Cationic Chalcone Antibiotics. Design, Synthesis, and Mechanism of Action

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Received July 20, 2004

This paper describes how the introduction of "cationic" aliphatic amino groups in the chalcone scaffold results in potent antibacterial compounds. It is shown that the most favorable position for the aliphatic amino group is the 2-position of the B-ring, in particular in combination with a lipophilic substituent in the 5-position of the B-ring. We demonstrate that the compounds act by unselective disruption of cell membranes. Introduction of an additional aliphatic amino group in the A-ring results in compounds that are selective for bacterial membranes combined with a high antibacterial activity against both Gram-positive and -negative pathogens. The most potent compound in this study (**78**) has an MIC value of 2 μM against methicillin resistant *Staphylococus aureus*.

Introduction

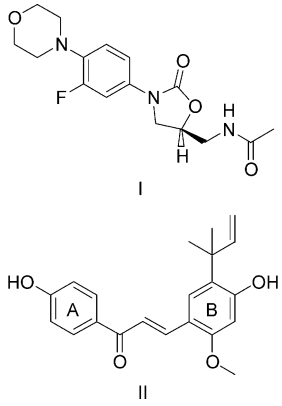
The rapid development of resistance in clinically important Gram-positive pathogens represents a serious public health threat. The proportion of *Staphylococus aureus* resistant to methicillin, oxacillin, or nafcillin (MRSA) continues to rise and is now more than 50% in intensive care units in the United States.[1] Even more critical is the development of vancomycin-resistant enterococci (VRE). Vancomycin belongs to the glycopeptide drug family and has for long been regarded as the last line of defense against drug-resistant strains, but now about 25% of all enterococcal infections in ICUs in the U.S. are resistant to vancomycin, and recently, the first case of fully vancomycin-resistant *S. aureus* has been reported.[1−3]

The last 10 years have seen only one significant novel class of non-β-lactams emerge from basic research, the oxazolidinones, of which the first drug, linezolid (**I**, Chart 1), was approved in year 2000.[4−6] However, the speed of resistance development in bacteria creates a continuous need for novel antibiotics; oxazolidinone resistant bacteria have already appeared[7,8]

One of the most promising groups of compounds for the future treatment of bacterial infections appears to be the membrane active cationic peptide antibiotics.[9−13] The main advantages are (a) the low likelihood of resistance development, as resistance requires alteration to the bulk membrane structure of the organism, (b) activity against multiresistant pathogens, as the compounds do not affect the classical antibiotic targets, (c) rapid bacterial killing, and (d) a broad spectrum of antibacterial activity.[14,15]

On the other hand, cationic peptide antibiotics face the problems related with the use of peptides as drugs: (a) large therapeutic doses as a consequence of high molecular weight and medium antibacterial activity; (b) complicated route of administration, as the compounds are not orally bioavailable; (c) stability in formulation and stability in vivo; and (d) toxicity of the peptide or degradation products.[16]

Chart 1. Linezolide (I) and Licochalcone A (II)



The oxygenated chalcone, licochalcone A (**II**, Chart 1), has previously been described as a moderate potent antibacterial compound with activity against Gram-positive bacteria.[17,18] In this paper, we describe the synthesis of a new group of compounds, the cationic chalcone antibiotics, which combine the advantages of cationic peptide antibiotics with the advantages of small molecules, potentially eliminating the peptide-related problems listed above.

A number of novel cationic antibacterial chalcones have been prepared by systematic introduction of aliphatic amino substituents in different positions of the chalcone rings. Due to the alkaline nature of the aliphatic amines, the compounds are expected to be protonated under physiological conditions.

Chemistry

The chalcones described were all prepared by base-catalyzed Claisen−Schmidt condensation[22] of acetophenones with appropriate benzaldehydes (e.g. Scheme 3) in a yield of 2−73%.

Acetophenones having aminoalkylamino substituents in the 2′- and 4′-positions (**1**, **2**) were easily prepared by noncatalyzed nucleophilic aromatic substitution us-

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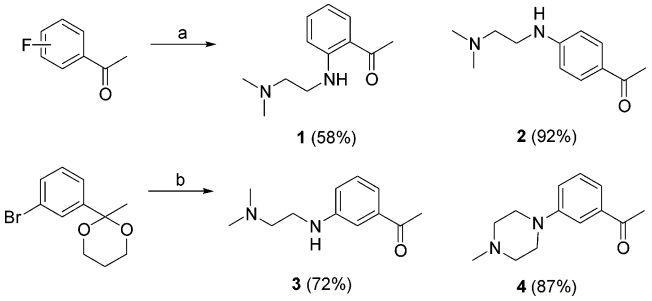
10.1021/jm049424k CCC: $30.25
Published on Web 03/04/2005

Scheme 1. Preparation of Aminoalkylamino-Substituted Acetophenones[a]



[a] Reagents and conditions: (a) K_2CO_3, *N,N*-dimethylethylenediamine, DMF, Δ; (b) (i) *N,N*-dimethylethylenediamine or 1-methylpiperazine, $Pd_2(dba)_3$, *rac*-BINAP, $NaOBu^t$, toluene Δ, (ii) 1 M HCl (aq).

ing 2′- or 4′-fluoroacetophenone (Scheme 1). Acetophenones with aminoalkylamino groups in the 3′-position (**3**, **4**) were synthesized by palladium-catalyzed reaction of 2-(3-bromophenyl)-2-methyl-[1,3]dioxane with the appropriate substituted aminoalkylamines followed by acidic workup as described for similar compounds by Buchwald et al.[21] (Scheme 1). The use of the unprotected 3′-bromoacetophenone was not feasible, as the yield was very low.

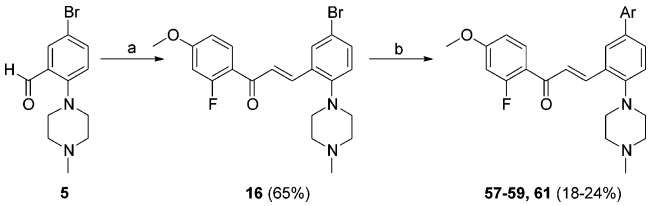
The nucleophilic aromatic substitution of 5-bromo-2-fluorobenzaldehyde with various aminoalkylamines afforded the benzaldehydes **5**−**9** (Scheme 2). Direct synthesis of the 5-bromo-2-(piperazin-1-yl)benzaldehyde (R = H, Scheme 2) using an excess of piperazine resulted in large quantities of polymeric material. An alternative route was successfully explored in which the Boc-protected benzaldehyde **7** was synthesized, coupled with the selected acetophenone, and deprotected to give the desired product (**78**, Table 4) by treatment with TFA.

Suzuki coupling[23] of the benzaldehydes **5**−**9** with various aryl boronic acids gave 5-aryl-substituted benzaldehydes (**10**−**15**).

In an attempt to explore the specific effect of the aromatic substituent in the 5-position of the B-ring, a number of chalcones with different aromatic rings in the 5-position were prepared from the corresponding 5-bromochalcone (**16**) under Suzuki conditions (Scheme 3, Table 2).

The 3-methylpiperazine benzaldehyde **17** was prepared by palladium-catalyzed amination using the 1,3-dioxane-protected 3-bromobenzaldehyde. Bromination

Scheme 3. Preparation of 5-Aryl Substituted Chalcones[a]



[a] Reagents and conditions:(a) 2′-F,4′-OMe-acetophenone, NaOH, ethanol; (b) (hetero)arylboronic acid, Na_2CO_3, $Pd(PPh_3)_2Cl_2$, DME/water, reflux; Ar: cf. Table 2.

of **17** using NBS in methylene chloride gave the desired 6-bromo isomer **18**, which was reacted with phenyl boronic acid to give the benzaldehyde **19** (Scheme 4).

Likewise palladium-catalyzed amination of 2-(3,5-dibromophenyl)-[1,3]dioxane with 1-methylpiperazine, deprotection, and subsequent Suzuki coupling with phenylboronic acid gave the 3-(4-methylpiperazin-1-yl)-5-phenylbenzaldehyde **21** (Scheme 4).

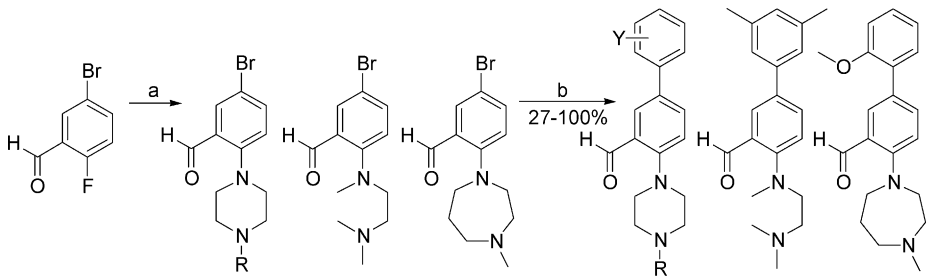
6-(4-Methylpiperazin-1-yl)biphenyl-3-carbaldehyde **23** and 3-(4-methylpiperazin-1-yl)biphenyl-4-carbaldehyde **25** were prepared by noncatalyzed nucleophilic aromatic substitution of the appropriate fluorobenzaldehyde, giving **22** and **24**, respectively, followed by Suzuki coupling using phenylboronic acid (Scheme 4).

