 2:1) afforded the crude allylic alcohol as a diastereomeric mixture (1:1) at C2 (33.8 mg, 34% from **26**).

^{1}H NMR (200 MHz, $CDCl_3$ /TMS): δ = 1.51 (1 H, m), 1.72 (2 H, m), 2.13 (1 H, m), 2.38 (1 H, br, OH), 2.40 (1 H, m), 2.68 (1 H, m), 2.90–3.48 (5 H, m), 3.53 (1 H, t, *J* = 9.0 Hz), 3.59 (1 H, t, *J* = 9.0 Hz), 3.81 (3 H, s), 3.93 (1 H, m), 4.02–4.40 (3 H, m), 4.47 (1 H, m), 5.40 (1 H, t, *J* = 9.0 Hz), 5.47 (1 H, m), 5.72 (1 H, m), 5.80 (2 H, m), 6.88 (2 H, m), 7.22 (2 H, m), 7.57 (2 H, m), 7.92 (2 H, m).

IR (film): ν = 3476, 2866, 2362, 1723, 1591, 1514, 1464, 1270, 1176, 1102, 843, 756, 667 cm^{-1}.

A solution of the above allylic alcohol (23.1 mg, 37.6 μmol) and Et_3N (77 μL, 550 μmol) in CH_2Cl_2 (1 mL) was treated with *p*-BrBzCl (24 mg, 110 μmol) and DMAP (1 mg, 5 μmol) at r.t. and the mixture was stirred for 1.5 h, diluted with Et_2O, washed with H_2O and dried ($MgSO_4$). Filtration, concentration and flash column chromatography (silica gel, hexane/EtOAc, 3:1) gave a mixture (1:1) of benzoates. A mixture of the benzoates in CH_2Cl_2 (1 mL)/ H_2O (50 μL) was treated with DDQ (18 mg, 75 μmol) and stirred at r.t. for 1 h. The mixture was quenched with aq sat. $Na_2S_2O_3$ (0.5 mL), diluted with Et_2O, washed with H_2O, brine and dried ($MgSO_4$). Filtration, concentration and flash column chromatography (silica gel, hexane/EtOAc, 2:1) gave **27** (6.2 mg, 24% for 2 steps) and **28** (8.3 mg, 33% for 2 steps) along with a mixture of **27** and **28** (7.1 mg, 28% for 2 steps).

(2*R*)-27

$[\alpha]_D^{24}$+16.8 (*c* = 0.31, $CHCl_3$).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.50 (1 H, m), 1.70 (2 H, m), 2.14 (1 H, m), 2.43 (1 H, m), 2.68 (1 H, ddd, *J* = 16.0, 8.0, 4.0 Hz), 3.19 (1 H, t, *J* = 9.0 Hz), 3.20–3.56 (5 H, m), 3.58 (1 H, t, *J* = 9.0, 2.0 Hz), 3.92 (1 H, m), 4.52 (1 H, m), 5.40 (1 H, t, *J* = 9.0 Hz), 5.42 (1 H, m), 5.55–5.88 (3 H, m), 7.45 (2 H, m), 7.57 (2 H, m), 7.72 (2 H, m), 7.87 (2 H, m).

IR (neat): ν = 2956, 2868, 1725, 1591, 1487, 1464, 1439, 1400, 1338, 1270, 1176, 1102, 1071, 1044, 1013, 977, 847, 801, 756, 683, 667 cm^{-1};

(2*S*)-28

$[\alpha]_D^{23}$+20.0 (*c* = 0.41, $CHCl_3$).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.50 (1 H, m), 1.70 (2 H, m), 2.14 (1 H, m), 2.43 (1 H, m), 2.68 (1 H, ddd, *J* = 16.0, 8.0, 4.0 Hz), 3.20 (1 H, t, *J* = 9.0 Hz), 3.19–3.50 (5 H, m), 3.59 (1 H, t, *J* = 9.0 Hz), 3.94 (1 H, m), 4.50 (1 H, m), 5.40 (1 H, m), 5.41 (1 H, t, *J* = 9.0 Hz), 5.57–5.87 (3 H, m), 7.57 (2 H, m), 7.59 (2 H, m), 7.86 (2 H, m), 7.94 (2 H, m).

IR (neat): ν = 3506, 2946, 2868, 1725, 1591, 1487, 1441, 1400, 1375, 1270, 1176, 1100, 1044, 1013, 977, 944, 847, 754, 683 cm^{-1};

ABC-Ring Fragment 5

A solution of **27** (12.1 mg, 17.8 μmol) and anhyd K_2CO_3 (22 mg, 160 μmol) in absolute MeOH (1 mL) was stirred at r.t. for 23 h. The mixture was concentrated and subjected to column chromatography on florisil (CH_2Cl_2/MeOH, 5:1) to give **5** (5.1 mg, 92%).

(2*R*)-5

Colorless solid.

