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Anti-staphylococcal activity and β-lactam resistance attenuating capacity of structural analogues of (−)-epicatechin gallate

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ABSTRACT

We examined the impact of gradual removal of hydroxyl groups from the A- and B-rings of (−)-epicatechin gallate on antibacterial activity and oxacillin resistance attenuation of an epidemic strain of methicillin resistant *Staphylococcus aureus*. Removal of both hydroxyls from the B-ring effected a large reduction in oxacillin MIC (from 512 to 0.25 mg/mL at a concentration of 12.5 mg/L); further hydroxyl deletion of the A-ring reduced the oxacillin effect but increased intrinsic anti-staphylococcal activity

Extracts of the tea plant, *Camellia sinensis*, contain abundant quantities of both galloyl and nongalloyl catechins; these polyphenols constitute around 20% of the dry leaf weight and contribute to the weak antibacterial activity of green tea preparations.[1] Subinhibitory concentrations of these secondary metabolites exert a range of effects on bacterial cells that have the potential to be harnessed for therapeutic and industrial use. For example, moderate concentrations of (−)-epicatechin gallate (ECg), and to a lesser extent (−)-epigallocatechin gallate (EGCg) and (−)-catechin gallate (Cg), disrupt the β-lactam-resistance machinery of methicillin resistant strains of *Staphylococcus aureus* (MRSA), inducing complete but reversible susceptibility to this important group of antibiotics.[2] Galloyl catechins also inhibit formation of staphylococcal biofilms,[3,4] reduce the secretion of toxins and other virulence-related proteins by *S. aureus* strains[5] and abolish halotolerance in staphylococcal strains associated with food spoilage and food poisoning.[6] Although the processes involved in these phenotypic modifications are as yet incompletely defined, there is strong evidence that they are dependent on the intercalation of the bioactive polyphenols into the bacterial cytoplasmic membrane (CM): the most potent modifier, ECg (Fig. 1), inserts into the staphylococcal bilayer, inducing a series of complex changes to the phospholipid palisade and leading to reduction in the efficiency of function of CM-embedded proteins such as the penicillin binding proteins responsible for peptidoglycan biosynthesis and β-lactam resistance.[4,7]

The interaction of galloyl catechins with model phospholipid membranes is partly governed by their relative hydrophobicity, with ECg showing a higher affinity than EGCg. ECg penetrates to a location deep within the phospholipid palisade[8] and in bilayers containing phosphatidylcholine (PC) there are strong initial cation-π interactions between the galloyl ring and quaternary amine of the head group.[9] ECg differs from EGCg only in the absence of a hydroxyl function at one of the *meta*- positions of the B-ring (Fig. 1) and we proposed,[10] on the basis of enhanced microbiological activity of ECg, that a further reduction in the degree of B-ring hydroxylation would enhance anti-MRSA effects by increasing the affinity of these analogues for lipid bilayers. In addition B-ring 4-hydroxyl deletion should also give compounds less prone to epimerization via a quinone methide-like intermediate due to the removal of this anchimeric assistance.[10] We demonstrated that a monohydroxylated 3-hydroxy B-ring **1** and a dihydroxylated 3,5-dihydroxy B-ring **2** ECg analogue sensitised MRSA strains to the β-lactam antibiotic oxacillin to a comparable extent compared to the natural product (Fig. 1).[10] In combination with hydrolytically more stable analogues possessing an amide linked gallate group **3**,[11] these modifications may provide a route to compounds of therapeutic interest. We now report evaluation of the anti-MRSA activity of further analogues of naturally occurring galloyl catechins with variable degrees of hydroxylation of the A- and B-rings (Fig. 1). We have synthesized, in enantiomerically pure form, the B-ring phenyl analogue **4** and the corresponding A-ring free hydroxyl compounds