The acetophenone **29** was prepared in a four-step synthesis starting with the reduction of 5-bromo-2-methoxybenzaldehyde using $NaBH_4$ to give the benzyl alcohol **26** (Scheme 5). The benzyl alcohol was converted to the benzyl chloride **27** by thionyl chloride, and subsequent reaction with aqueous dimethylamine gave the amine **28**. Conversion of **28** to the desired acetophenone **29** was performed under Heck conditions[24,25] using butoxyethene followed by hydrolysis.

The amide **30** was synthesized from the corresponding carboxylic acid using thionyl chloride and dimethylamine. Borane reduction of the amide gave the amine **31**, which was converted to the acetopheneone **32** in a similar manner as described for **30** (Scheme 5).

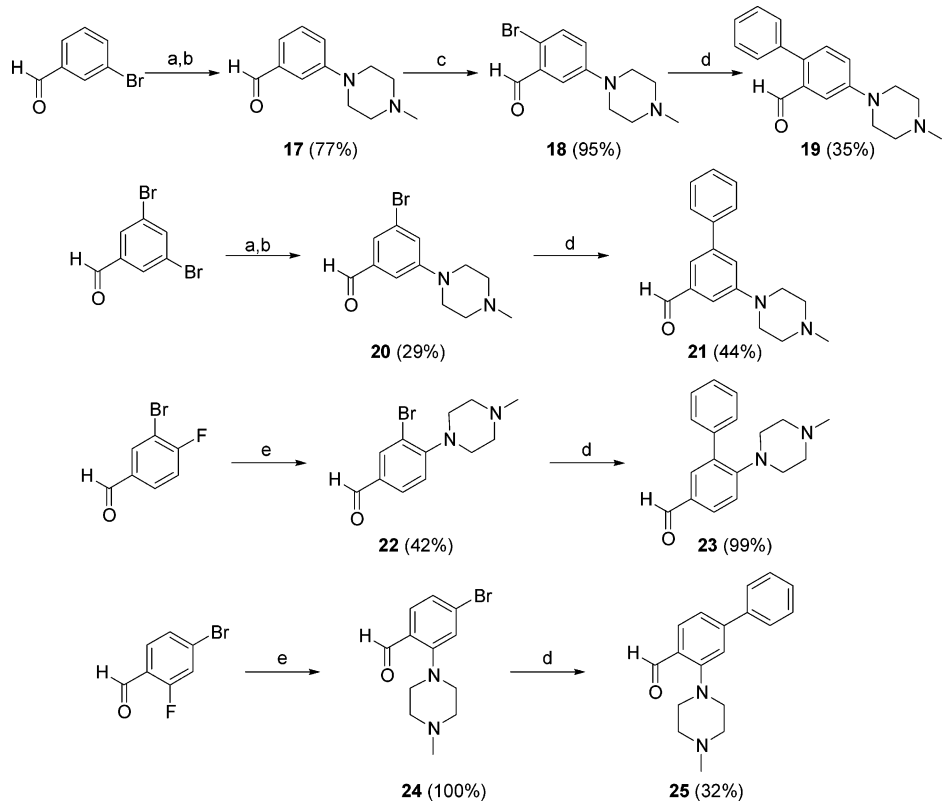
Reaction of 4-bromobenzyl bromide with aqueous dimethylamine afforded the amine **33**, which was converted into the phenyl analogue **34** under Suzuki conditions (Scheme 6). Directed ortho-metalation of **34** using *n*-butyllithium in refluxing diethyl ether and subsequent quenching with *N,N*-dimethylformamide gave the desired benzaldehyde **35**.

Scheme 2. Preparation of 2,5-Disubstituted Benzaldehydes[a]



[a] Reagents and conditions: (a) K_2CO_3, aminoalkylamine, DMF; (b) arylboronic acid, Na_2CO_3, $Pd(PPh_3)_2Cl_2$, DME/water, reflux.

Scheme 4. Preparation of 2,4-, 3,5-, 3,6-, or 4,5-Disubstituted Benzaldehydes To Be Used in the Chalcone Synthesis Presented in Table 5[a]



[a] Reagents and conditions: (a) 1,3-propandiol, p-TsOH, toluene; (b) (i) 1-methylpiperazine, $Pd_2(dba)_3$, *rac*-BINAP, $NaOBu^t$, toluene, Δ, (ii) HCl_{aq}; (c) NBS, CH_2Cl_2; (d) phenylboronic acid, Na_2CO_3, $Pd(PPh_3)_2Cl_2$, DME/water, reflux; (e) K_2CO_3, 1-methylpiperazine, DMF.

The quaternary amines (**84**−**86**) (Chart 2) were prepared by the treatment of the corresponding tertiary amines with iodomethane in a suitable solvent.

Results and Discussion

The oxygenated chalcone licochalcone A acts nonselectively at both prokaryotic and eukaryotic cell membranes (Table 6), making the compound unfit for the treatment of bacterial infections. Licochalcone A is, due to high lipophilicity, expected to interact with the central lipophilic part of the membrane. The surface of eukaryotic cell membranes is exclusively composed of neutral zwittterionic phospholipids, whereas bacterial membranes contain large amounts of negatively charged phospholipids.[11,20] Introduction of a positive charge in the chalcone would make the basis for a strong electrostatic interaction with the surface of the bacterial membrane, while the lipophilic interaction between the chalcone and the interior of the membrane would be retained. This would lead to a more potent compound with reduced affinity for eukaryotic cells.

The major parameter used in the evaluation of the compounds prepared is the in vitro antibacterial activity against a panel of Gram-positive and -negative pathogens. In addition, the bacterial selectivity of the compounds was examined. The selectivity is expressed as the degree of hemolysis of human erythrocytes at 4 times the minimum inhibitory concentration value (MIC value), which is the concentration level needed to obtain a secure antibacterial efficacy in vivo.

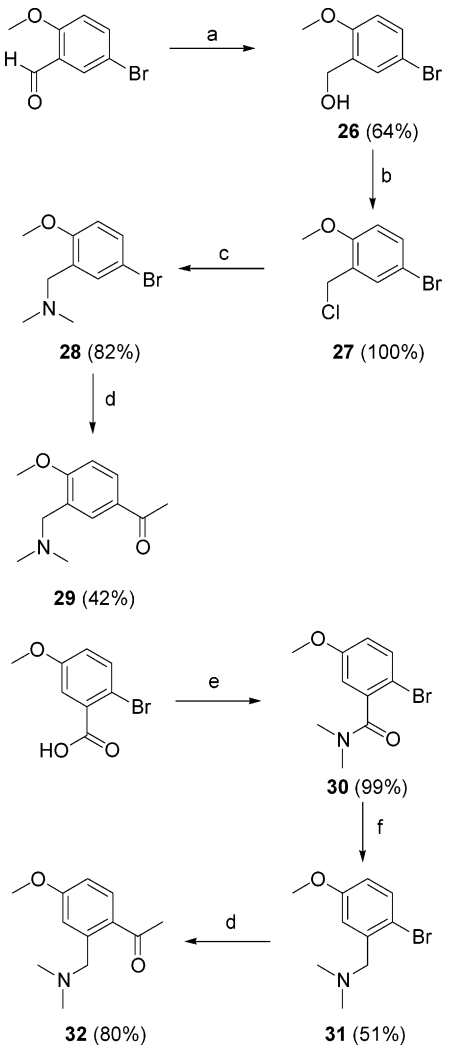
Series A. Aliphatic Amines in the A-Ring. A number of chalcones having aliphatic amino substituents in the A-ring and diverse substituents in the B-ring have been prepared (Table 1). The position of the aliphatic amine in the A-ring only has a marginal effect on the activity, as compounds having the same amino substituents in the 2′-, 3′-, or 4′-position are equipotent (**36**−**38**). The distance between the aliphatic amino group and the aromatic ring is not important for the activity, as compounds having a dimethylethylendiamine, dimethylaminoethoxy, or dimethylaminomethyl group have comparable activity (**38**−**40**). On the other hand, the lipophilicity of the B-ring substituents appears to be the most important property with regard to modulating the activity (**41**−**43**, **45**), whereas the position of the substituents has a minor effect on the activity (**43**, **44**). Generally, the compounds exhibit a broad Gram-positive antibacterial spectrum (Table 1) but do not affect Gram-negative pathogens (MIC > 300 μM).

All compounds in this series showed high protein binding (>97%), low aqueous solubility, and a high degree of hemolysis (80−100%) at 4 times the MIC. Compared to licochalcone A (100% haemolysis) only a marginally improvement has been achieved. These results prompted us to investigate compounds having aliphatic amines in the B-ring.

Series B. Aliphatic Amines in the B-Ring. Introduction of an aliphatic amine in the B-ring and a lipophilic group in the A-ring results in compounds that show the same selectivity profile and antibacterial activity as seen for the compounds in series A (compounds **43** and **46**, Table 1).

