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Cytosporone E: racemic synthesis and preliminary antibacterial testing

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Abstract—The antibiotic cytosporone E (isolated from the broth of the endophytic fungi CR 200 (*Cytospora sp.*) and CR 146 (*Diaporthe sp.*)) was synthesized as a racemic mixture. The key step in the synthesis is the Meyers *ortho*-alkylation of a chiral aromatic oxazoline. Preliminary antibiotic activity shows antibiosis against Gram-positive bacteria but not Gram-negative bacteria as previously reported.

1. Introduction

There is a continuing search for new and more effective antibiotics as bacteria become resistant to the current antibiotic arsenal. In 2000 Clardy and co-workers[1] isolated the secondary metabolites cytosporone A–E as racemates from the broth of two endophytic fungi; CR 200 (*Cytospora sp.*) and CR146 (*Diaporthe sp.*). Two of these, cytosporone D (**1**) and E (**2**) (Fig. 1), were shown to have equipotent antibacterial activity against representative strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and the fungus *Candida albicans*. These activities are significant because as bacteria develop resistance to available antibiotics, new antibacterial compounds must be discovered. For example, *Staphylococcus aureus* (bacteria that can cause pneumonia, sepsis, osteomyelitis, meningitis, arthritis, and toxic shock syndrome) shows widespread resistance to amoxicillin and decreased susceptibility to vancomycin is of great concern.[2] The intestinal bacterium *Enterococcus faecalis*, a frequent cause of nosocomial infections, including urinary tract, and wound infections, is increasingly resistant to vancomycin, which has been considered the last line of defense.[3,4]

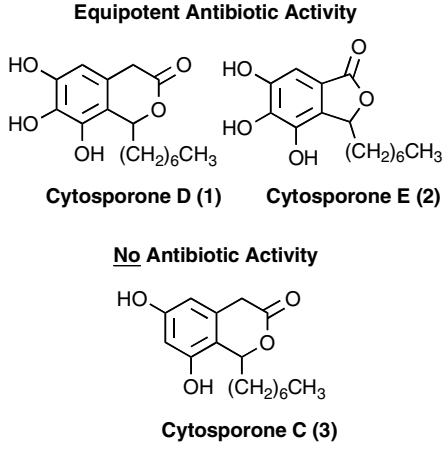

Figure 1. Selected cytosporones.

This report focuses on the synthesis of cytosporone E, which has a phthalide carbon backbone, a structure common in many naturally occurring biologically active substances.[5] Cytosporone D is equipotent to E; however it is interesting to note that it has been proposed that the trihydroxy functionality is likely the source of antibiosis.[1] This was proposed by comparing cytosporone D to cytosporone C (**3**), which only differ by the central phenolic hydroxy moiety.

Keywords: Phthalide; Antibiotic; Meyers *ortho*-Alkylation; Chiral oxazoline; Endophytic fungus.
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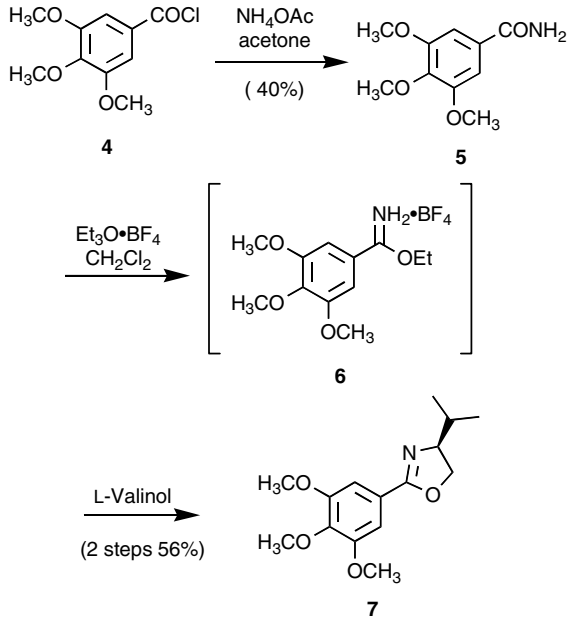
2. Results and discussion