^{1}H NMR (600 MHz, Pyridine-d_5/TMS): δ = 1.47 (1 H, m, H-15), 1.49 (1 H, m, H-14), 1.56 (1 H, m, H-15), 2.04 (1 H, m, H-14), 2.52 (1 H, m, H-8), 2.70 (1 H, ddd, *J* = 15.9, 8.0, 4.0 Hz, H-8), 3.18 (1 H, ddd, *J* = 10.5, 9.1, 4.3 Hz, H-13), 3.26 (1 H, t, *J* = 9.1 Hz, H-12), 3.29 (1 H, m, H-16), 3.44 (1 H, td, *J* = 8.8, 4.0 Hz, H-9), 3.73 (1 H, t, *J* = 8.8 Hz, H-10), 3.89 (1 H, m, H-16), 3.91 (2 H, m, H-1), 4.04 (1 H, dd, *J* = 9.1, 8.8 Hz, H-11), 4.67 (1 H, m, H-2), 4.82 (1 H, m, H-5), 5.74 (1 H, m, H-7), 5.88 (1 H, dt, *J* = 11.3, 3.2 Hz, H-6), 6.33 (2 H, m, H-3,4), 6.43 (1 H, br, OH), 6.67 (1 H, br, OH), 7.13 (1 H, br, OH).

^{13}C NMR (150 MHz, Pyridine-d_5/TMS): δ = 25.65 (CH_2, C-15), 29.83 (CH_2, C-14), 34.81 (CH_2, C-8), 67.39 (CH_2, C-1), 67.59 (CH_2, C-16), 73.26 (CH, C-2), 74.55 (CH, C-11), 75.24 (CH, C-13), 76.51 (CH, C-9), 78.63 (CH, C-5), 83.06 (CH, C-12), 88.16 (CH, C-10), 127.16 (CH, C-7), 131.61 (CH, C-3 or C-4), 132.76 (CH, C-3 or C-4), 136.14 (CH, C-6).

MS (EI): m/z (rel. int.,%) = 294 (8), 282 (12), 281 (14), 280 (5), 278 (6), 263 (6), 253 (17), 252 (100), 251 (73), 238 (15), 139 (23), 138 (11), 127 (14), 113 (15), 109 (15), 101 (10), 97 (15), 71 (58).

HRMS (EI): m/z calcd for $C_{16}H_{24}O_6$ (M^+) 312.5171, found 312.1571.

ABC-Ring Fragment 6

A solution of **28** (10.6 mg, 15.6 μmol) and anhyd K_2CO_3 (22 mg, 160 μmol) in absolute MeOH (1 mL) was stirred at r.t. for 23 h. The mixture was concentrated and subjected to flash column chromatography (silica gel, CH_2Cl_2/MeOH, 5:1) to give **6** (3.5 mg, 72%).

(2*S*)-6

Colorless solid.

^{1}H NMR (600 MHz, Pyridine-d_5): δ = 1.46 (1 H, m, H-15), 1.48 (1 H, m, H-14), 1.56 (1 H, m, H-15), 2.04 (1 H, m, H-14), 2.53 (1 H, m, H-8), 2.71 (1 H, ddd, *J* = 15.9, 8.0, 4.0 Hz, H-8), 3.18 (1 H, ddd, *J* = 10.5, 9.1, 4.3 Hz, H-13), 3.26 (1 H, t, *J* = 9.1 Hz, H-12), 3.29 (1 H, m, H-16), 3.44 (1 H, td, *J* = 8.8, 4.0 Hz, H-9), 3.73 (1 H, t, *J* = 8.8 Hz, H-10), 3.88 (1 H, m, H-16), 3.96 (2 H, m, H-1), 4.04 (1 H, dd, *J* = 9.1, 8.8 Hz, H-11), 4.68 (1 H, m, H-2), 4.84 (1 H, m, H-5), 5.74 (1 H, m, H-7), 5.88 (1 H, dt, *J* = 11.3, 3.2 Hz, H-6), 6.36 (2 H, m, H-3,4), 6.43 (1 H, br, OH), 6.68 (1 H, br, OH), 7.14 (1 H, br, OH).

^{13}C NMR (150 MHz, Pyridine-d_5): δ = 25.66 (CH_2, C-15), 29.84 (CH_2, C-14), 34.82 (CH_2, C-8), 67.42 (CH_2, C-1), 67.59 (CH_2, C-16), 73.26 (CH, C-2), 74.56 (CH, C-11), 75.25 (CH, C-13), 76.53 (CH, C-9), 78.67 (CH, C-5), 83.07 (CH, C-12), 88.14 (CH, C-10), 127.15 (CH, C-7), 131.53 (CH, C-3 or C-4), 132.65 (CH, C-3 or C-4), 136.23 (CH, C-6).

MS (EI): m/z (rel. int.,%) = 294 (8), 282 (12), 281 (12), 263 (4), 253 (16), 252 (100), 251 (71), 238 (19), 139 (19), 138 (10), 127 (12), 113 (13), 109 (14), 97 (14), 84 (11), 83 (11), 71 (54).

HRMS (EI): m/z calcd for $C_{16}H_{24}O_6$ (M^+) 312.5171, found 312.1579.

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(17) (2*R*, 5*R*)-**29**
UV (EtOH): λ_{max} = 245 nm (log ε = 4.8).
CD (EtOH): λ_{ext} = 257 nm (Δε = –1.5), 242 nm (+11.9).
(2*S*, 5*R*)
UV (EtOH): λ_{max} = 245 nm (log ε = 4.8).
CD (EtOH): λ_{ext} = 253 nm (Δε+19.4), 236 nm (–4.6).

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