ACS Infectious Diseases

Article

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ACS Infect. Dis., **Just Accepted Manuscript** • DOI: 10.1021/acsinfecdis.9b00378 • Publication Date (Web): 10 Jun 2020

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Anti-bacterial anacardic acid derivatives

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We report on the antibacterial activity of five phenolic lipids derived from anacardic acid characterized by increasing alkyl chain lengths with C6, C8, C10, C12, or C14 carbon atoms. The compounds were profiled for their physicochemical properties, transport across epithelial monolayers, cytotoxicity and antibacterial activity as compared to common antibiotics. No cytotoxicity was reported in cell lines of fibroblast, hepatic, colorectal, or renal origin. C10 and C12 significantly increased survival in a *Galleria mellonella* model infected with multi-drug resistant *Staphylococcus aureus* (MRSA) or Vancomycin-resistant *Enterococci* (VRE) as compared to the untreated control group. Future studies are required to corroborate these findings in relevant animal model systems of infection.

Keywords: Methicillin resistant Staphylococcus aureus; VRE; Galleria mellonella; biofilm inhibition; anacardic acid

Sub-therapeutic doses of antibiotics selectively pressure bacteria towards resistance. Such sub-therapeutic exposure is particularly probable for bacterial biofilms, within which antibiotics form a decreasing concentration gradient hence lower (and then potentially sub-therapeutic) antibiotic concentrations might be established in vicinity of the bacteria.[1] It is for this among other effects that multi-drug-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococci* (VRE) have formed.[2] A Study from 2019 estimated that around 600,000 infections with antibiotic-resistant bacteria (63.5% considered nosocomial infections) lead to 30,000 deaths annually and cause 800,000 disability-adjusted life-years in the European Union alone.[3] Within this context, biofilm disrupting molecules with antiseptic activity are of increasing interest.[4] Although current disinfectants such as Chlorhexidine, Octenidine or Polyhexanide have antiseptic activity and a low tendency to induce bacterial resistances, their application is only limited to local administration as of cytotoxic effects and tissue irritation. This limitation motivated us to screen for novel starting off a series of amphiphilic compounds derived from anacardic acid. Anacardic acid is naturally found in the cashew nut shell

(*Anacardium occidentale*), or the shell of the pistachio nut (*Pistachia vera*). Anacardic acid's antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Streptococcus mutans* has been reported and was attributed to its amphiphilic structure.[5-7] Additionally, anacardic acid has several other reported activities such as inhibition of histone acetyl transferase (HAT)[8], inhibition of matrix metalloproteinases (MMP)[9] and the chelation of metal ions as well as antioxidant properties.[10] We built off these findings synthesizing derivatives of anacardic acid by elongating their lipophilic alkyl tail in analogy to a previous study on derivatives of *ortho*-hydroxybenzoic acid.[11] The resulting compounds were physically and chemically characterized and their antiseptic activity, cytotoxicity and ability for biofilm reduction was characterized in comparison to relevant antibiotics including amoxicillin, ciprofloxacin, vancomycin, and linezolid and to octenidine, a locally administered anti-infective.[12] Lastly, the two most promising candidates were further assessed *in vivo* using a *Galleria mellonella* infection model. *G. mellonella* larvae demonstrate an innate immune response similar to vertebrates and have proofed themselves as reliable *in vivo* model for novel antibiotic drug candidates and toxicological studies.[13]

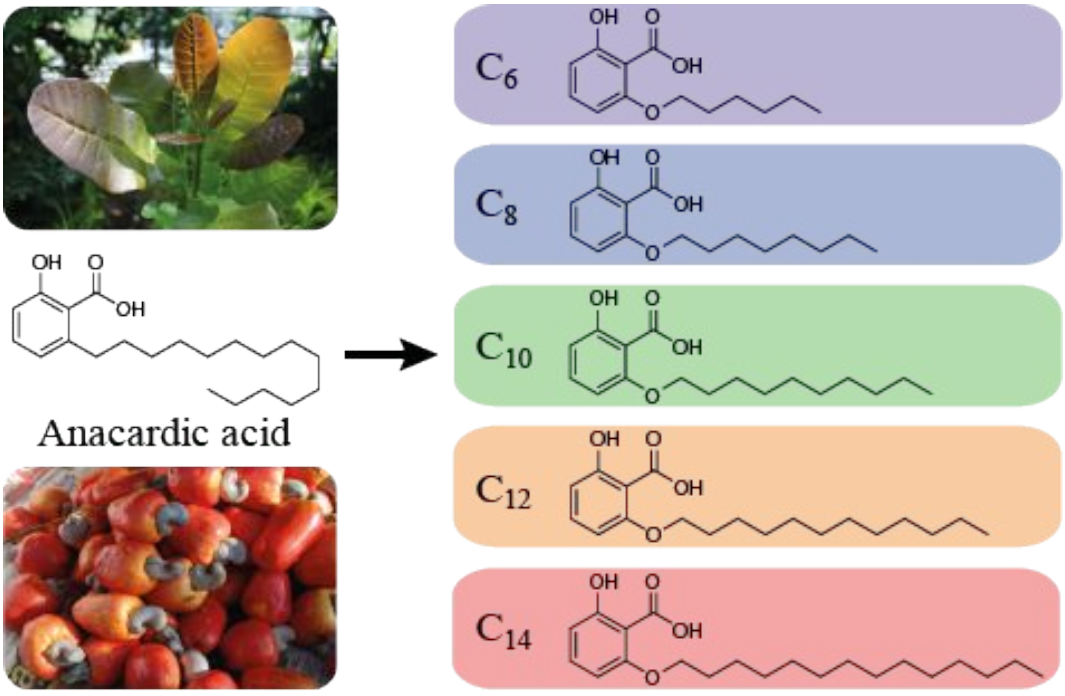


Figure 1 (left) Fruit and plant of the cashew nut (*Anacardium occidentale*) and (right) the synthetic compounds derived from anacardic acid and used in this study.