It was envisioned that having a bulky group and an aliphatic amine in the same ring would result in a

Scheme 5. Preparation of 2,4- and 3,4-Subsituted Acetophenones[a]



[a] Reagents and conditions: (a) $NaBH_4$, ethanol; (b) $SOCl_2$, CH_2Cl_2; (c) Me_2NH (aq); (d) (i) butoxyethene, palladium acetate, 1,3-bis(diphenylphosphino)propane, K_2CO_3, DMF/H_2O, 80 °C; (ii) HCl_{aq}; (e) (i) $SOCl_2$, (ii) Me_2NH (aq); (f) BH_3·THF.

change in the way the compounds interact with the membrane, making them more selective.

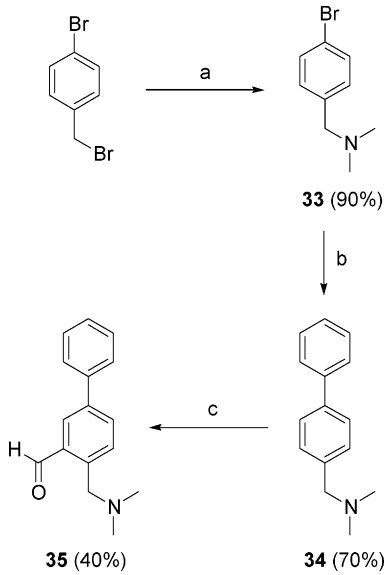
Previous papers on bioactive chalcones highlight the importance of bulky substituents in the 5- position.[17,26,27] We have prepared a number of analogues with increasingly bulky substituents in the 5-position, while the aliphatic amine in the 2-position (**47**−**52**, Table 2) was kept. An increase of the bulk in the 5-position gradually increases the activity of the compounds (**47**−**52**). Analogues with a phenyl group (**50**) or a 3,5-dimethylphenyl group (**51**) in the 5-position are more than 60 times as potent as the nonsubstituted analogue **47**.

In addition, the substitution pattern of the phenyl ring in the 5-position have been evaluated (Table 2, compounds **51**−**61**). In general, nonpolar substituents are tolerated in the 2″- or 3″-position (**53**, **57**, **58**), while introduction of polar substituents dramatically reduces potency (**60**, **61**). Substituents in the 4″-positon appear to be detrimental to the activity (**59**). The preferred aromatic substituent in the 5-position in this study is 3,5-dimethylphenyl (**51**).

The distance between the aliphatic amino group and the B-ring is of importance for the activity. An aliphatic nitrogen atom spaced to the B-ring by a single methylene group (**56**) reduces the activity, whereas an aliphatic nitrogen atom in a more distant position results in potent compounds independent of the linker (**52**−**54**). As expected, a complete masking of the aliphatic amino group as an amide (**55**) totally eliminates the activity.

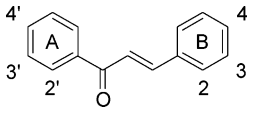
Having identified the 2-amino-5-phenyl combination as the preferred substitution pattern, a further investigation of the structure−activity relationships was initiated. A library of analogues with different polar and nonpolar substituents (Br, OBu, NH_2, F) in the A-ring was synthesized according to a statistical design (data

Scheme 6. Preparation of 2-Dimethylaminomethyl-5-phenylbenzaldehyde[a]

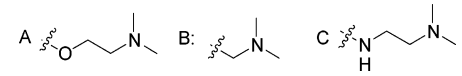


[a] Reagents and conditions: (a) Me_2NH (aq); (b) phenylboronic acid, Na_2CO_3, $Pd(PPh_3)_2Cl_2$, DME/H_2O; (c) (i) n-BuLi, diethyl ether, reflux (6 h), (ii) DMF.

Table 1. In Vitro Antibacterial Activity of Chalcones with Aliphatic Amines in Ring A against a Panel of Representative Gram-Positive Bacteria[a]

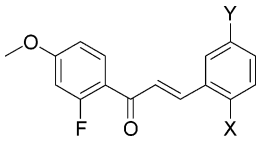


	substituents		MIC (μM)			
compd	A-ring	B-ring	A[b]	B[c]	C[d]	log *P*[e]
LicA			40	40	75	
36	2-C	2,4-Cl	40	40	75	1.4
37	3-C	2,4-Cl	40	40	40	1.4
38	4-C	2,4-Cl	40	40	40	1.4
39	4-A	2,4-Cl	40	40	40	1.4
40	4-B	2,4-Cl	40	40	75	1.4
41	4-C	H	300	NA	NA	0
42	4-C	4-Cl	75	75	75	0.7
43	4-C	4-OPh	20	20	5	2.1
44	4-C	3-OPh	20	20	5	2.1
45	4-C	4-N(Bu)$_2$	10	10	10	3.4
46	4-OPh	4-C	20	20	10	2.1

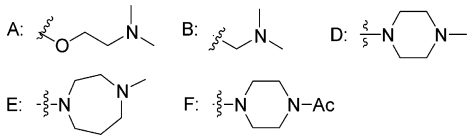


[a] Each value represents the mean of three experiments. [b] *S. aureus* ATCC33591 (resistant to methicillin). [c] *Enterococcus faecium* #17501 (vancomycin-resistant clinical isolate). [d] *E. faecium* ATCC292121. [e] log *P* is calculated as $\Sigma\pi$ of substituents on the B-ring.

Table 2. In Vitro Antibacterial Activity of Chalcones with Aliphatic Amines in Ring B against a Panel of Representative Gram-Positive Bacteria[a]



compd	substituents X	substituents Y	MIC (μM) A[b]	B[c]	C[d]
47	2-A	H	300	300	300
48	2-A	Me	75	150	150
49	2-A	t-Bu	10	20	20
50	2-A	Ph	5	10	10
51	2-A	3,5-(Me)$_2$-Ph	5	5	5
52	2-A	2-OMe-Ph	10	10	10
53	2-D	2-OMe-Ph	10	10	20
54	2-E	2-OMe-Ph	10	10	20
55	2-F	2-OMe-Ph	NA	NA	NA
56	2-B	Ph	150	150	150
57	2-D	2-CF_3-Ph	5	10	20
58	2-D	3-CF_3-Ph	5	5	10
59	2-D	4-CF_3-Ph	NA	NA	NA
60	2-D	3-pyridyl	75	75	75
61	2-D	3-(CH_2OH)-Ph	NA	NA	NA



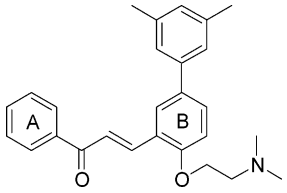
[a] Each value represents the mean of three experiments. [b] *S. aureus* ATCC33591 (resistant to methicillin). [c] *E. faecium* #17501 (vancomycin-resistant clinical isolate). [d] *E. faecium* ATCC29212.

not shown).[9] Surprisingly, the substituents on the A-ring have little or no influence on the activity, indicating that the A-ring is less important for binding. Compounds having aniline as the A-ring (**62**−**64**) appear to be more selective for the bacterial membrane (20−30% hemolysis at 4 times the MIC as compared to 50−100% for the rest). This may be due to a different orientation of the compounds in the eukaryotic cell membrane as a consequence of the increased polarity of the A-ring. In addition, the protein binding is lower than for the rest of the compounds (75−88% as compared to 94−97%), but the solubility is relatively low (approximately 0.2 mg/mL).

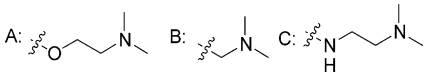
Series C. Aliphatic Amines in the A -and B-Ring. On the basis of the observations described above and in an attempt to take full advantage of the negatively charged bacterial membrane, a number of compounds with aliphatic amines in both the A- and B-ring were prepared. It was the hope that this would lead to an improvement of the activity, selectivity, protein binding, and solubility of the compounds.

(a) Effect of Changing the Aliphatic Amine in the A-Ring. A series of chalcones with diverse aliphatic amines in the A-ring and the (2-dimethylaminoethoxy-5-(3,5-dimethylphenyl) B-ring was prepared (Table 3, **65**−**75**). The data shows that the position and type of linker between the aliphatic nitrogen and the A-ring have relatively small effect on the antibacterial activity. In the 2′-position, a one-atom linker to the aliphatic nitrogen in combination with a methoxy group in the 4′-position is the optimal combination (**68**), showing slightly better activity than the compounds having longer linkers or no 4′-methoxy group (**65**−**67**). The 3′-position favors a three-atom linker to obtain the most potent compounds (**69**, **70**), while a one-atom linker, especially in combination with a 4′-methoxy group is unfavorable (**71**, **72**). A one-atom linker appears to be the preferred in the 4′-position (**75**). A majority of the compounds prepared for this series are active against both Gram-positive and -negative organisms (*Escherichia coli*).