Our synthesis of the cytosporone E focuses on using the powerful Meyers *ortho*-alkylation of chiral aromatic oxazolines as the key step to complete the phthalide backbone. Ohzeki and Mori's[6] racemic synthesis of this compound relied on the Snieckus's *ortho*-lithiation of an achiral aromatic amide. These two methods are complimentary; however, the chiral oxazoline methodology is foreseen to directly yield the chiral benzylic center in a future chiral synthesis of the antibiotic.[7]

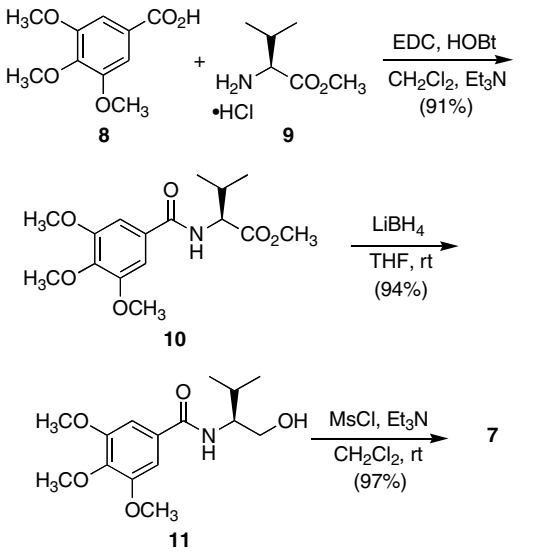
Formation of the aromatic oxazoline was initially approached via imidate ester **6** as described by Meyers et al.[8] (Scheme 1). Amide formation was accomplished by converting 3,4,5-trimethoxybenzoyl chloride (**4**) to amide **5** using ammonium acetate[9] in 40% yield. The amide was then reacted with Meerwein's reagent ($Et_3O{\cdot}BF_4$) to give an intermediate imidate ester **6**, which then reacted with L-valinol to yield chiral oxazoline **7** in 56% yield from the amide. The overall yield of the oxazoline was 29% from **4**; therefore, we approached the synthesis of the oxazoline from a different angle.

The revised synthesis[10] of oxazoline **7** (Scheme 2) started with 3,4,5-trimethoxybenzoic acid (**8**) and was coupled with L-valine methyl ester hydrochloride (**9**) using EDC, HOBt, triethylamine to afford ester **10** in 91% yield. Reduction of the methyl ester using lithium borohydride afforded alcohol **11** in 94% yield. Cyclization was accomplished using methane sulfonyl chloride and triethylamine to give the desired oxazoline **7** in 97% yield. The three steps gave an improved overall 83% yield from **8**.

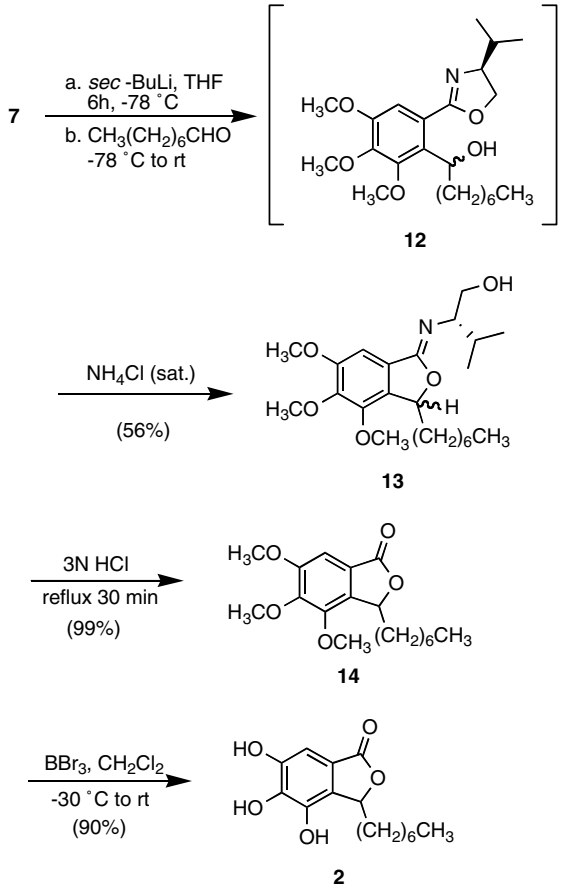
With oxazoline **7** in hand we proceeded to complete the Meyers *ortho*-alkylation (Scheme 3). This was accomplished via *ortho*-lithiation with *sec*-BuLi at −78 °C for