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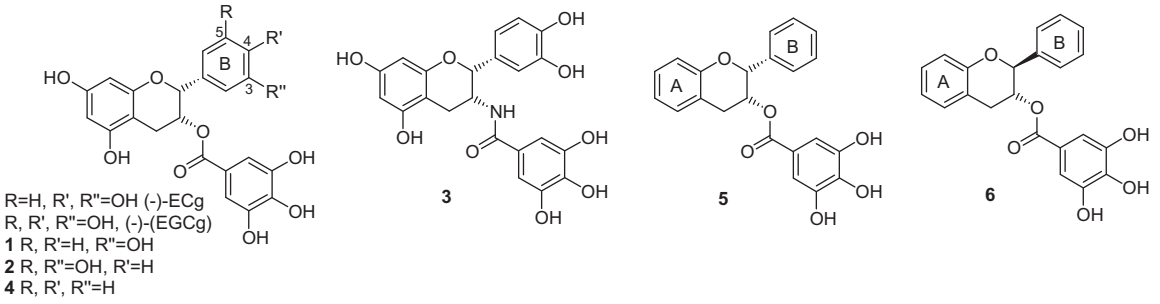
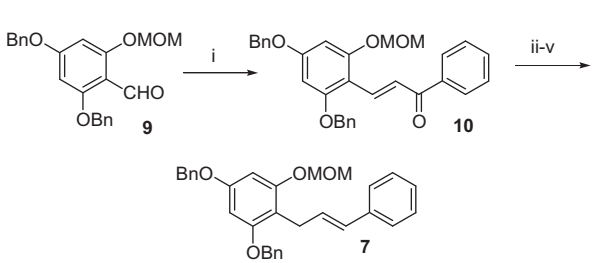


Figure 1.

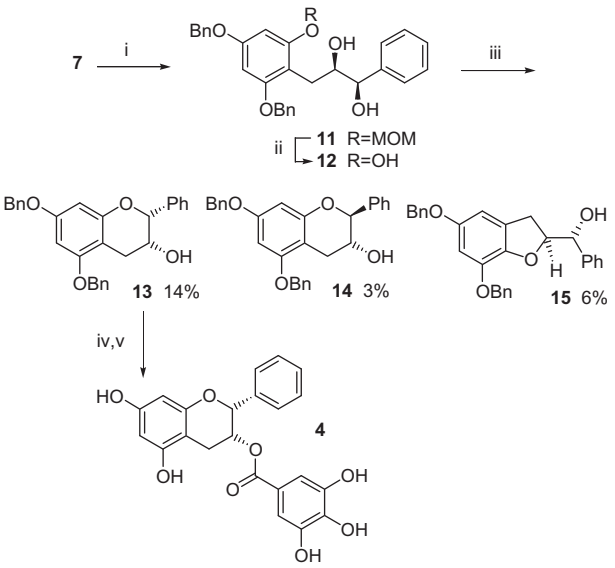
in the epicatechin **5** and catechin **6** relative stereochemistry to compare their capacity to suppress the resistance of MRSA isolates to the β-lactam antibiotic oxacillin.

The enantiomer of **4** has previously been synthesized in undetermined enantiomeric purity by Chan and co-workers.[12] In the synthesis reported here the introduction of absolute and relative stereochemistry is almost identical to the route developed by Chan and co-workers,[12] but we have quantified the enantioselectivity by chiral HPLC with comparison to racemic standards.[13] We also chose to begin our synthesis in an analogous fashion to our syntheses of **2** and **3**,[10] and this synthesis differs from Chan's in the route to the benzyl styrene **7** (Scheme 1). Aldol condensation of **9**[10] with acetophenone gave enone **10** in 70% yield (Scheme 1).[14] As we had noted before, reduction of this type of enone system to the saturated alcohol and subsequent elimination to give the alkene was surprisingly capricious.[10] Reduction of the conjugated alkene with catechol borane[14] and subsequent reduction of the ketone with lithium aluminium hydride gave the corresponding saturated alcohol in 61% yield over two steps. Dehydration by first bromination and then elimination with DBU gave **7** in gram quantities in good overall yield (Scheme 1).

Control of absolute stereochemistry was achieved by enantioselective Sharpless dihydroxylation that had previously been developed by Chan[12] and which we had used successfully ourselves.[10] Treatment of **7** using AD-mixβ®[15] gave **11** in 46% yield in an initial 76% ee as measured by chiral HPLC of the corresponding dimethyl acetal (Scheme 2).[16] Tritration of the solid with an Et_2O/EtOAc mix upgraded the material to 96% ee in 38% isolated yield. Removal of the MOM ether gave triol **12** which contained ~8% of a dimethyl acetal impurity. This was removed by recrystallisation (Et_2O) to give pure **12** in 81% yield. This material corresponded to the intermediate in Chan's synthesis of the enantiomer of **4**.[12] Cyclisation to the 2,3-*cis*-substituted C-ring followed the procedure used by Chan that we had also used in our previous syntheses.[10] In our hands treatment of **12** with trimethyl orthoformate and then ring opening with acetyl bromide[17] gave a mixture of products. Optimisation experiments led us to prepare acetyl bromide in situ from