Table 3. In Vitro Antibacterial Activity of Chalcones with Aliphatic Amines in Rings A and B against a Panel of Representative Gram-Positive and -Negative Bacteria[a]



compd	substituents: A-ring	MIC (μM) A[b]	B[c]	C[d]	D[e]
62	2-NH_2	5	5	10	NA
63	3-NH_2	10	10	10	NA
64	4-NH_2	10	10	20	NA
65	2-C	10	10	10	10
66	2-A	10	10	10	40
67	2-B	10	10	5	75
68	2-B, 4-OCH_3	5	5	5	75
69	3-C	5	10	10	20
70	3-A	5	10	10	20
71	3-B	10	10	10	40
72	3-B, 4-OCH_3	20	20	20	40
73	4-C	10	10	20	20
74	4-A	10	10	20	40
75	4-B	5	10	20	20

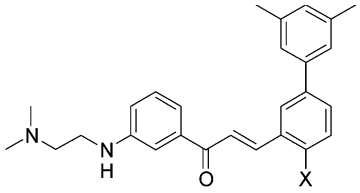


[a] Each value represents the mean of three experiments. [b] *S. aureus* ATCC33591 (resistant to methicillin). [c] *E. faecium* #17501 (vancomycin-resistant clinical isolate). [d] *E. faecium* ATCC29212. [e] *E. coli* ATCC25922.

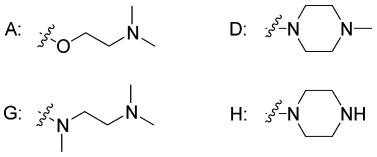
(b) Effect of Changing the Amine in the B-Ring. To investigate the effect of introduction of different amines in the 2-postion of the B-ring, a number of analogues were prepared retaining the most favorable A-ring (Table 4). Chalcones having the *N*-methylpiperazine (**76**) and *N*,*N*-dimethylaminoethoxy (**69**) groups in the 2-position are equipotent, whereas the introduction of the *N*,*N*′,*N*′-trimethylethylendiamine group (**77**) results in a less potent compound. The most potent class of compounds has a piperazine in the 2-position (**78**). The high activity of this secondary amine may reflect the fact that the latter compound is more basic and consequently has a higher degree of protonation in the assay.[28] Given the mechanism of action described below, this fact should be favorable for the binding in the membrane.

(c) Position of the Amine and the Phenyl Group in Ring B. The effect of moving the amine and/or the phenyl group was investigated (**79**−**83**, Table 5). Moving the phenyl group to the 4-position of the B-ring (**80**) only marginally changes the activity. The same pattern is seen when moving the amine to the 3-position and keeping the phenyl in the 5-position (**81**). However, having the amine in the 3-position and a phenyl in 6-position (**82**) or an amine in the 4-position and phenyl in the 5-position (**83**) significantly reduce the activity.

Table 4. In Vitro Antibacterial Activity of Chalcones with Aliphatic Amines in Rings A and B against a Panel of Representative Gram-Positive and -Negative Bacteria[a]

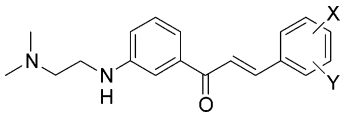


compd	substituents: X	MIC (μM) A[b]	B[c]	C[d]	D[e]
69	2-A	5	10	10	20
76	2-D	5	5	5	10
77	2-G	10	10	10	20
78	2-H	2	5	5	5

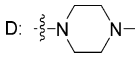


[a] Each value represents the mean of three experiments. [b] *S. aureus* ATCC33591 (resistant to methicillin). [c] *E. faecium* #17501 (vancomycin-resistant clinical isolate). [d] *E. faecium* ATCC29212. [e] *E. coli* ATCC25922.

Table 5. In Vitro Antibacterial Activity of Chalcones with Aliphatic Amines in Rings A and B against a Panel of Representative Gram-Positive and -Negative Bacteria[a]



compd	substituents X	Y	MIC (μM) A[b]	B[c]	C[d]	D[e]
79	2-D	5-Ph	20	20	40	20
80	2-D	4-Ph	20	40	40	40
81	3-D	5-Ph	20	40	40	75
82	3-D	6-Ph	75	75	75	NA
83	4-D	5-Ph	75	75	150	NA



[a] Each value represents the mean of three experiments. [b] *S. aureus* ATCC33591 (resistant to methicillin). [c] *E. faecium* #17501 (vancomycin-resistant clinical isolate). [d] *E. faecium* ATCC29212. [e] *E. coli* ATCC25922.

The reason for the lower activity of these compounds may be an unfavorable orientation of the chalcone in the cell membrane (**82**) or a result of bringing the lipophilic and hydrophilic parts of the molecule close together, making the incorporation of the molecule in cell membrane difficult (**83**).

The idea of introducing two aliphatic amines in the molecule in order to improve the in vitro performance has proven successful. The most noteworthy feature of the compounds in series C is that, in addition to retaining the potency seen in the best compounds of series A and B, they are, in general, practically non-hemolytic (0−10%) at 4 times the MIC. One of the most active compounds (**78**) is as potent in our in vitro assay as linezolide (MIC = 2 μM against *S. aureus*) and the degree of hemolysis is negligible (<4%) even at a concentration of 150 μM. Furthermore, the compounds in series C have acceptable protein binding (50−87%)

Table 6. Mechanism of Action: In Vitro Activity and Effect on Bacterial Membrane Integrity (BacLight) and Hemolysis of Selected Analogues[a]

compound	MIC *S. aureus* (μM)	% membrane integrity	% hemolysis (4 × MIC)
control		100	
licochalcone A	40	0	100
39 (series A)	40	0	100
53 (series B)	10	0	80
68 (series C)	5	2	<1
78 (series C)	2	3	0
ciprofloxacin	2	102	0

[a] Each value represents the mean of three experiments.

and a very favorable aqueous solubility at physiological pH (>1 mg/mL). Due to the promising results, this group of compounds is currently under further investigation

Mechanism of Action

The mechanism of action has been investigated and validated by four different methods.

(a) Hemolysis. Human erythrocytes were exposed to different concentration of the test compound and the proportion of lysed cell was measured.[29] The amino chalcones from series A and B like licochalcone A have pronounced hemolytic properties, which support the underlying thesis of this work that licochalcone A and the novel amino chalcones share the same mechanism of action (Table 6).

(b) Kinetics of Bacterial Killing. Time−kill experiments were carried out in order to investigate the kinetics of the killing of the bacteria (Figure 1). The extremely fast killing kinetics seen for licochalcone A (Figure 1A) indicates an immediate bacteriocidal effect on the membrane structure of the cell. Furthermore, the three amino chalcones (**40**, **53**, **76**) were shown to have bactericidal profile even at concentrations corresponding to the MIC value (Figure 1B−D). For the compounds belonging to series A (e.g. **40**) and series C (e.g. **76**), a concentration-dependent bacterial killing was observed. The compound from series B (e.g. **53**) shows a slower bacterial killing that appears to be time-dependent. This observation may reflect that the compounds due to different orientation in the membrane induce slightly different mechanisms of membrane damage, as also seen for cationic peptides.[16]

(c) BacLight assay.[29] The integrity of the bacterial membranes was investigated by the use of the BacLight fluorescence assay. The bacteria are labeled with a mixture of SYTO9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain propidium iodide. The SYTO9 stain generally labels all bacteria in a population, whereas propidium iodide only penetrates damaged membranes, labeling solely the bacteria with damaged membranes. The difference in spectral characteristics and ability to penetrate healthy bacterial cells can be used to investigate the degree of membrane damage. We have investigated the membrane-damaging effect of licochalcone A and selected compounds representing the three series of compounds presented in this paper (**39**, **53**, **68**, **78**). As seen in Table 6, all compounds tested produce almost complete membrane damage within 15 min (≥97% permeability compared 0% for the nondrug control), strongly supporting the hypothesis that damage of the membrane