Materials and Methods

Materials

Dulbecco's Modifed Eagle's Medium (DMEM; #D6429-500mL), high glucose, Minimum Essential Medium Eagle (MEM; #M2279-500mL), Hanks' Balanced Salts powder (HBSS) without phenol red, MEM Non-essential Amino Acid Solution (100x), L-Alanyl-L-glutamine solution (200 mM, sterile),

sodium pyruvate solution (100 mM, sterile), Penicillin-Streptomycin solution (Penicillin 10000 units mL^{-1} and Streptomycin 10 mg mL^{-1}, sterile), Endo Agar powder, Fluorescein sodium salt, BioReagent, suitable for fluorescence, Lucifer Yellow CH dilithium salt, Ginkgolic acid C13:0 and (15:0) Anacardic acid were purchased from Sigma Aldrich (Schnelldorf, Germany). Trypticasein Soy Broth (TSB) powder, Müller-Hinton-Bouillon (MH) powder and Brain Heart Infusion (BHI) powder were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Mowiol 4-88 for histology was purchased from AppliChem GmbH (Darmstadt, Germany), 4',6-diamino-2-phenylindole (DAPI) from Invitrogen (Carlsbad, CA), mouse antibody against human e-cadherin MAb from R&D systems (Minneapolis, MN) and antimouse antibody Alexa Fluor 488 from Life Technologies (Darmstadt, Germany) and hexadeuterio-dimethyl sulfoxide (DMSO-d_6, 99.8% D) from Euriso-top (Saarbrücken, Germany). Ciprofloxacin hydrochloride and Vancomycin hydrochloride and Linezolid were purchased from Sigma-Aldrich. Amoxicillin was a gift from TAD Pharma GmbH (Cuxhaven, Germany). Deionized water was obtained from our in-house Merck Millipore water purification system (Darmstadt, Germany). Other chemicals and reagents used within this study were purchased from Sigma Aldrich if not noted otherwise. The cell lines used for cell culture were obtained from ATCC with HEK293 (ATCC ID# CRL-1573), Hep G2 (ATCC ID# HB-8065), Caco-2 (ATCC ID# HTP-37, and NIH 3T3. The origin of the bacterial strains is shown below (**Table 1**).

Table 1 Tested bacterial strains, including information on species, source and culture conditions.

Species	Code	Source	Anaerobic	Medium	Comment
Staphylococcus aureus	JE2	Clin. isolate	No	MH	MRSA
Staphylococcus aureus	Lac*	Clin. isolate	No	MH	MRSA
Staphylococcus aureus	ST228	Clin. isolate	No	MH/TSB	Biofilm-forming
Staphylococcus epidermidis	RP62a	Clin. isolate	No	MH	-
Enterococcus faecalis	51229	ATCC	No	BHI	VRE
Enterococcus faecium	UL602570	Clin. isolate	No	BHI	VRE
Streptococcus agalactiae	13813	ATCC	No	BHI	-

Species	Code	Source	Anaerobic	Medium	Comment
Clostridium difficile	DSM27543	DSM	Yes	BHI	-

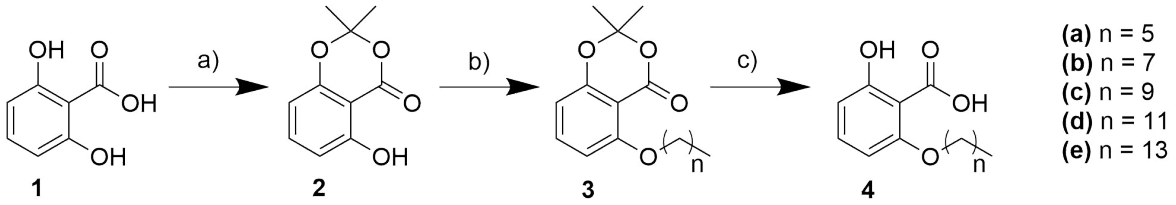
Methods

Proton nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance spectra (^{1}H-NMR) of all compounds were recorded on a Bruker Avance 400 MHz spectrometer (Karlsruhe, Germany) operating at 400.13 MHz (**Figure S22-32**) and additionally ^{13}C-NMR spectra of all final products (**Figure S33-37**). The spectra were referenced to the residual solvent signal of DMSO-d_6 at 2.54 ppm.[14]

Synthetic procedures

Synthetic procedures 1, 2 and 3 were obtained from previously described methods with small changes to obtain higher yields (**Scheme 1**).[11, 15, 16]



Scheme 1 Schematic route of synthesis used within this study. a) Synthetic Procedure 1; $SOCl_2$, DMAP, acetone, diethyl ether, 0 °C, 1 h, 25 °C, 1 day. b) Synthetic Procedure 2; 1-bromoalkane, K_2CO_3, acetonitrile, 50 °C, 4 days. c) Synthetic Procedure 3; KOH, tetrahydrofuran, 60 °C, 1 day.