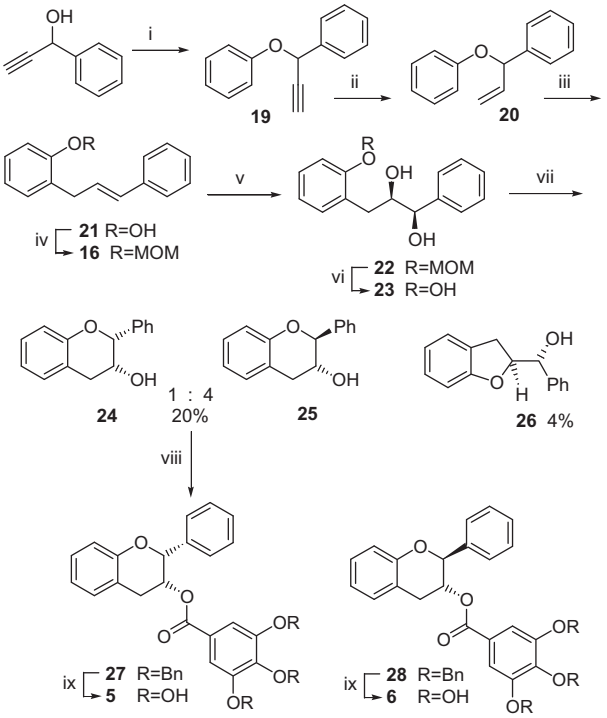


Scheme 1. Reagents and conditions: (i) KOH aq (25%), PhCOMe, EtOH, THF, rt, 16 h, 70%; (ii) Catechol borane, THF, −78 °C to rt, 99% crude; (iii) $LiAlH_4$, THF, 0 °C, 61% crude; (iv) PPh_3, Br_2, Et_3N, CH_2Cl_2, 0 °C to rt, 91%; (v) DBU, PhMe, 110 °C, 16 h, 81%.



Scheme 2. Reagents and conditions: (i) AD-mix-β®, *t*-BuOH, H_2O, $MeSO_2NH_2$, 0 °C, 5 days, 46% @76% ee, 38% @96% ee; (ii) HCl, MeOH, Et_2O, reflux, 6.5 h, 81%; (iii) $HC(OMe)_3$, PPTS cat., CH_2Cl_2, rt; w/up then AcBr, 4 Å mol sieves, CH_2Cl_2, rt; K_2CO_3, acetone, rt, 48 h; w/up then $NaBH_4$, MeOH, DME rt, 75 min, **13** 14%, **14** 3% and **15** 6% (over four steps); (iv) tri-*O*Bn gallic acid chloride, DMAP, Et_3N, CH_2Cl_2, rt, 16 h, 61%; (v) H_2, 10% $Pd(OH)_2$/C, EtOAc, rt, 6 h, 89%.

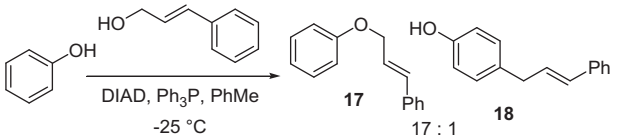
phosphorus tribromide and acetic anhydride to minimise any HBr formation. A small excess of acetic anhydride was used to sequester any water present. Treatment of the mixture of alkyl bromides with K_2CO_3/acetone to induce cyclisation and then removal of the formate ester with sodium borohydride gave a diastereomeric mixture of pyrans **13** (14%) and **14** (3%) and the dihydrobenzofuran **15** (6%) (Scheme 2). The relative stereochemistry of **13** was assigned in part on the multiplicity of the 1H NMR signal of the Ar*CH*O proton in **14** (the corresponding signal in **13** is overlapping with $PhCH_2$ signals) which has $J = 7.9$ Hz (δ 4.79, 1H, d), indicative of a *trans* relationship of the two pyran substituents. The multiplicity of this signal in the final compound **4** also correlates with other systems (vide infra). The relative stereochemistry of **15** is based on an assumed double inversion mechanism, resulting in retention of stereochemistry in the formation of the furan ring. The double inversion mechanism is widely accepted to account for the retention of stereochemistry seen in the expected cyclisation to **13**.[17] However the isolation of a small amount of diastereoisomer **14** suggests that epimerization of the intermediate bromide centre is possible at some point in the reaction sequence. Completion of the synthesis was achieved by DMAP catalysed coupling with 3,4,5-tribenzoyloxy benzoic acid chloride (61%) followed by global hydrogenolysis with $Pd(OH)_2$ catalysis