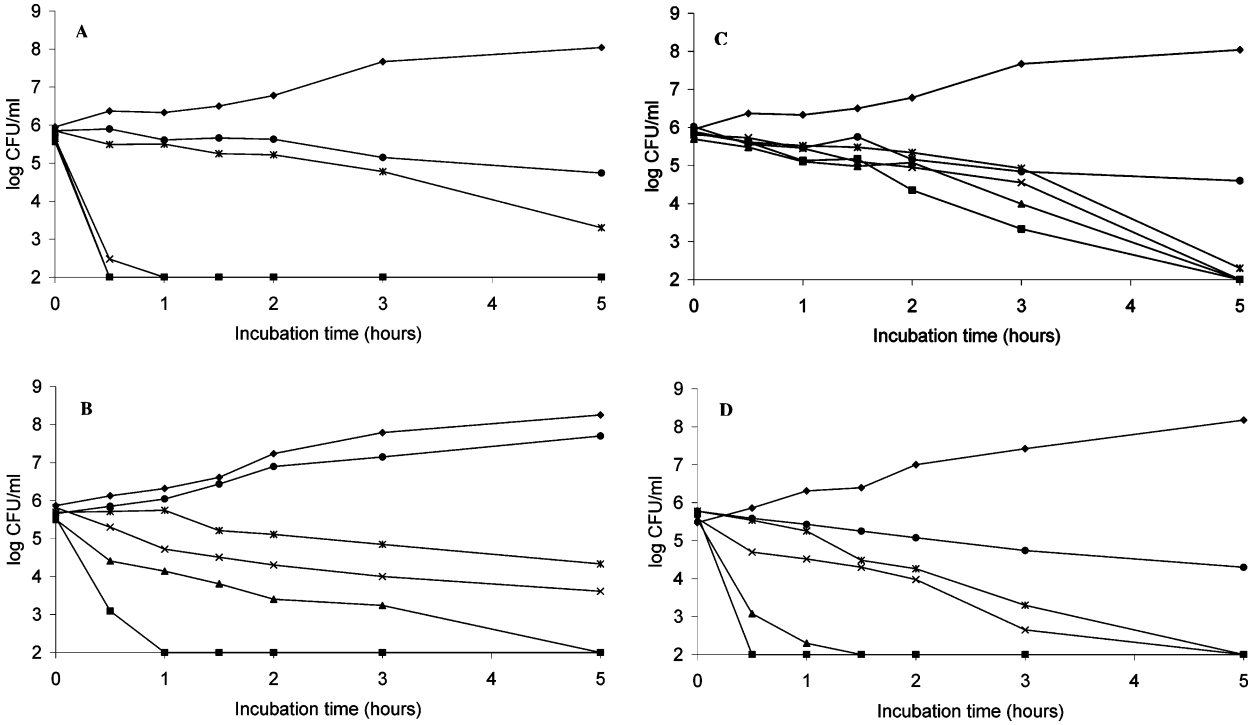
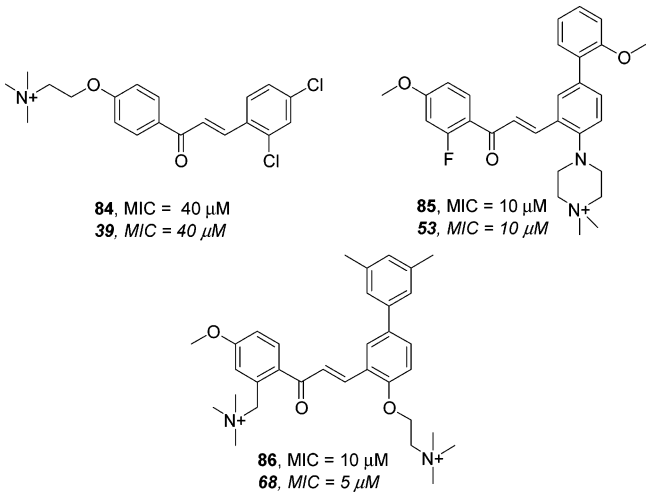


Figure 1. Time−kill kinetics and dose−response effect of licochalcone A and selected analogues representing the three series against *S. aureus* (ATTC 29213): (A) licochalcone A, (B) **40**, (C) **53**, (D) **76**. Compound concentrations: 8 × MIC (■), 4 × MIC (▲), 2 × MIC (×), MIC (∗), 0.5 × MIC (●), and control (◆).

Chart 2. In Vitro Activity of the Quaternary Chalcones Analogoues against *S. aureus*[a]



[a] The activity of the corresponding tertiary analogues is written in italics. Each value represents the mean of three experiments.

is the mechanism of action. On the other hand, the degree of hemolysis is related to the series of compounds as described previously. Series A, exemplified by compound **39**, and series B, exemplified by compound **53**, causes >80% hemolysis at 4 times the MIC, whereas compounds belonging to series C (**68**, **78**) show less than 10% hemolysis, even though the bacterial membrane is completely disrupted, indicating a significant degree of selectivity. The data demonstrate that the compounds from the three series have the same mechanism of action on bacterial cells, but at the same time they have dramatically different affinity for the eukaryotic membranes. Ciprofloxazine, which was included as a negative control, does not affect the bacterial membrane or cause hemolysis in our assays.

(c) Synthesis of Quaternary Amine Analogues. To conclusively prove the suggested mechanism of action, a number of quaternary amine analogues were synthesized and their activities were compared with those of the corresponding tertiary amine analogues (Chart 2). It is highly unlikely that the quaternary compounds, being charged at any time, can penetrate the bacterial membrane. As seen in Chart 2, the compounds are equipotent to the parent compounds, fully supporting the suggested mechanism of action.

Conclusion

In this report, we describe the synthesis and antibacterial effect of a novel class of chalcones having aliphatic amino substituents. The mechanism of action has been investigated and it is shown that the compounds act by disruption of the cell membrane. We describe how generally membrane damaging chalcones (e.g. licochalcone A) were converted into a class of chalcones that selectively disrupt bacterial membranes without affecting eukaryotic cell membranes via the introduction of aliphatic amino groups in both rings of the chalcone, resulting in compounds being cationic at physiological pH. The antibacterial spectrum has been expanded, as many of the compounds are equally potent against Gram-positive and -negative pathogens. Due to the novel mechanism of action, the compounds are active against drug resistant pathogens such as MRSA and VRE, making them very attractive for further development.

In addition, the protein binding has been reduced to an acceptable level and the aqueous solubility has been improved more than 500 times, making administration and absorption feasible.

Experimental Section

Chemistry. Thin-layer chromatography (TLC) was performed on silica gel F_{254} plates (Merck). All compounds were detected using UV light. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC-300 F spectrometer, using $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts are given in ppm (δ) using TMS as the internal standard, and coupling constants (J) are given in hertz. Mass spectra (GC−MS) were recorded using an Agilent Technologies 6890N and were all >95% pure. Mass spectra (LC−MS) were recorded using a Waters Alliance HPLC-system coupled to a Quatro Micro triple quadropol mass spectrometer (Micromass) operating in positive (ESI) mode. Separation was performed on a XTerra MS C_{18} column (150 × 2.1 mm i.d., 3.5 μm particle size). All intermediate compounds were characterized by either GC−MS or LC−MS and all final compounds were confirmed by LC−MS. The purity of the compounds (>95%) was determined using a Waters Alliance 2690 separation module and Waters 996 PDA-detector (Waters, Milford, MA). Separation was performed on a XTerra MS C_{18} column (150 × 2.1 mm i.d., 3.5 μm particle size) (Waters Milford, MA).

For system I, initial conditions were 40% mobile phase A (acetonitrile) and 60% mobile phase B (10 mM ammonium acetate pH 9.5). During the first 20 min, the mobile phase was changed via a linear gradient to 90% A and 10% B.

For system II, initial conditions were 70% mobile phase A (0.1% (v/v) formic acid in MilliQ-water) and 30% mobile phase B (methanol). During the first 10 min, the mobile phase was changed via a linear gradient to 10% A and 90% B, and this gradient was maintained for 10 min.

The accurate mass measurements (HRMS) were obtained on a VG AutoSpec mass spectrometer (Micromass, Manchester, UK) equipped with an EI source. The instrument was operated at a resolution of 10000 fwhm.

Column chromatography (CC) was performed on Merck silica gel 60 (0.063−0.200 mm). All solvents and reagents were obtained from Fluka or Aldrich and used without further purification except DMF and THF, which were stored over 3 Å molecular sieves.

General Procedure A for the Aromatic Nuclephilic Substitution of Fluoroacetophenones (1, 2). Example: 1-[2-(2-Dimethylaminoethylamino)phenyl]ethanone (1). A solution of 2′-fluoroacetophenone (5.1 mL, 40 mmol), *N,N*-dimethylethylenamine (11.1 g, 50 mmol), and K_2CO_3 (11.1 g, 50 mmol) in dry DMF (20 mL) was refluxed under an atmosphere of argon for 18 h. The solvent was removed in vacuo and H_2O (50 mL) was added to the resulting residue. The aqueous phase was extracted with Et_2O (2 × 100 mL) and the organic phase was dried (K_2CO_3) and evaporated in vacuo to yield the desired product, which was used without further purification.

General procedure A gave the title compound as a yellow oil in 58% yield (5.0 g). ^1H NMR ($CDCl_3$): δ 8.95 (bs, 1H), 7.73 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H), 7.35 (td, J = 8.0 Hz, J = 1.6 Hz, 1H), 6.69 (bd, J = 8.0 Hz, 1H), 6.58 (td, J = 7.1 Hz, J = 1.6 Hz, 1H), 3.30 (q, J = 6.5 Hz, 2H), 2.58 (t, J = 6.5 Hz, 2H), 2.56 (s, 3H), 2.30 (s, 6H). GC−MS: 206 (M).

General Procedure B for the Buchwald-type Coupling of 3′-Bromoacetophenones (3, 4). Example: 1-[3-(2-Dimethylaminoethylamino)phenyl]ethanone (3). A solution of 3′-bromoacetophenone (19.9 g, 100 mmol), 1,3-propandiol (9.1 g, 120 mmol), and p-TsOH (0.1 g) in toluene (200 mL) was refluxed, azeotropically removing H_2O using a Dean−Stark water separator. After 18 h, the solution was washed with 5% Na_2CO_3 (100 mL) and dried (K_2CO_3) and the solvent removed in vacuo. The resulting 1,3-dioxane was used without further purification.