Synthetic Procedure 1

2,6-Dihydroxybenzoic acid **1** (6.16 g, 40 mmol) and N,N-dimethylpyridin-4-amine (244 mg, 2 mmol) were dissolved in 30 mL diethyl ether under nitrogen atmosphere. The resulting dispersion was cooled to 0 °C. Acetone (3.02 g, 3.85 mL, 52 mmol) and thionyl chloride (6.18 g, 3.77 mL, 52 mmol) were carefully added. The reaction was stirred for 1 h at 0 °C and for 24 h at room temperature. The reaction mixture was diluted with 20 mL aqueous saturated $NaHCO_3$-solution and extracted with diethyl ether (3 x 20 mL). The combined organic phases were washed with brine (3 x 10 mL), dried over $MgSO_4$ and concentrated under reduced pressure.

Synthetic Procedure 2

5-Hydroxy-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one **2** (776 mg, 4 mmol) and potassium carbonate (1.10 g, 8 mmol) were suspended in 10 mL acetonitrile under argon atmosphere. The reaction was cooled

to 0 °C and 1-bromoalkane (5.2 mmol) was slowly added. The mixture was stirred for 30 min at 0 °C and for 96 h at 50 °C. The reaction mixture was diluted with 30 mL deionized water and extracted with ethyl acetate (3 x 40 mL). The combined organic phases were washed with deionized water (2 x 30 mL), 1 M hydrochloric acid (1 x 30 mL), brine (2 x 20 mL) and dried over Mg_2SO_4. The solvent was evaporated under reduced pressure.

Synthetic Procedure 3

5-(Alkyloxy)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one **3a/3b/3c/3d/3e** (2.3 – 3.3 mmol) were dissolved in 10 mL tetrahydrofuran and 10 mL 5 M potassium hydroxide aqueous solution was added. The reaction was stirred for 24 h at 70 °C. The mixture was acidified with 6 M hydrochloric acid and extracted with ethyl acetate (3 x 30 mL). The combined organic phases were washed with deionized water (1 x 30 mL), brine (2 x 30 mL) and were dried over Mg_2SO_4. The organic solution was concentrated under reduced pressure.

High Resolution Mass Spectrometry (HRMS)

HRMS of all final products was measured by electrospray ionization mass spectrometry (ESI-MS) on a micrOTOF-Q III (Bruker Daltonics, Billerica, MA) equipped with an Apollo II ESI Ion Source. The instrument has a resolution of 20,000 and a mass accuracy of 5 ppm with external calibration. Briefly, samples were injected with a constant flow of 4 µL min^{-1}. The spectra were recorded continuously in the range from 50 to 2500 m/z with capillary voltage set to -4.5 kV, end plate voltage -4 kV, nitrogen nebulizer pressure 0.3 bar, dry gas flow 4 L min^{-1} and dry temperature 200 °C. Funnel 1 was 100 Vpp and Funnel 2 RF 200 Vpp, Hexapole RF 200 Vpp; Quadrupole ion energy 5 eV, Quadrupole low mass 50 m/z; collision energy 10 eV, collision RF 300 Vpp, transfer time 70 µs, pre pulse storage 10 µs. The instrument was calibrated with sodium formate which was measured directly after the sample measurements. The spectra were recorded with the Compass Software from Bruker Daltonics containing the OTOF Control 3.4 software for controlling the instrument. For data evaluation the Data Analysis software DA 4.2 from Bruker Daltonics was used.

Critical Micelle Concentration (CMC)

CMC values were determined using a Krüss K12 Tensiometer (Krüss GmbH, Hamburg, Germany) equipped with a heating device to maintain 25 °C. Surface tension was measured at different concentrations, using the Wilhelmy plate method.[17] Briefly, 70 mL deionized water and a magnetic stirring bar were added to a 120 mL glass vial and 1 mL aliquot of an aqueous stock solution was added and stirred for 30 sec between each measuring point. Concentrations of the used stock solutions were 500, 150, 50, 100 and 100 µmol L^{-1} for $\mathbf{C_6}$, $\mathbf{C_8}$, $\mathbf{C_{10}}$, $\mathbf{C_{12}}$ and $\mathbf{C_{14}}$, respectively. The surface tension (σ) was calculated by the software, measuring the force applied to a platinum plate which was slowly immersing from the aqueous solution. CMC values are presented as the concentration in µmol L^{-1} at the

crossing-point of two trend lines obtained by linear regression of the two linear sections before and after the CMC. Experiments were done in triplicate and results are displayed as mean ± standard deviation.