Scheme 3. Reagents and conditions: (i) PhOH, DIAD, Ph_3P, PhMe. −25 °C, 73%; (ii) H_2, Lindlar cat., EtOH, rt, 86%; (iii) PhMe, 110 °C, 56%; (iv) NaH·MOMCl, THF, 99%; (v) AD-mixβ-®, *t*-BuOH, H_2O, $MeSO_2NH_2$, 0 °C, 5 days, 67% @ 96% ee; (vi) HCl, MeOH, Et_2O, reflux, 7.5 h, 99%; (vii) $HC(OMe)_3$, PPTS cat., CH_2Cl_2, rt; w/up then AcBr, 4 Å mol sieves, CH_2Cl_2, rt; K_2CO_3, acetone, rt, 48 h; w/up then $NaBH_4$, MeOH, *t*-BuOMe rt, 75 min, **24**/**25** 20% and **26** 4% (over four steps); (viii) tri-OBn gallic acid chloride, DMAP, Et_3N, CH_2Cl_2, rt, 16 h, **27** 5%, **28** 65%; (ix) H_2, 10% $Pd(OH)_2$/C, EtOAc, rt, **5** 4 h, 91%, **6** 5 h, 63%.

(89%) to give **4** (96% ee). The stereochemical integrity of the 2,3-*cis* substitution was proven from the multiplicity of the 1H NMR signal of ArC*H*O (δ 5.31, 1H, br s) which agrees with our previous analysis[12] of the relative stereochemistries of **1** and **2**. Absolute stereochemistry was assumed from the Sharpless mnemonic for AD-mixβ®[15] and the fact that the optical rotation exhibits the same sense of optical rotation as naturally occurring (−)-ECg.

The asymmetric synthesis of the novel A ring analogues **5** and **6** relied again on the Sharpless dihydroxylation/*ortho*-ester cyclisation methodology that had previously been developed by Chan[12] and which we had used successfully ourselves.[10] We investigated whether we could access the key alkene **16** using the Mitsunobu chemistry also reported by Chan.[12] Unfortunately in this particular case treatment of phenol and cinnamyl alcohol with di-isopropyl azodicarboxylate (DIAD) and triphenyl phosphine led mainly to the direct substitution product **17** and the electrophilic aromatic substitution product **18** (Eq. 1).



(1)

It turned out that the use of a propargyl alcohol circumvented the selectivity issue producing the desired ether **19** as the main product in 73% yield (Scheme 3). Classic Lindlar reduction of the alkyne **19** to the alkene **20** occurred uneventfully (86%) and required refluxing in toluene for 48 h to promote a Claisen rearrangement to deliver the required alcohol **21** in 56% yield. We noted that this material was unstable for longer periods of heating and slowly decomposed when stored neat at −20 °C. Protection with MOMCl (99%) made the product **16** much more stable. The rest of the synthesis followed the established route.[10,12] Sharpless asymmetric dihydroxylation with AD-mixβ®[15] gave **22** in 67% yield in 96% ee as measured by chiral HPLC of the corresponding dimethyl acetal.[16] Removal of the MOM group gave the triol **23** and subjection of this to the optimised conditions for orthoformate formation, conversion to the corresponding alkyl bromide, cyclisation and removal of the formate esters gave a mixture of products. We isolated the desired A-ring compounds **24**/**25** as an inseperable *trans*/*cis* mixture in a ratio of 4:1, the desired product being the minor component. Clearly the double inversion mechanism is breaking down in these simpler unactivated benzylic cases. We also isolated a dihydrobenzofuran byproduct **26**, as described above. Esterification of the mixture **24**/**25** led to separable protected esters **27** (5%) and **28** (65%). Global hydrogenolysis gave the desired A-ring modified compounds **5** (91%) and **6** (63%) in 96% ee.