Scheme 1. Initial synthesis of the chiral aromatic oxazoline.



Scheme 2. Revised synthesis of the chiral aromatic oxazoline.



Scheme 3. Formation of cytosporone E.

6 h followed by addition of *n*-octanal. A mild acidic work-up (satd NH_4Cl) rearranges[8] the intermediate alcohol **12** to afford a 1:1 diastereomeric mixture of imino lactone **13** in 56% yield. We are currently investigating methods to improve the alkylation and the diastereoselectivity of this reaction.[7] Hydrolysis of this

Table 1. Cytosporone activity against representative strains of Gram-negative bacteria

Gram-negative bacteria[a]	Diameter of the zone of inhibition (mm ± 1 mm)		
	μg Cytosporone E/disk		
	40	60	80
E. coli	None	None	None
P. aeruginosa	None	None	None
M. smegmatis	None	None	None
C. freundii	None	None	None

[a] Purchased from Difco Laboratories, Detroit, MI. The ATCC® strains are as follows: *Esherichia coli* 25922; *Pseudomonas aeruginosa* 27853; *Mycobacterium smegmatis* wild type; *Citrobacter freundii* 8090.

Table 2. Cytosporone activity against representative strains of Gram-positive bacteria

Gram-negative bacteria[a]	Diameter of the zone of inhibition (mm ± 1 mm)		
	μg Cytosporone E/disk		
	40	60	80
E. faecalis	10 mm	11 mm	10 mm
S. aureus	13 mm	13 mm	13 mm
S. epidermidis	13 mm	14 mm	14 mm
B. subtilis	12 mm	12 mm	11 mm

[a] Purchased from Difco Laboratories, Detroit, MI. The ATCC® strains are as follows: *Enterococcus faecalis* 19433; *Staphylococcus aureus* 25923; *Staphylococcus epidermidis* 12228; *Bacillus subtilis* wild type.

intermediate in refluxing 3 N HCl affords the 4,5,6-trimethoxyphthalide (**14**) in 99% yield. Removal of the methoxy groups was conducted using boron tribromide[11] at −30 °C to room temperature to afford cytosporone E (**2**) in 90% yield (^{1}H NMR and ^{13}C NMR coincide with the natural product[1]). The overall six-step yield of cytosporone E from 3,4,5-trimethoxybenzoic acid is 41%.

It was noted[1] that cytosporone E was effective against Gram-positive and Gram-negative strains of bacteria. Our initial screenings of representative strains of Gram-positive and Gram-negative bacteria contradict the initial results. Table 1 illustrates that cytosporone E is not effective against Gram-negative bacteria at 40, 60, or 80 μg of antibiotic per disc. However, Table 2 shows that the antimicrobial is effective against Gram-positive bacteria at 40, 60, and 80 μg of antibiotic per disc.

3. Conclusion

Racemic cytosporone E was synthesized in 41% yield starting from 3,4,5-trimethoxybenzoic acid using the Meyers *ortho*-alkylation of a chiral aromatic oxazoline as the key step to form the phthalide carbon framework. It has been noted that cytosporone E has antibiotic activity against both Gram-positive and Gram-negative bacteria. However, our samples were found to only be active against Gram-positive bacteria.