Partition Coefficient (logDpH7.4)

Partition coefficients was determined following OECD Guidelines on partition coefficient, HPLC method.[18] Briefly, an Agilent 1200 series HPLC consisting of a G1322A degasser, G1311A quarternary pump, G1329A autosampler, G1316A column oven and a G1314 VWD detector equipped with a Synergi™ Max-RP, 80 Å, 150 x 4.6 mm, 4µm column from Phenomenex (Torrance, CA). Mobile phase consisted of 10 mmol L^{-1} phosphate buffer pH 7.4 and methanol 30:70 and was used in an isocratic method for a total run time of 20 min with a flow of 1.5 mL/min. Temperature was set to 30 °C, injection volume to 40 µL and the detection wavelength to λ = 254 nm. Samples were prepared by dissolving 4 mg of the compound in 1 mL methanol, diluting it 1:1000 with the same solvent and filtering it through 0.2 µm, 4 mm PTFE filters from Whatman (Maidstone, UK). A calibration curve was prepared from measuring thiourea (dead-time), acetanilide, benzene, biphenyl, anthracene and triphenylamine, using their calculated capacity factor k (Equation 1) ($R^2 = 0.99$). Partition coefficients logP were calculated from $logD_{pH\ 7.4}$ (Equation 2).

Equation 1

$$k = \frac{t_R - t_o}{t_0}$$

k - capacity factor
t_R - retention time
t_0 - dead-time

Equation 2

$$logP = logD_{pH} - log(\frac{1}{1 + 10^{pH\ -\ pKa}})$$

logP - partition coefficient
logD - distribution coefficient
pKa - acid dissociation constant

Solubility Determination

Solubility was determined using a shake flask method.[19] 5-10 mg of compound were placed in a 1.5 mL reaction vial (Eppendorf, Hamburg, Germany). 1 mL of phosphate buffer saline (PBS) pH 7.4 ± 0.05 was added and placed in a ThermoMixer F1.5 (Eppendorf). The aliquots were shaken at 800 RPM for 48 h at 25 °C. After centrifugation at 17880 rcf for 10 min at 25 °C in a Universal 302 R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), 0.25 mL were carefully withdrawn and immediately diluted with 0.75 mL methanol. Absorbance was measured UV-metric at 254 nm with an Evolution 201 UV-

Visible Spectrophotometer (Thermo Scientific, Waltham, MA), if necessary further dilution was done with methanol. Standard curves were conducted in methanol from 100 µmol L^{-1} – 5 µmol L^{-1} on the same instrument, all yielding coefficients of determination $R^2 \geq 0.99$. Results were calculated as means with standard deviation (n = 3).

Dissociation Constant (pKa)

Dissociation constants were determined using a UV-metric titration assay on a Sirius T3 titration platform from Pion Inc. (Woburn, MA) following the manufacturer instructions.[20] Briefly, 0.001 mL of a 150 mmol L^{-1} stock solution in dimethyl sulfoxide (DMSO) was placed into a reaction vial and 1.5 mL ionic strength adjusted water (ISA water) was added automatically. The starting pH 12 value was automatically adjusted by addition of 0.5 mol L^{-1} potassium hydroxide solution and stirred for 2 min. The solution was stepwise titrated to pH 2 by addition of 0.5 mol L^{-1} hydrochloric acid solution aliquots. After each addition a UV-spectrum and the respective pH value was recorded by the UV-dip probe and the Ag/Cl electrode. The titration from pH 2 - 12 was repeated three times per experiment and each experiment was performed in triplicate. The spectrum between 250 nm and 450 nm was used in the range of pH 2 – 6 to identify the protonated and deprotonated species and calculate the pK_a value. Analysis was done using the SiriusT3Refine software (Version 1.1.3.0) from Pion Inc.

High Pressure Liquid Chromatography (HPLC)

HPLC analysis was performed on a Hitachi LaChrom Ultra equipped with a UV Detector L-2400U, Column Oven L-2300, Autosampler L-2200U and two pumps L-2160. ZORBAX Eclipse XDB-C18 5 µm 4.1x150 mm column from Agilent Technologies (Waldbronn, Germany) was used at 23 °C and 0.01 mL injection volume with a detection wavelength of 254 nm was set. An isocratic method was used with acetonitrile and water (9:1) + 0.1% trifluoroacetic acid (TFA). Run time was up to 25 min.

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS analysis was performed on a Shimadzu LC-20A-Prominence HPLC instrument (Shimadzu, Kyoto, Japan) coupled with a UV-detector set to $\lambda = 254$ nm, an electrospray ionization (ESI) and single quadrupole mass analyzer (QMS) operating in positive mode. Samples were prepared by dissolving 1 mg of compound in 1 mL methanol (LC/MS grade) and further diluting it, resulting in a final concentration of 0.1 mg mL^{-1}. Separation after injection of 0.02 mL was achieved by using a Phenomenex-Synergy-Fusion-RP, 4.6 x 150 mm, 5 µm, column (Phenomenex, Torrance, CA) and a methanol/water gradient. In detail, mobile phase A was deionized water with 0.1% (v/v) formic acid and mobile phase B was methanol with 0.1% (v/v) formic acid. For separation %B-content of the mobile phase was continuously increased from 5 to 90% over 18 min.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry was measured with a DSC 8000 from Perkin Elmer (Waltham, MA). Briefly, 3 – 4 mg substance were weighed into aluminum pans with a pinhole in the lid and analyzed at a scanning rate (heating and cooling rate) of 20 °C min^{-1} from -50 °C to 100 °C. The mass of the crucible was noted before and after the experiment to calculate any loss of mass during the process. Measurement of three cycles was performed for every sample and the mean melting point was calculated.