The stereochemical assignment of the C2 and C3 substitution in **5** and **6** follows from the 1H NMR chemical shifts and multiplicities of the ArC*H*O signal in each (**5** δ 5.43, 1H, s, and **6** δ 5.41, 1H, d, *J* = 6.1 Hz) which correlates with **4** and agrees with our previous analysis.[12] Absolute stereochemistry was assumed from the Sharpless mnemonic for AD-mixβ®.[15]

Minimum inhibitory concentration (MIC) of each compound against the epidemic MRSA clinical isolate EMRSA-16 was determined by the CLSI (formerly NCCLS) broth microplate assay as previously described.[2] For MIC determination, compounds were dissolved in 60% v/v EtOH prior to dilution in broth; at the concentration used the solvent had no effect on bacterial viability. The capacity of the compounds to modulate the β-lactam resistance of EMRSA-16 was evaluated by determination of the MIC at a fixed concentration in combination with oxacillin. Assays were performed in 96-well microtitre trays with a bacterial inoculum of around 10^4 colony-forming units in 100 μl of Mueller–Hinton (MH) broth (Oxoid, Basingstoke, UK) supplemented with 2% w/v NaCl. Doubling dilutions of oxacillin were used.

S. aureus EMRSA-16 is highly resistant to the β-lactam agent oxacillin, with a MIC of 512 mg/L (Table 1). ECg possesses barely discernable anti-staphylococcal activity against EMRSA-16 (Table 1) and other Gram-positive bacteria.[1] However, the presence of 12.5 mg/L ECg in the oxacillin assay elicits a reduction in MIC to 1 mg/L, essentially abrogating the β-lactam resistance machinery of EMRSA-16. This effect is not confined to EMRSA-16; in a previous study we showed that ECg is able to abolish resistance to oxacillin and a range of other β-lactam drugs in forty MRSA strains isolated from all major regions of the globe.[2]

Compound **2** displayed an identical anti-EMRSA-16 profile to ECg, indicating that reduction of B-ring substitution to a single hydroxyl function at the 3-position did not influence

Table 1
Minimum inhibitory concentrations (MICs) and β-lactam modifying capacity of (−)-epicatchin gallate (ECg) and structural analogues[a]

Compound	MIC (μg/mL)	Oxacillin MIC (μg/mL) Compound (μg/mL)	
		12.5	6.25
Oxacillin	512	–	–
ECg	128	1	–
1	128	1	–
2	128	1	–
4	64	0.25	2
5	16	NG[b]	16
6	16	NG[b]	4

[a] Assays were performed in duplicate and repeated; no variation was noted.
[b] No growth.

anti-staphylococcal activity or its capacity to abrogate β-lactam resistance (Table 1). Similarly, compound **3**, with an unnatural 3,5-dihydroxylation of the B-ring, is indistinguishable from ECg and **2** in these assays, strongly suggesting that these two analogues insert into the staphylococcal cytoplasmic membrane phospholipid bilayer in a way comparable to ECg and we are currently investigating biophysical aspects of membrane intercalation of these analogues. However, the B-ring phenyl analogue **4** produced a more pronounced, albeit still weak, anti-staphylococcal effect and displayed an enhanced capacity to reduce oxacillin resistance in EMRSA-16 at a concentration of 12.5 mg/L. This latter result was unexpected, as we had assumed that structural analogues of ECg would be unable to reduce the oxacillin MIC below 1 mg/L, which we considered to represent the limit of resistance abrogation with this compound class.[10,11] This is clearly not the case and analogue **4** provides an opportunity to develop more potent resistance modifiers with no significant bactericidal or bacteriostatic properties, a desirable feature when considering control of evolution and emergence of resistance to therapeutic agents of this type.[18]

This initial data for analogue **4** suggests an increased affinity for the staphylococcal membrane compared to ECg and compounds **1** and **2**. This contention is supported by the capacity of **4** to considerably enhance, over a range of concentrations, the oxacillin resistance modifying capacity of ECg; in fact, appropriate concentrations of ECg in combination with **4** reduce the MIC of EMRSA-16 to 0.125 mg/L (Fig. 2). Further reduction in hydroxylation by deletion of the two hydroxyl moieties on the A-ring of the B-ring phenyl analogue, yielding **5**, appeared to alter the nature of the interaction with the staphylococcal membrane; a substantial increase in anti-staphylococcal activity was evident, accompanied by a reduction in β-lactam resistance modifying potential (Table 1). The Cg analogue **6** showed a similar profile but exhibited an enhanced resistance modifying effect at a concentration of 6.25 mg/L compared to the ECg analogue **5**. This contrasts with the resistance modifying capacity of the corresponding natural products; ECg is a significantly more potent modifier than Cg.[2] Due to the increased bacterial growth inhibiting activity of **5** and **6** (Table 1), these compounds could not be examined at concentrations above 6.25 mg/L. Thus, increases in the lipophilicity of domains governed by the A- and B-rings result in the appearance of a more overt anti-staphylococcal effect, in all probability due to damage to the cytoplasmic membrane, rather than the more subtle effects that result from membrane intercalation of the natural products.[4,7]