2-(3-Bromophenyl)-2-methyl-[1,3]dioxane (20.4 g, 79 mmol), dimethylethylenediamine (11.0 mL, 95 mmol), $Pd_2(dba)_3$ (365 mg, 0.4 mmol, 1 mol % Pd), *rac*-BINAP (500 mg, 0.8 mmol), and $NaOBu^t$ (10.7 g, 111 mmol) were stirred under an atmosphere of argon in toluene (100 mL) at 80 °C for 18 h. The dark brown mixture was poured into an ice cold solution of HCl (aq, 1 M, 200 mL) and stirred vigorously for 2 h at 25 °C. The solution was cooled to 0 °C and pH was adjusted to 13 using 6 M NaOH (aq) and extracted with Et_2O (4 × 100 mL). The organic phase was dried (K_2CO_3) and the solvent was removed in vacuo. The resulting crude oil was purified by CC using EtOAc−Et_3N 20:1. General procedure B gave the title compound as a yellow oil in 72% yield (11.2 g). ^1H NMR ($CDCl_3$): δ 7.21 (t, J = 7.9 Hz, 1H), 7.15−7.12 (m, 2H), 6.85 (bd, $J \approx$ 8 Hz, 1H), 5.66 (t, J = 5.3 Hz, 1H), 3.13 (q, J = 6.6 Hz, 2H), 2.50 (s, 3H), 2.44 (t, J = 6.6 Hz, 2H), 2.25 (s, 6H). GC−MS: 206 (M).

General Procedure C for the Aromatic Nuclephilic Substitution of Fluorobenzaldehydes (5−9, 22, 24). Example: 5-Bromo-2-(4-methylpiperazin-1-yl)benzaldehyde (5). To a stirred solution of 5-bromo-2-fluorobenzaldehyde (40 g, 197 mmol) in dry DMF (200 mL) were added K_2CO_3 (81.7 g, 590 mmol) and 1-methylpiperazine (30 g, 290 mmol), and the mixture was left overnight at 100 °C. The reaction mixture was cooled to room temperature, quenched with H_2O (200 mL), and extracted with Et_2O (3 × 100 mL). The combined organic phases were washed with H_2O, dried (Na_2SO_4), and evaporated in vacuo. The resulting crystals were recrystallized (heptane) to give the desired product.

General procedure C gave the title compound as yellow crystals in 54% yield (30 g). ^1H NMR ($CDCl_3$) δ 10.24 (s, 1H), 7.91 (d, J = 2.4 Hz, 1H), 7.61 (dd, J = 8.7 Hz, J = 2.4 Hz, 1H), 7.02 (d, J = 8.7 Hz, 1H), 3.11 (m, 4H), 2.63 (m, 4H), 2.39 (s, 3H). GC−MS: 282 (M).

General Procedure D for the Suzuki Coupling of Substituted Benzaldehydes (10−15, 19, 21, 23, 25, 34). Example: 3′,5′-Dimethyl-4-(4-methylpiperazin-1-yl)biphenyl-3-carbaldehyde (10). To a solution of 5-bromo-2-(4-methylpiperazin-1-yl)benzaldehyde **5** (13.5 g, 47.6 mmol) and 3,5-dimethylphenylboronic acid (8.6 g, 57.2 mmol) in DME (130 mL) was added a solution of Na_2CO_3 (5.2 g, 143 mmol) in H_2O (70 mL). The mixture was flushed with argon for 2 min followed by addition of $Pd(PPh_3)_2Cl_2$ (1.0 g, 3 mol %). The reaction was heated to reflux and left overnight under an atmosphere of argon. The solution was cooled, washed with 2 M Na_2CO_3 (aq), extracted with Et_2O (3 × 40 mL), and purified by CC.

General procedure D gave the title compound as yellow crystals in 74% yield (10.9 g). ^1H NMR (DMSO-d_6): δ 10.22 (s, 1H), 7.91 (d, J = 2.4 Hz, 1H), 7.87 (dd, J = 8.5 Hz, J = 2.4 Hz, 1H), 7.28−7.24 (m, 3H), 6.98 (bs, 1H), 3.12 (m, 4H), 2.56 (m, 4H), 2.33 (s, 6H), 2.25 (s, 3H). GC−MS: 280 (M).

General Procedure E for the Buchwald-type Coupling of Bromobenzaldehydes (17, 20). Example: 3-(4-Methylpiperazin-1-yl)benzaldehyde (17). A solution of 3-bromobenzaldehyde (18.5 g, 100 mmol), 1,3-propandiol (9.1 g, 120 mmol), and p-TsOH (0.1 g) in toluene (200 mL) was refluxed, azeotropically removing H_2O using a Dean−Stark water separator. After 18 h, the solution was washed with 5% Na_2CO_3 (100 mL) and dried (K_2CO_3) and the solvent removed in vacuo. The resulting 1,3-dioxane was used without further purification.

2-(3-Bromophenyl)-[1,3]dioxane (14.0 g, 54 mmol), 1-methylpiperazine (6.6 g, 64.8 mmol), $Pd_2(dba)_3$ (0.25 g, 0.3 mmol, 1 mol % Pd), *rac*-BINAP (0.52 g, 0.8 mmol), and $NaOBu^t$ (9.1 g, 91.8 mmol) were stirred under argon in toluene (100 mL) at 100 °C for 18 h. The dark brown mixture was poured into an ice cold solution of HCl (aq, 1 M, 200 mL) and stirred vigorously for 2 h at 25 °C. The solution was cooled to 0 °C and pH was adjusted to 13 using 6 M NaOH (aq) and extracted with Et_2O (4 × 100 mL). The organic phase was dried (K_2CO_3) and the solvent was removed in vacuo. The resulting crude oil was purified by flash chromatography using EtOAc−Et_3N 20:1.

General procedure E gave the title compound as brown oil in 77% yield (17 g). ^1H NMR (DMSO-d_6): δ 9.94 (s, 1H), 7.45−7.40 (m, 2H), 7.31−7.27 (m, 2H), 3.21 (bt, J = 4.9 Hz, 4H), 2.46 (bt, J = 4.9 Hz, 4H), 2.22 (s, 3H). GC−MS: 204 (M).

2-Bromo-5-(4-methylpiperazin-1-yl)benzaldehyde (18). A solution of compound **17** (10 g, 49 mmol) in CH_2Cl_2 (150 mL) was slowly added a solution of NBS (22 g, 98 mmol) in CH_2-

Cl_2 (450 mL). The mixture was stirred for 10 min at rt and washed with 2 M NaOH (aq), and the organic phase was removed in vacuo. The resulting oil was purified by CC, giving the title compound as black oil (90% pure) in 95% yield. The product was used without further purification. ^{1}H NMR ($CDCl_3$): δ 10.29 (s, 1H), 7.47 (d, J = 8.7 Hz, 1H), 7.40 (d, J = 3.4 Hz, 1H), 7.00 (dd, J = 8.7 Hz, J = 3.4 Hz, 1H), 3.26 (bt, $J \approx$ 6 Hz, 4H), 2.58 (bt, $J \approx$ 6 Hz, 4H), 2.36 (s, 3H). GC−MS: 282 (M).

(5-Bromo-2-methoxyphenyl)methanol (26). To a solution of 5-bromo-*o*-anisaldehyde (21.5 g, 0.10 mol) in 96% EtOH (100 mL) was added $NaBH_4$ (3.8 g, 0.10 mol). The mixture was stirred overnight, and the solvent was removed in vacuo. The residue was dissolved in EtOAc and washed with H_2O, and the organic phase was evaporated in vacuo. The resulting oil was purified by CC giving the title compound as white crystals (13.9 g, 64%). ^{1}H NMR (DMSO-d_6): δ 7.47 (bd, J = 2.7 Hz, 1H), 7.37 (dd, J = 8.7 Hz, J = 2.7 Hz, 1H), 6.90 (d, J = 8.7 Hz, 1H), 5.18 (t, J = 5.6 Hz, 1H), 4.46 (d, J = 5.6 Hz, 2H), 3.77 (s, 3H). GC−MS: 216 (M).

4-Bromo-2-chloromethyl-1-methoxybenzene (27). A solution of compound **26** (13.9 g, 64 mmol) in CH_2Cl_2 (100 mL) was slowly added $SOCl_2$ (5.1 mL, 70 mmol) and the mixture was stirred for 2 h at rt. The solvent and excess of $SOCl_2$ were removed in vacuo, giving **27** as red oil (15 g, 100%), which was used without further purification. ^{1}H NMR (DMSO-d_6): δ 7.60 (d, J = 2.5 Hz, 1H), 7.51 (dd, J = 8.7 Hz, J = 2.5 Hz, 1H), 7.01 (d, J = 8.7 Hz, 1H), 4.68 (s, 2H), 3.84 (s, 3H). GC−MS: 234 (M).