X-Ray Powder Diffraction (XRD)

X-Ray powder diffraction was measured on a silicon single crystal holder and analyzed with a Bruker Discover D8 powder diffractometer (Karlsruhe, Germany) using Cu-Kα radiation (unsplit $K\alpha_1$+$K\alpha_2$ doublet, mean wavelength λ = 154.19 pm) at 40 mA and 40 kV, a focusing Goebel mirror and a 1.0 mm microfocus alignment (1.0 mm pinhole with 1.0 mm snout). The scattered X-ray beam went through a receiving slit with 7.5 mm opening, a 2.5° axial Soller slit and detection was performed with a LynxEye-1D-Detector (Bruker AXS) using 192 channels. Measurements were done in reflection geometry in coupled two theta/theta mode with a step size of 0.025° in 2θ and 0.25 s measurement time per step in the range of 5 – 60° (2θ). Data collection and processing was carried out with the software packages DIFFRAC.Suite (V2 2.2.69.0) and DIFFRAC.EVA 2.1 from Bruker.

Caco-2 Permeation Assay

The Caco-2 permeation assay was performed as described before with small variations.[21] Transportation was studied in apical-basolateral direction and cells were used in passage 51 – 54. In brief, cells were incubated in culture medium (DMEM high glucose, 10% (v/v) FBS and 1% (v/v) non-essential Amino acid (NEAA) solution) at 37 °C, 5% CO2. Passages were performed at 80% confluence in 1:2 - 1:4 ratios and three passages were necessary to develop the phenotype. Afterwards cells were seeded in the trans-wells in a 2.6 x 10^5 cells cm^{-2} density and maintained for 27 days. Culture medium for maintenance (DMEM high glucose, 10% (v/v) FBS, 1% (v/v) NEAA, 1% (v/v) penicillin G/streptomycin solution) was changed every second to third day. Trans-epithelial electric resistance (TEER) values were measured with an EVOM2 STX3 chopstick electrode connected to an EVOM2 epithelial voltammeter from World Precision Instruments (Sarasota, Florida) to follow the monolayer development. Transportation medium was HBSS at pH 7.4 altered by adding 25 mmol L^{-1} 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid (HEPES) and 0.35 g L^{-1} $NaHCO_3$. 100 mmol L^1 stock solutions of all substances were prepared in DMSO and diluted with transportation medium resulting in test solutions of 100 µmol L^{-1} with a maximum of 0.1% (v/v) DMSO in all cases. Fluorescein- and Lucifer Yellow solutions were used as controls. Trans-wells were washed with HBSS, removing residual culture medium, and 0.5 mL of test solution was applied in the apical chamber (donor). The basolateral chamber was filled with 1.2 ml HBSS (acceptor). Immediately after apical application, 0.1 mL was withdrawn from the donor chamber to determine the initial concentration (c_0). The plates were incubated at 37 °C, 0% CO_2 while gently shaking at 150 RRM. 0.1 mL samples were withdrawn from the receiving chamber after 30, 60, 90, and 120 min and immediately replaced with warm transportation buffer. Additionally,

after 120 min a 0.1 mL sample was taken from the apical chamber as well to calculate the mass balance (c_{rest}). TEER values were measured directly before and after the experiment to monitor the integrity of the monolayer. Monolayers with a TEER value ≥ 165 Ω cm² were considered integer. Apical and basolateral samples were stored at 4 °C and analyzed by HPLC on the next day. Fluorescein and Lucifer Yellow (LY) were analyzed by fluorimetry. Experiments were performed in triplicate and results were calculated and reported as apparent permeation coefficient (P_{app}). Representative cell monolayers were cut out and stained to evaluate their integrity.

Fluorimetric analysis of Fluorescein and LY

Analysis was performed using a LS50 B luminescence from Perkin Elmer (Waltham, MA) equipped with a 96-well plate adapter as described before.[22] Briefly, samples were sufficiently diluted with HBSS buffer and 0.09 mL were pipetted into white Nunc™ F96 MicroWell™ plates from ThermoFisher Scientific (Waltham, MA). Parameters for Fluorescein measurement were set to extinction wavelength λ = 450 nm, extinction slit 2.5, emission wavelength λ = 514 nm, emission slit 15 and read time to 0.4 s. A standard curve was conducted in a range of 1 µmol L^{-1} to 0.05 µmol L^{-1} (R^2 = 0.99). LY analysis was performed on the same LS50 B luminescence setting the parameters to extinction wavelength λ = 470 nm, extinction slit 2.5, emission wavelength λ = 535 nm, emission slit 2.5 and read time to 5 s. A standard curve was conducted in a range of 10 µmol L^{-1} to 0.1 µmol L^{-1} (R^2 = 0.98).