4. Experimental

The NMR spectra were recorded on a Varian Mercury 300 VXR NMR (^{1}H at 300 MHz and ^{13}C at 75 MHz). A Mattson Genesis II FT-IR using an ATR sampling method was used to obtain infrared spectra. Melting point in an open ended capillary was uncorrected and recorded using a Mel-Temp II apparatus. Optical rotation was measured using an Autopol IV Automatic Polarimeter. Elemental analyses were performed by Quantitative Technologies Inc. of Whitehouse, NJ, USA. THF and Et_2O were distilled from lithium aluminum hydride or sodium/benzophenone ketyl; CH_2Cl_2 was purified from molecular sieves, Et_3N was distilled from CaH_2, acetone was distilled from K_2CO_3, and all other reagents were purchased and used as obtained. Column chromatography separations were obtained on Silica gel (230–400 mesh). All reactions were done with flame-dried glassware and under N_2 gas unless otherwise noted. EDC is 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, and HOBt is 1-hydroxybenzotriazole hydrate.

4.1. 3,4,5-Trimethoxybenzamide 5

To a solution of 3,4,5-trimethoxybenzoyl chloride (10.4 g, 44. mmol) in acetone (150 mL) was added in one portion ammonium acetate (10.5 mL, 136 mmol). After stirring for 48 h at rt the mixture was filtered and the filtrate was concentrated in under reduced pressure. The brown residue was recrystallized with hot methanol (150 mL) to yield a white solid (3.79 g, 40% yield). This material matched the known compound. Mp 174–176 °C, lit.[12] 177 °C. ^{1}H NMR ($CDCl_3$): δ 7.04 (s, 2H), 3.91 (s, 6H), 3.89 (s, 3H), 1.83 (s, 2H); ^{13}C NMR ($CDCl_3$): δ 169.2, 153.2, 141.4, 128.7, 104.9, 61.2, 56.6.

4.2. (4*S*)-4-Isopropyl-2-(3,4,5-trimethoxyphenyl)-2-oxazoline 7 via imidate ester 6

4.2.1. Formation of Meerwein's salt ($Et_3O{\cdot}BF_4$).[13] To a stirred solution of $BF_3{\cdot}OEt_2$ (14.3 mL, 113 mmol) in diethyl ether (29.0 mL) in a three neck RB flask equipped with a reflux condenser was added epichlorohydrin (6.68 mL, 85.2 mmol) dropwise over 1 min. Upon addition the reaction was refluxed for 1 h and then cooled to rt and allowed to stand overnight to produce colorless crystals. The ether was cannulated off and fresh anhydrous ether (15 mL) was added to rinse the crystals and then this was cannulated off. This step was repeated two more times. The crystals were dried under high vacuum to afford 12.3 g (76% yield) of triethyl oxonium fluoroborate. (This yield varied between 75% and 90%.) These were used directly in the formation of the imidate ester above.

4.2.2. Oxazoline 7 formation. To a stirred solution of triethyl oxonium fluoroborate (12.3 g, 63.2 mmol) in 1,2-dichloroethane (50 mL) was added a solution of amide **6** (17.6 g, 63.2 mmol) in 1,2-dichloroethane (250 mL). After stirring the combined solutions for 24 h a solution of L-valinol (7.17 g, 69.5 mmol) in 1,2-dichloroethane