Based upon our observation that reduction of B-ring hydroxylation in the series of compounds EGCg, ECg, **1** and **2** enhanced anti-MRSA effects, we synthesized, in single enantiomer form, the epicatechin gallate analogue with free B-ring hydroxylation **4** and the corresponding A-ring free hydroxyl compounds in the epicatechin **5** and catechin **6** relative stereochemistry (Fig. 1). The introduction of absolute chirality relied upon the asymmetric Sharpless dihydroxylation reaction and the creation of the C-ring relative stereochemistry by an *ortho*-ester/bromide cyclisation strategy. Previously this strategy has been shown to give good compound yields. However, the lack of electron-releasing hydroxyl groups in the A and B rings compromised the efficiency of the cyclisation strategy in the cases presented here. There is a clear need for alternative synthetic routes to deliver these compounds and other analogues which will be the subject of our future work. Biological assays showed that **4** displayed an enhanced capacity to reduce oxacillin resistance in EMRSA-16 at a concentration of 12.5 mg/L, reducing the oxacillin MIC from 512 to 0.25 mg/L. Complete deletion of A and B ring hydroxyl groups in either epicatechin or catechin stereochemistry resulted in a reduction in β-lactam resistance-modifying potential and an increase in intrinsic anti-staphylococcal activity (Table 1). These results support our hypothesis that reduction of B-ring hydroxylation enhances intercalation of the epicatechin B-ring into the staphylococcal cytoplasmic membrane, compromising the β-lactam resistance machinery. Ongoing work is aimed at the design and characterisation of ompounds of therapeutic potential inspired by these results and by membrane intercalation studies.

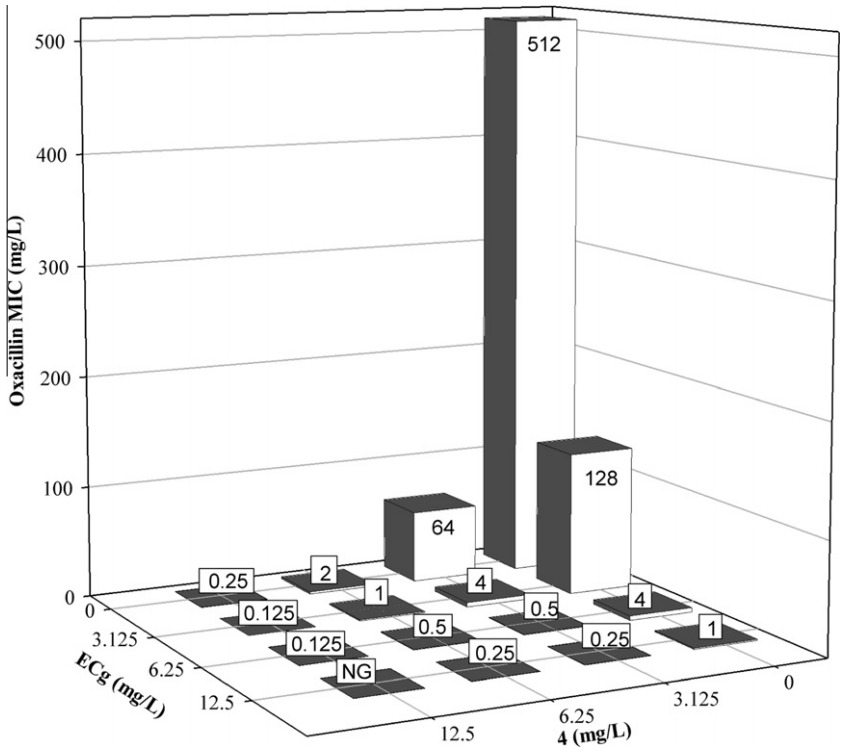


Figure 2. Effect of combinations of ECg and **4** on the minimum inhibitory concentration (MIC) of oxacillin for EMRSA-16.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.116.

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16. Enantiomeric purity measured by HPLC analysis of the corresponding dimethyl acetal (see Supplementary data) against a racemic sample on a Chiralcel OD column hexane/*i*-PrOH (90:10): **12** 0.5 mL min^{-1}, *R,R* (major) 12.8 min, *S,S* 14.3 min. **23** 0.1 mL min^{-1}, *R,R* (major) 4.4 min, *S,S* 5.7 min Absolute configuration was assumed from the Sharpless mnemonic (see Ref. 16).
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