Staining and Visualization of Caco-2 Monolayers

Preparation of the Caco-2 monolayers attached to the trans well-system was done by single placing representative trans wells in a 6-well plate and staining with 4',6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488 as previously described.[23] In detail, monolayers were washed two times apical and basolateral with PBS pH 7.4 and exposed to cold methanol for 10 min (washing was always performed with PBS pH 7.4). They were washed again three times and blocked using 0.5 mL Roti®-Block (Carl Roth, Karlsruhe, Germany) in PBS for 1 h at room temperature. Inserts were washed three times and mouse antibody against human e-cadherin, diluted 1:100 in PBS 4 °C, was added and incubated overnight at 4 °C. On the next morning the cells were washed five times, followed by the secondary antimouse antibody Alexa Fluor 488, diluted 1:200 in blocking solution and incubation 90 min at room temperature in the dark. The cells were washed four times follow by DAPI, diluted 1:1000 in PBS. After incubation for 10 min at room temperature in the dark, cells were washed three times. Stained monolayers were cut out from the transwell-inserts using a scalpel and embedded with Mowiol 4-88 on glass microscope slides. Samples were stored in the dark until further use. Representative pictures were taken using a Leica DMIRE2 confocal microscope (Leica Camera AG, Wetzlar, Germany) equipped with a HCX PL APO 63x/1.40 Oil lbd BL objective from Leica operated with the Leica confocal software. Excitation was done by (blue channel) and (green channel). The average of four pictures in a resolution of 1024x1024 pixels with a 2.0x zoom was taken and a series of 20 pictures per stack was collected for every sample.

Cell Proliferation Assay (WST-1)

Cytotoxicity was studied in human embryotic kidney cells (HEK 293), human liver cells (Hep G2), human epithelial colorectal cells (Caco-2) and murine fibroblasts (NIH 3T3) in their respective culture medium (**Table 2**).[23] After thawing, cells were passaged at least three times before the experiments were performed to develop their phenotype. Compound stock solutions were prepared in DMSO and diluted with culture medium. For the experiments 0.1 mL cell suspension in the appropriate seeding density (**Table 2**) was transferred into 96-well plates and incubated overnight at 5% CO_2 and 37 °C. 0.1 mL diluted substance solution was added and incubated for 24 h. Final DMSO concentrations did not exceed 0.5%. After incubation Cell Proliferation Reagent WST-1 (WST-1) was diluted (1:1 v/v) with culture medium and 0.02 mL were added per well. The absorbance was measured after 2 h at 450 nm, using 630 nm as reference wavelength. The experiments on NIH 3T3 were performed in quadruplicate in three separate replicates (n = 12) covering a concentration range from 100 µmol L^{-1} to 195 nmol L^{-1}. Cytotoxicity on HEK 293, Hep G2 and Caco-2 was screened in triplicate in one repetition (n = 3) covering a concentration range from 100 µmol L^{-1} to 6.25 µmol L^{-1}. The IC_{50} values were calculated if possible, using controls without compound and cells without WST-1 Cell-types used in the cell proliferation assay (WST-1). For overview, please see below.

Table 2 Tested cell-types including information on culture medium and seeding density.

Cell-type	Culture medium	Seeding density
HEK 293	DMEM high glucose, +10% (v/v) FCS, +1% (v/v) P/S	4 x 10^4 cells mL^{-1}
Hep G2	MEM, +10% (v/v) FCS, +1% (v/v) P/S, +1% (v/v) NEAA, +2 mmol L^{-1} glutamine, + 1 mmol L^{-1} sodium pyruvate, +1.5 g L^{-1} sodium bicarbonate	1.25 x 10^5 cells mL^{-1}
Caco-2	DMEM high glucose, +10% (v/v) FCS, +1% (v/v) NEAA, +1% (v/v) P/S	2 x 10^5 cells mL^{-1}
NIH 3T3	DMEM high glucose, +10% (v/v) FCS, +1% (v/v) P/S	5 x 10^4 cells mL^{-1}

Reverse Mutation assay using Bacteria (Ames test)