(40 mL) was added. After refluxing for 24 h the reaction was quenched by pouring it into 500 mL of 5% $NaHCO_3$. The layers were separated and the aqueous phase was extracted three times with CH_2Cl_2. The combined organic layers were washed with brine, dried over Na_2SO_4, and then concentrated under reduced pressure. The crude oil was purified by flash chromatography (5–25% EtOAc/hexanes) to afford oxazoline **7** as a light yellow oil in 72% yield (10.0 g, 45.5 mmol). ^{1}H NMR ($CDCl_3$): δ 7.21 (s, 2H), 4.40 (m, 1H), 4.12 (m, 2H), 3.91 (s, 6H), 3.88 (s, 3H), 1.89 (m, 1H), 1.03 (d, J = 6.83 Hz, 3H), 0.93 (d, J = 6.83 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 163.0, 152.9, 140.5, 123.1, 105.3, 72.6, 70.0, 60.9, 56.2, 32.7, 19.0, 17.9. IR (oil) 1653, 1587, 1505, 1128 cm^{-1}. $[\alpha]_D^{20}$ −0.404, $[\alpha]_{365}^{20}$ −1.79, $[\alpha]_{436}^{20}$ −0.938, $[\alpha]_{633}^{20}$ −0.334 (*c* 1.0, CH_2Cl_2). Anal. Calcd for $C_{15}H_{21}NO_4$: C, 64.50; H, 7.58; N, 5.01. Found: C, 64.01; H, 7.70; N, 4.94.

4.3. Methyl (2*S*)-2-(3,4,5-trimethoxybenamido)-3-methylbutanoate 10

To a 50 mL round bottom flask was added 3,4,5-trimethoxybenzoic acid (**8**) (0.447 g, 2.11 mmol) and 9 mL of CH_2Cl_2. This suspension was stirred at 0 °C, and then EDC (0.809 g, 4.22 mmol) and HOBt (0.570 g, 4.22 mmol) were added. In a second 50 mL round bottom flask was added L-valine methyl ester hydrochloride (**9**) (0.500 g, 3.17 mmol), 9 mL of CH_2Cl_2 and Et_3N (0.588 mL, 4.22 mmol). (The solution goes from having a suspension to clear and then back to having a white suspension.) After 10 min the methyl ester solution was cannulated into the 3,4,5-trimethoxybenzoic acid solution. Upon mixing the ice bath was removed and the reaction was stirred for 24 h. Then the CH_2Cl_2 was evaporated under reduced pressure and the residue was dissolved in a 4:1 mixture of EtOAc/H_2O mixture. The organic layer was then washed two times with 1 M HCl, one time each with satd $NaHCO_3$, H_2O, and brine. The organic layer was dried over Na_2SO_4, filtered, and then concentrated under reduced pressure. The residue was then purified using flash chromatography (15% EtOAc/hexanes) to yield **10** as an oil in 91% (0.625 g, 2.03 mmol). Mp 132–135 °C. ^{1}H NMR ($CDCl_3$): δ 7.03 (s, 2H), 6.58 (br d, J = 8.2 Hz, 1H), 4.76 (dd, J = 8.6 and 4.9 Hz, 1H), 3.92 (s, 6H), 3.89 (s, 3H), 3.79 (s, 3H), 2.28 (m, 1H), 1.02 (d, J = 7.3 Hz, 3H), 0.99 (d, J = 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 172.8, 166.9, 153.2, 141.1, 129.5, 104.4, 60.9, 57.5, 56.3, 52.2, 31.6, 19.0, 18.0. IR (solid) 3288, 1747, 1630, 1124 cm^{-1}. $[\alpha]_D^{20}$ +0.218, $[\alpha]_{365}^{20}$ +0.616, $[\alpha]_{436}^{20}$ +0.466, $[\alpha]_{633}^{20}$ +0.180 (*c* 1.0, CH_2Cl_2). Anal. Calcd for $C_{16}H_{23}NO_6$: C, 59.06; H, 7.13; N, 4.31. Found: C, 59.10; H, 7.27; N, 4.18.