(5-Bromo-2-methoxybenzyl)dimethylamine (28). A solution of compound **27** and dimethylamine (2 M solution in THF, 23 mL, 45 mmol) was stirred for 18 h at rt. The organic solvent was removed in vacuo and the residue was extracted with Et_2O and washed with 2 M NaOH (aq), giving the product as orange oil (4.0 g, 82%). ^{1}H NMR (DMSO-d_6): 7.42 (d, J = 2.5 Hz, 1H), 7.38 (dd, J = 8.6 Hz, J = 2.5 Hz, 1H), 6.94 (d, J = 8.6 Hz, 1H), 3.76 (s, 3H), 3.36 (s, 2H), 2.15 (s, 6H). GC−MS: 243 (M).

1-(3-Dimethylaminomethyl-4-methoxyphenyl)ethanone (29). A solution of compound **28** (7.0 g, 29 mmol), butoxyethene (10 g, 100 mmol), $Pd(OAc)_2$ (200 mg, 0.9 mmol), 1,3-bis(diphenylphosphino)propane (1.8 mmol), and K_2CO_3 (30 mmol) in DMF (50 mL) and H_2O (3 mL) under an atmosphere of argon was heated at 80 °C overnight. The mixture was poured into a solution of 2 M HCl (aq) and stirred for 1 h. The solution was adjusted to basic pH using a solution of 2 M NaOH (aq) and extracted with CH_2Cl_2. The organic phase was evaporated on Celite and the residue was purified by CC to give the title product as orange oil in 42% yield (2.5 g). ^{1}H NMR ($CDCl_3$): δ 7.90 (s, 1H), 7.88 (dd, J = 8.9 Hz, J = 2.3 Hz, 1H), 6.89 (d, J = 8.9 Hz, 1H), 3.88 (s, 3H), 3.44 (s, 2H), 2.55 (s, 3H), 2.25 (s, 6H). GC−MS: 207 (M).

2-Bromo-5-methoxy-*N*,*N*-dimethylbenzamide (30). A mixture of 2-bromo-5-methoxybenzoic acid (30 g, 130 mmol) and $SOCl_2$ (15 mL, 200 mmol) was refluxed for 1 h. Excess $SOCl_2$ was removed in vacuo, and the product was dissolved in Et_2O (100 mL) and added to dimethylamine (aq, 8 M, 400 mL, 3.2 mol). The reaction was stirred vigorously for 1 h and extracted with Et_2O. Evaporation in vacuo gave the desired product as a dark oil (33 g, 99%) that was used without further purification. ^{1}H NMR ($CDCl_3$): δ 7.43 (dt, J = 9.0 Hz, J = 1.5 Hz, 1H), 6.81−6.75 (m, 2H), 3.79 (s, 3H), 3.13 (s, 3H), 2.87 (s, 3H). GC−MS: 257 (M).

(2-Bromo-5-methoxybenzyl)dimethylamine (31). Borane (1 M solution in THF, 300 mL, 0.30 mol) in THF (150 mL) was slowly added compound **30** (33 g, 0.13 mol). The reaction was stirred for 5 h under an atmosphere of argon followed by careful addition of MeOH. Evaporation in vacuo followed by addition of 6 M HCl (aq) resulted in a suspension that was stirred for 18 h. The mixture was made alkaline using 2 M NaOH (aq) and extracted with Et_2O to give the desired product as a clear oil after distillation (16.1 g, 51%). Bp: 100−103 °C/0.05 bar. ^{1}H NMR (DMSO-d_6): δ 7.46 (d, J = 8.6 Hz, 1H), 7.00 (d, J = 2.9 Hz, 1H), 6.80 (dd, J = 8.6 Hz, J = 2.9 Hz, 1H), 3.75 (s, 3H), 3.41 (s, 2H), 2.20 (s, 6H). GC−MS: 243 (M).

1-(2-Dimethylaminomethyl-4-methoxyphenyl)ethanone (32). Same procedure as described for compound **29** (36 mmol) gave the title compound as a yellow oil in 80% yield. ^{1}H NMR (DMSO-d_6): δ 7.46 (d, J = 8.6 Hz, 1H), 7.05 (d, J = 2.9 Hz, 1H), 6.88 (dd, J = 8.6 Hz, J = 2.9 Hz, 1H), 3.81 (s, 3H), 3.59 (s, 2H), 2.46 (s, 3H), 2.11 (s, 6H). GC−MS: 207 (M).

(4-Bromobenzyl)dimethylamine (33). To a solution of 4-bromobenzyl bromide (50 g, 200 mmol) in dioxane (100 mL) was added dimethylamine (8 M solution in H_2O, 100 mL, 800 mmol), and the mixture was stirred for 3 h at rt. The mixture was extracted with Et_2O to give 37 as yellow oil (38.4 g, 90%). Bp: 68−70 °C/2 mbar. ^{1}H NMR (DMSO-d_6): δ 7.49 (d, J = 7.5 Hz, 2H), 7.24 (d, J = 7.5 Hz, 2H), 3.33 (s, 2H), 2.12 (s, 6H). GC−MS: 213 (M).

4-Dimethylaminomethylbiphenyl-3-carbaldehyde (35). To a solution of compound **34** (11.6 g, 55 mmol) in Et_2O was added n-BuLi (2.5 M in THF, 65 mmol), and the mixture refluxed for 6 h under an atmosphere of argon. The solution was cooled on ice and DMF (60 mmol) was added. The reaction was stirred overnight, quenched with H_2O, and extracted with EtOAc. The desired product was isolated as yellow oil after distillation (5.3 g, 40%). Bp: 130−145 °C/0.015 mbar. ^{1}H NMR (DMSO-d_6): δ 10.38 (s, 1H), 8.03 (d, J = 2.0 Hz, 1H), 7.89 (dd, J = 7.9 Hz, J = 2.0 Hz, 1H), 7.73−7.69 (m, 2H), 7.53−7.39 (m, 4H), 3.76 (s, 2H), 2.17 (s, 6H). GC−MS: 239 (M).

General Procedure F for the Synthesis of Substituted Chalones by Claisen Condensation (16, 36−52−56, 60, 62−83). To a solution of acetophenone (2 mmol) and benzaldehyde (2 mmol) in 96% EtOH (10 mL) was added 8 M NaOH (aq, 0.3 mL), and the reaction was stirred for 18 h at 25 °C. The mixture was evaporated on Celite and the product was isolated by CC. When indicated, the compounds were used as the free base, but in some cases the compounds were isolated as the fumarate salt or the oxalate salt. The aminochalcone was dissolved in MeOH:Et_2O (1:9 v/v, 10 mL), and a solution of fumaric acid or oxalic acid in MeOH:Et_2O (1:9 v/v) was added. Alternatively, the aminochalcones were isolated as their HCl salt. The aminochalcone was dissolved in a small amount of 96% EtOH, and a 3 M solution of concentrated HCl in EtOH was added. The desired compound was precipitated by addition of Et_2O. The resulting crystals were filtered off and, when indicated, recrystallized from CH_3CN.

Example: (*E*)-3-(2,4-Dichlorophenyl)-1-[2-(2-dimethylaminoethylamino)phenyl]propenone (36). General procedure F (3 mmol) gave the oxalate salt of title compound as yellow crystals in 69% yield. ^{1}H NMR (DMSO-d_6): δ 9.13 (t, J = 5.0 Hz, 1H), 8.24 (t, J = 6.9 Hz, 2H), 8.08 (d, J = 13.9 Hz, 1H), 7.90 (d, J = 16.0 Hz, 1H), 7.72 (d, J = 7.2 Hz, 1H), 7.56−7.40 (m, 2H), 6.93 (d, J = 7.0 Hz, 1H), 6.70 (t, J = 5.2 Hz, 1H), 3.70−3.58 (m, 2H), 3.20 (t, J = 6.0 Hz, 2H), 2.75 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 190.1, 164.1, 150.7, 135.7, 135.1, 134.8, 132.4, 131.5, 129.7, 129.3, 127.8, 126.6, 117.9, 114.7, 111.7, 54.9, 42.6, 37.2. LC-MS: 363.1 (M+1). HR-MS ($C_{19}H_{20}Cl_2N_2O$) Calcd.: 362.0953. Found: 362.0972.

General Procedure G for the Synthesis of Substituted Chalones by Suzuki Couplings (57−59, 61). Example: (*E*)-1-(2-Fluoro-4-methoxyphenyl)-3-[4-(4-methylpiperazin-1-yl)-2′-trifluoromethylbiphenyl-3-yl]propenone (57). A solution of compound **16** (0.40 g, 0.92 mmol) and 2-(trifluoromethyl)phenylboronic acid (1.11 mmol) in DME (10 mL) was added a solution of Na_2CO_3 (290 mg, 2.8 mmol) in H_2O (5 mL). The mixture was flushed with argon for 2 min followed by addition of $Pd(PPh_3)_2Cl_2$ (19 mg, 3 mol %). The reaction was heated at reflux overnight under an atmosphere of argon. The solution was cooled, washed with 2 M Na_2CO_3 (aq), extracted with Et_2O (3 × 20 mL), and purified by CC.