The potential to cause mutations was assessed in *Salmonella typhimurium* and *Escherichia coli* following OECD Guidelines for the Testing of Chemicals and previous published protocols.[24-26] Four histidine-auxotrophic *S. typhimurium* strains (TA 98, TA 100, TA 1535 and TA 1537) and one tryptophan-auxotrophic *E. coli* strain (WP2) were tested w/ or w/o addition of metabolic activity by S9 homogenate (Trinova Biochem GmbH, Giessen, Germany). Two different methods were tested, the plate incorporation method and pre-incubation method. Prior the experiments bacteria were cultured in broth containing 25 g L^{-1} Nutrient medium No.2 for 10 h at 37 °C while gently shaking up to a bacterial density of approximately 10^9 cells ml^{-1} in late exponential phase of growth. The final concentrations of the tested compounds were 500.7, 158.5, 50.1, 15.9, and 5.0 µg per agar plate. Briefly, for the plate incorporation method, 2000 µL top agar, 100 µL test solution (dissolved test compound or control) 100 µL bacterial suspension and 500 µL S9 Mix (w/ metabolic activity) or S9 Mix substitution buffer were mixed at 45 °C and poured on minimal-agar plates supplemented with 0.05 mmol L^{-1} histidine (for *S. typhimurium* strains) or 0.05 mmol L^{-1} L-tryptophan (for *E. coli* strain). After solidification agar plates were incubated at 37 °C for 40 – 72 h. For the pre-incubation method, 100 µL test solution, 100 µL bacterial suspension and 500 µL S9 Mix or S9 Mix substitution buffer were mixed and incubated at 37 °C for 20 min. After this pre-incubation 2000 µL top agar were added, the solution was mixed, poured on minimal-agar plates and after solidification incubated at 37 °C for 40 – 72 h. Negative control was DI water, solvent control was DMSO. Positive controls w/o metabolic activity were 5 µg 2-nitrofluorene for TA 98, 10 µg sodium azide for TA 100, 1.5 µg sodium azide for TA 1535, 50 µg 9-aminoacridine for TA 1537 and 0.05 µg methyl methanesulfonate for WP2. Positive controls w/ metabolic activity was 2-aminoanthracene: 1 µg, 1 µg, 3 µg, 3 µg and 20 µg for TA 98, TA 100, TA 1535, TA 1537, and WP2, respectively. Revertant colonies were counted and the mutation factor (MF) was calculated (Equation 3). The test compound was considered mutagenic if there was a dose dependent increase in number of revertants and/or a biologically relevant positive response for one or more concentrations in at least one strain. Biologically relevant increase was defined as a revenant count at least twice as high for test strains TA 98, TA 100 and E. coli WP2 and at least three times higher for test strains TA 1535 and TA 1537 compared to the solvent control.[27] Experiments were performed in triplicate. For an overview of used solutions see below (**Table 3**).

Equation 3

$$MF = \frac{\text{Mean value of revertant counts (test item)}}{\text{Mean value of revertant counts (solvent control)}}$$

MF - mutation factor

Table 3 Solutions used for the Ames test.

Solution	Composition	Treatment
Top agar	6.0 g L^{-1} bacto agar, 5.0 g L^{-1} sodium chloride in DI water	Sterile; autoclaved for 20 min at 121 °C
S9 Mix	Prepared from S9 liver homogenate as specified by Trinova Biochem GmbH. 5% S9 Mix for *S. typhimurium* strains 10% S9 Mix for *E. coli* strain	-
S9 Mix substitution buffer	120 mL 0.2 mmol L^{-1} NaH_2PO_4 x H_2O, 880 mL 0.2 mmol L^{-1} Na_2HPO_4, pH adjusted to 7.4	Sterile, autoclaved for 20 min at 121 °C
Vogel-Bonner-salts	10 g L^{-1} $MgSO_4$ x 7 H_2O, 100 g L^{-1} citric acid, 175 g L^{-1} $NaNH_4HPO_4$ x 4 H_2O, 500 g L^{-1} K_2HPO_4 in DI water	Sterile; autoclaved for 20 min at 121 °C
Vogel Bonner Medium E agar plates	15 g L^{-1} agar agar, 20 mL L^{-1} Vogel-Bonner salts solution, 50 mL L^{-1} glucose solution (40%) in DI water	Sterile; autoclaved for 20 min at 121 °C

Biofilm-inhibition Assay

Biofilm-formation was quantified by crystal violet staining.[28] *Staphylococcus aureus* (ST228 – clinical isolate) was plated on Mueller-Hinton agar plates and stored at 4 °C until usage. Bacteria were cultured overnight in tryptic soy broth (TSB) at 37 °C and adjusted to an optical density (OD_{600}) of 0.05 on the next day. Compound stock solutions were prepared in DMSO and diluted with TSB resulting in a maximum DMSO concentration of 1% (v/v), which did not affect bacterial growth or biofilm-formation (data not shown). 0.2 mL bacterial solution and 0.05 mL compound solution were added into each well of a 96-well plate and cultured overnight at 37 °C. Controls were 0.2 mL bacterial solution plus 0.05 mL TSB (negative control) and 0.25 mL TSB without bacteria (positive control). On the next day, optical density at 595 nm (OD_{595}) was measured using a Multiskan Ascent (Thermo Electron Corporation, Waltham, MA) plate reader, afterwards all wells were carefully decanted and washed three times with phosphate buffer saline (PBS) pH 7.4. Plates were placed on a heating device at 60 °C for 1

h and afterwards stained with 0.1 mL crystal violet 0.1% (m/v) in water. After 15 min at room temperature (RT) the crystal violet solution was decanted, the plates washed with DI water and dried overnight at RT. 0.1 mL ethanol was added into each well and gently shaken in an orbital shaker for 20 min. Absorbance at 492 nm was measured in the same UV plate reader as above and used for calculation of the relative biofilm formation. All experiments were performed three times with four replicates.