4.4. *N*-((*S*)-1-Hydroxy-3-methylbutan-2-yl)-3,4,5-trimethoxybenzamide 11

To a stirred solution of ester **10** (0.626 g, 1.92 mmol) in THF (28.6 mL) at 0 °C was added $LiBH_4$ (1.92 mL of a 2 M solution in THF, 3.85 mmol) dropwise and then warmed to rt. After 20 h the reaction was quenched with 2 M HCl and then concentrated under reduced pressure. The residue was dissolved in a 4:1 mixture of EtOAc/H_2O. The separated aqueous layer was extracted three times with EtOAc. The combined organic layers were washed with 1 M NaOH, brine, dried over Na_2SO_4, and then concentrated under reduced pressure. The crude oil was purified using flash chromatography (35–70% EtOAc/hexanes) afforded alcohol **11** in 94% yield (0.533 g). Mp 163–168 °C. ^{1}H NMR ($CDCl_3$): δ 7.00 (s, 2H), 6.28 (br d, J = 8.2 Hz, 1H), 3.91 (s, 6H), 3.88 (s, 3H), 3.82 (m, 2H), 2.59 (br s, 1H), 2.02 (m, 1H), 1.04 (d, J = 5.7 Hz, 3H), 1.02 (d, J = 5.8 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 168.1, 153.1, 141.0, 130.0, 104.4, 63.7, 60.9, 57.6, 56.3, 29.2, 19.6, 19.1. IR (solid) 3291, 1630, 1128 cm^{-1}. $[\alpha]_D^{20}$ −0.266, $[\alpha]_{365}^{20}$ −0.884, $[\alpha]_{436}^{20}$ −0.516, $[\alpha]_{633}^{20}$ −0.228 (*c* 1.0, CH_2Cl_2). Anal. Calcd for $C_{15}H_{23}NO_5$: C, 60.59; H, 7.80; N, 4.71. Found: C, 60.72; H, 8.12; N, 4.63.

4.5. (4*S*)-4-Isopropyl-2-(3,4,5-trimethoxyphenyl)-2-oxazoline 7 via amide alcohol 11

To a solution of alcohol **11** (0.158 g, 0.531 mmol) in CH_2Cl_2 (2.70 mL) at 0 °C was added Et_3N (2.34 mL, 1.68 mmol) then MsCl (0.0822 mL, 1.06 mmol) dropwise then warmed to rt. After 16 h the solvent was removed under reduced pressure. The oily residue was redissolved in a 4:1 EtOAc/H_2O solution. The aqueous layer was extracted three times with EtOAc and then the combined organic layers were washed with brine and dried over Na_2SO_4. After concentrating the organic layer under reduced pressure, the oily residue was purified via flash chromatography (30–37% EtOAc/hexanes) to afford oxazoline **7** as an oil in 97% yield (0.143 g). Spectral data matched that of the oxazoline via the imidate ester. See Section 4.2.

4.6. (*S*,*Z*)-2-(1-Heptyl-5,6,7-trimethoxyisobenzofuran-3(1*H*)-ylideneamino)-3-methylbutan-1-ol 13

To a stirred solution of oxazoline **7** (0.105 mg, 0.376 mmol) in THF (2.51 mL) at −78 °C was added *sec*-BuLi (0.418 mL of a 1.08 M solution in hexanes, 0.451 mmol). After 6 h, *n*-octanal (0.070 mL, 0.451 mmol) was added dropwise at −78 °C. After 45 min at −78 °C the reaction was allowed to warm to rt overnight. The reaction was quenched with the addition of NH_4Cl and stirred for 15 min at rt and then diluted with Et_2O. The aqueous layer was extracted one time with Et_2O then the combined organic layers were washed with brine and dried over Na_2SO_4 and concentrated under reduced pressure. The crude oil was purified by flash chromatography (30% EtOAc/hexanes) to afford **13** as a 1:1 mixture of diastereomers as an oil in 56% yield (0.086 g). Characterization of the top diastereomer will be reported for simplicity. (Based on TLC 80% EtOAc/hexanes the top diastereomer has an R_f = 0.38 and the bottom diastereomer has an R_f = 0.23.) ^{1}H NMR ($CDCl_3$): δ 7.16 (s, 1H), 5.45 (dd, J = 7.4 and 3.1 Hz, 1H), 3.93 (s, 3H), 3.92 (s, 3H), 3.89 (s, 3H), 3.78–3.70 (m, 3H), 2.49 (br s, 1H), 2.06 (m, 1H), 1.87 (m, 1H), 1.60 (m, 1H), 1.22 (m, 10H), 0.97 (d, J = 6.6 Hz, 3H), 0.91 (d, J = 6.8 Hz, 3H), 0.88 (t, J = 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 168.8, 155.1, 147.4, 144.5, 131.7, 125.9, 101.1, 82.3, 64.6, 63.7, 61.0,