General procedure G gave the title compound as yellow oil in 20% yield. ^{1}H NMR ($CDCl_3$): δ 8.14 (dd, J = 16.1 Hz, J = 2.1 Hz, 1H), 7.86 (t, J = 8.7 Hz, 1H), 7.74 (d, J = 7.4 Hz, 1H), 7.63−7.30 (m, 6H), 7.07 (d, J = 8.7 Hz, 1H), 6.77 (dd, J = 8.7 Hz, J = 2.4 Hz, 1H), 6.61 (dd, J = 16.1 Hz, J = 2.4 Hz, 1H), 3.85 (s, 3H), 3.08 (m, 4H), 2.65 (bs, 4H), 2.38 (s, 3H). ^{13}C NMR

(DMSO-d_6) δ 190.2, 164.8 (J = 12.5 Hz), 163.4 (J = 151.3 Hz), 153.5, 143.4 (J = 25.4), 133.3, 132.5 (J = 5.6 Hz), 130.5, 130.1 (J = 186 Hz), 128.7, 127.5, 126.7, 126.4, 124.7, 124.0, 123.6, 123.2, 120.4, 119.3 (J = 13.1 Hz), 111.6, 100.1 (J = 23.3 Hz), 55.4, 55.0, 51.5, 43.9. HR-MS ($C_{28}H_{26}F_4N_2O$) Calcd.: 498.1930. Found: 498.1941.

General Procedure H for the Synthesis of Chalcones with one Quartinary Amine (84, 85). Example: (*E*)-4-{3-[3-(2-Fluoro-4-methoxyphenyl)-3-oxopropenyl]-2′-methoxybiphenyl-4-yl}-1,1-dimethylpiperazin-1-ium Iodide (85). Iodomethane (25 mg, 0.17 mmol) was added to a solution of compound **53** (23 mg, 0.05 mmol) in THF (10 mL) and stirring was continued for 16 h. The precipitate was collected by filtration and was washed with THF to give the product as yellow crystals in 56% yield. ^{1}H NMR (DMSO-d_6): δ 7.81 (d, J = 16.8 Hz, 1H), 7.71−7.60 (m, 2H), 7.48 (dd, J = 8.4 Hz, J = 1.8 Hz, 1H), 7.32−7.17 (m, 4H), 7.04 (d, J = 7.5 Hz, 1H), 6.96 (t, J = 7.5 Hz, 1H), 6.79 (dd, J = 9.0 Hz, J = 1.9 Hz, 1H), 6.72 (dd, J = 13.0 Hz, J = 2.2 Hz, 1H), 3.73 (s, 3H), 3.64 (s, 3H), 3.48 (m, 4H), 3.18 (m, 4H), 3.10 (s, 6H). ^{13}C NMR (DMSO-d_6) δ 188.5, 164.1 (J = 11.5 Hz), 161.0 (J = 250.2 Hz), 154.7, 152.6, 140.4, 132.9, 132.8, 132.5, 131.8 (J = 4.4 Hz), 130.7, 127.9 (J = 7.5 Hz), 127.5, 127.4, 125.8 (J = 5.1 Hz), 120.3, 118.6 (J = 13.7 Hz), 118.3, 112.7, 111.9, 101.0 (J = 25.6 Hz), 58.1, 56.7, 52.7, 50.0, 45.7. LC-MS: 475.2 (M).

(*E*)-3-[4-(2-Trimethylammoniumethoxy)-3′,5′-dimethylbiphenyl-3-yl]-1-(2-trimethylammonium-4-methoxyphenyl)propenone Diiodide (86). A solution of **68** (69 mg, 0.16 mmol) in iodomethane (2.3 g, 16 mmol) was stirred for 16 h. Iodomethane was removed under reduced pressure and the residue was washed with THF to give the product as yellow crystals in 66% yield (70 mg). ^{1}H NMR (DMSO-d_6): δ 8.17 (d, J = 2.3 Hz, 1H), 8.13 (d, J = 8.7 Hz, 1H), 7.97 (d, J = 15.7 Hz, 1H), 7.82−7.70 (m, 2H), 7.36−7.26 (m, 5H), 7.00 (bs, 1H), 4.90 (s, 2H), 4.63 (bs, 2H), 3.93 (s, 3H), 3.91 (m, 2H), 3.22 (s, 9H), 3.04 (s, 9H), 2.35 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 192.6, 161.2, 155.7, 138.8, 137.8, 133.7, 133.1, 132.5, 129.8, 129.2, 128.6, 126.0, 124.2, 123.7, 123.1, 121.8, 114.9, 113.3, 63.9, 63.8, 62.1, 55.9, 53.1, 52.5, 20.9. LC-MS: 516.3 (M).

Determination of MIC. MIC was determined in triplicate in a microdilution assay using Mueller−Hinton agar as described by the National Committee for Clinical Laboratory Standards (NCLLS).[31] This method was modified to include an uninoculated dilution series of test compounds to facilitate MIC determination if the test compound should precipitate. MIC was determined as the lowest concentration of test compound able to inhibit visible growth of bacteria.

Kinetics of Bacterial Killing. For the determination of the killing curve of a test compound, a dilution series of test compound was made using 2 mL of MH-broth cultures in microtiter plates and an inoculum of approximately 5×10^5 CFU/mL as described by Amsterdam.[32] At the time points indicated, 100 μL samples were withdrawn from the test tubes, serially diluted, and spotted in duplicate on unselective agar plates to determine CFU. Test compounds with bactericidal activity were capable of decreasing surviving colony counts (CFU/mL) when incubated with bacteria.

Determination of Protein Binding. Protein binding was assessed by determining in parallel the MIC in unsupplemented Mueller−Hinton broth (MH) and Mueller−Hinton broth supplemented with 40 mg/mL bovine serum albumin (BSA, Sigma) using 2-fold dilution steps. The free fraction (FF) of test compound in albumin-supplemented broth was determined as the ratio between the MIC in unsupplemented broth and in BSA-supplemented broth. Protein binding was determined as 1 − FF.

Determination of Bacterial Membrane Damaging Activity. Membrane damaging activity was assessed using the BacLight kit (Molecular Probes) essentially as described by Hilliard et al.[29] The assay employs two fluorescent dyes, SYTO-9 and propidium iodide, that compete for binding to the bacterial nucleic acid. SYTO-9 labels all cells, in contrast to propidium iodide, which only labels cells with damaged membranes.

An overnight culture of *S.aureus* ATCC29213 was diluted 1:20 in Mueller−Hinton broth and grown under aeration until reaching an OD_{450} of 0.6−0.7. The bacterial suspension was washed once in sterile H_2O and then resuspended in one-fifth of its original volume. Then, the resulting bacterial suspension was diluted 1:20 in either test compound at a concentration of 4 × MIC or in DMSO diluted in H_2O at the same concentration as used to dissolve the test compound (usually 1%). Ciprofloxacin at 2 μg/mL (corresponding to 4 × MIC) was included as a control. The suspensions were incubated at 37 °C for 15 min under gentle agitation. Then a 10 μL sample was removed and immediately diluted 1:100 in Mueller−Hinton broth for CFU and cell viability determination, and the remaining suspensions was washed and resuspended in distilled H_2O at an OD_{600} of approximately 0.4. A 100 μL volume of the resulting bacterial suspension was added in triplicate to a microtiter plate and an equal volume of BacLight reagent was added. Plates were then incubated in the dark for 15 min and then green fluorescence (SYTO-9) was read at 530 nm and red fluorescence was read at 630 nm using a Bio-Tek FL600 fluoremeter with an excitation wavelength of 485 nm. The ratio of green to red fluorescence was normalized to the untreated control and expressed as percentage of the control.

Determination of Hemolysis Activity. Hemolytic activity was determined using horse erythrocytes (supplied by State Serum Institut, Copenhagen) in an assay modified after Hilliard et al.[29] Briefly, horse erythrocytes were washed three times in Dulbeccos phosphate-buffered saline (D-PBS) and resuspended at a final concentration of 5×10^8 RBC/mL in D-PBS. To aliquots of the cell suspension were added 4 volumes of either test compound at a concentration 4 × MIC in D-PBS/1% DMSO or D-PBS/1% DMSO (negative control yielding 0% hemolysis) or 0.1% NH_4OH or sterile H_2O (positive controls). Cell suspensions were then incubated at 37 °C for 90 min under gentle agitation. At the end of incubation, samples were centrifuged at 1300*g* for 5 min, and hemoglobin release was determined by measuring A_{540} of the supernate using A_{670} as reference. As sterile H_2O consistently gave higher A_{540}-determinations than the 0.1% NH_4OH control, the sterile H_2O positive control was chosen as positive control yielding 100% hemolysis.

Determination of Solubility. Solubility of the compounds was determined by preparing a saturated solution of compound in 0.3 M phosphate buffer (pH 7.4 ± 0.3) in a brown glass tube. The suspensions were rotated slowly for 24 h. Aliquots were centrifuged for 10 min at 14 000 rpm, and supernatants were diluted in 40% (v/v) acetonitrile in water prior to HPLC analysis (system I). Concentrations of analytes were quantified against a standard curve and used as a term of solubility.

Supporting Information Available: Experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM049424K