60.6, 56.4, 34.0, 31.8, 30.3, 29.3, 29.1, 24.7, 22.6, 19.7, 19.4, 14.1. IR (oil) 3210, 1688, 1614, 1478, 1346, 1108 cm^{-1}. Anal. Calcd for $C_{23}H_{37}NO_5$: C, 67.78; H, 9.15; N, 3.44. Found: C, 67.64; H, 9.39; N, 3.22.

4.7. 3-Heptyl-4,5,6-trimethoxyphthalide 14

To a stirred solution of **13** (0.079 g, 0.194 mmol) in THF (0.200 mL) was added 3 M HCl (2.8 mL) and then refluxed at 110 °C for 30 min. The reaction was cooled and diluted with CH_2Cl_2 and the two layers were separated. The aqueous layer was extracted three times with CH_2Cl_2. The organic layers were combined and dried over Na_2SO_4 and then concentrated under reduced pressure. Flash column chromatography (30% EtOAc/hexanes) afforded **14** as a light yellow oil in 99% yield (0.062 g). ^1H NMR ($CDCl_3$): δ 7.12 (s, 1H), 5.46 (dd, $J = 2.9$ and 7.8 Hz, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.92 (s, 3H), 2.25–2.14 (m, 1H), 1.76–1.63 (m, 1H), 1.46–1.22 (m, 10H), 0.87 (t, $J = 6.9$ Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 170.6, 155.5, 147.6, 146.8, 135.4, 121.4, 102.5, 80.2, 61.1, 60.8, 56.4, 33.4, 31.7, 29.2, 29.1, 24.7, 22.6, 14.0. IR (oil) 1766, 1618, 1108 cm^{-1}. Anal. Calcd for $C_{18}H_{26}O_5$: C, 67.06; H, 8.13. Found: C, 66.99; H, 8.28.

4.8. Cytosporone E 3

To a solution of **14** (0.218 g, 0.676 mmol) in CH_2Cl_2 (2.25 mL) at −30 °C was added BBr_3 (2.70 mL of a 1.0 M solution in CH_2Cl_2, 2.70 mmol). After 1 h the reaction is warmed to rt for 2 h after which it was poured into ice-water and the aqueous layer was extracted with CH_2Cl_2 twice. The combined organic layers were washed with brine and dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash chromatography (5% MeOH/$CHCl_3$) to afford Cytosporone E in 90% yield (0.170 g). ^1H and ^{13}C NMR spectra were identical with those of the natural product.

4.9. Antibiotic testing

The antibiotic testing against representative Gram-positive and Gram-negative bacteria was accomplished using the Kirby–Bauer method. Cytosporone E, in a solution of 1:1 ethanol–sterile water, was applied to sterile standardized filter paper discs to correspond to the varying desired amounts of 40, 60, and 80 μg of antibiotic per disc. The discs were placed in an incubator at 37 °C for 40 min to allow the ethanol to evaporate. The discs were then aseptically applied to the surface of agar plates that had been heavily inoculated with the test organisms; then the plates were incubated overnight. The diameter of the clear zone that resulted (i.e., the zone of inhibition) was measured in millimeters. The zone of inhibition results from Cytosporone E diffusing into the medium surrounding the disc. As a standard, one disc was treated only with the solvent system and carried through the process described above. This disc showed no activity as expected if all the ethanol had evaporated. The antibiotic activity of each sample was determined in triplicate.

Acknowledgements

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7. The current chiral oxazoline yielded a 1:1 mixture of diastereomers in the alkylation step (see Scheme 3), and we are currently investigating different chiral oxazolines to improve this step and finish the chiral synthesis of cytosporone E.
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