Minimal Inhibitory Concentration (MIC)

MIC values were determined according to CLSI guidelines.[29] Briefly, for aerobic bacteria these were frozen in the respective growth medium containing 20% (v/v) glycerol. These were streaked and incubated on agar-plates before testing and used within two weeks to prevent auto-lysis or mutation. The day before the experiment, a colony was picked and cultured overnight at 37 °C in 3 mL of the respective growth medium on an orbital shaker. On the next morning 0.1 mL of this bacterial suspension was withdrawn and added to 30 mL of growth medium and cultured at 37 °C on an orbital shaker operating at 150 RPM. After 2 – 5 hours depending on the bacterial growth, optical density at a wavelength of 600 nm (OD_{600}) was measured using a Eppendorf Biophotometer plus (Eppendorf, Hamburg, Germany), adjusted to OD_{600} = 0.05 with growth medium and used within 30 min. 100 or 50 mmol L^{-1} stock solutions in DMSO were prepared and diluted to 200 µmol L^{-1} with growth medium. A serial dilution was prepared in sterile, transparent U-bottom 96-well plates (VWR, Darmstadt, Germany), each well containing 0.1 mL of the respective concentration. 0.1 mL of the bacterial suspension (OD_{600} = 0.05) was added resulting in a tested concentration range of 100 µmol L^{-1} to 195 nmol L^{-1} where the maximum DMSO concentration did not exceed 0.2% (v/v). Negative control was medium w/o compound and bacteria while growth control was bacteria w/o compound. The 96-well plates were cultured overnight at 37 °C and visually evaluated for bacterial growth on the next morning. The MIC values were defined as the lowest concentration w/o visible growth and were reported as cautious as possible by stating the highest observed value from the total of six repetitions in three independent experiments. All experiments were performed three times in duplicate. Representative dilutions were plated on agar-plates for every bacterial strain.

MIC values in anaerobic bacteria were determined inside an Anaerobic Chamber (Coy Laboratory Products Inc, Grass Lake, MI) with an anaerobic atmosphere composed of 85% N_2, 10% H_2 and 5% CO_2. Briefly, 96-well plates containing compound solutions, media and agar-plates were put into the chamber the day before the experiment to remove any residual oxygen. Bacteria were grown under static conditions at 37 °C within the chamber. Preparation and dilution of the solutions as well as evaluation of the MIC values was done as described above.

MIC plating on Agar-plates

According to CLSI guidelines MIC values were confirmed after visual detection.[30] 0.01 mL of the well with the detected MIC value and the next two concentrations above and below that value were

withdrawn and diluted with 0.09 mL of the respective growth medium (1:10). 0.01 mL droplets of these five dilutions were separately placed side by side on an agar-plate and the plate was carefully inclined to allow the droplet to flow across it in a straight line. Plates were cultured overnight at 37 °C and bacterial growth was evaluated on the next day by counting the colonies, if any were present.

Galleria mellonella assay

Galleria mellonella were purchased (Feeders & more GmbH, Au in der Hallertau, Germany) with an overall weight of 0.45 ± 0.10 g for the *E. faecium* UL602570-group and 0.31 ± 0.07 g for the *S. aureus* Lac*-group. All *G. mellonella* larvae were used within 4 days post-delivery to avoid maturing and pupating. Each group contained 20 *G. mellonella* larvae which were housed separately in petri dishes with small amounts of wooden chips. Control groups were non-infected animals treated with phosphate buffer saline pH 7.4 (PBS) and infected animals treated with PBS. Bacteria were streaked on Agar-plates of the respective medium and used within one week. Cultures in 3 mL growth medium were incubated at 37 °C on the evening before the experiment and 0.2 mL of these cultures were transferred into 30 mL growth medium on the next morning and incubated at 37 °C (under gentle shaking). Optical density was adjusted in PBS and the resulting bacterial suspension was mildly centrifuged at 3500 rcf for 5 min. The supernatant was carefully withdrawn, and the bacteria resuspended in PBS. The suspension was again centrifuged at 3500 rcf for 5min, supernatant removed and resuspended in PBS to remove any residual bacterial toxins. Resulting bacterial concentrations were 2.5 x 10^8 CFU mL^{-1} for ST228 and 1.25 x 10^7 CFU mL^{-1} for UL602570, respectively. Galleria were infected by injection of 0.02 mL bacterial suspension (or 0.02 mL PBS for the non-infected group) into the lower part of their body using a BD Micro-Fine™ + Demi 0.3 mL, 0.30 mm (30G) x 8 mm syringe. Compound stock solutions were prepared in DMSO and further dilutions were done in PBS resulting in final concentrations of 200 µmol L^{-1} for all compounds. All PBS media and compound dilutions in PBS used for treatment contained residual 5% DMSO (v/v). 1 hour post infection, 0.02 mL of compound solution was injected in the same body part of the *G. mellonella* and the animals were placed at 37 °C. Observation period was set to 94 h and dead animals were counted (darkened color and no visible movement) and removed from the petri dish every 6 h in the first 48 hours and after that every 12 h until 96 hours post infection. Results for all groups are presented as a survival plot.

Statistics

Data evaluation and statistics was done using GraphPad Prism version 6.04 from GraphPad Software, Inc. (San Diego, CA). Robust regression and Outlier removal (ROUT) identification was performed for all data containing 10 or more data sets ($n \geq 10$) using a ROUT coefficient Q of 1%. Data sets were compared using a One-way ANOVA test, differences among groups were calculated using Dunnett's multiple comparison test and represented by asterisks (* p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001).

Results

A series of 5 compounds, inspired by the naturally occurring anacardic acid, were synthesized systematically extending the aliphatic alkane residue from 6 to 14 carbon atoms (**Figure 1**). Namely, 2-(hexyloxy)- (**C_6**), 2-(octyloxy)- (**C_8**), 2-(decyloxy)- (**C_{10}**), 2-(dodecyloxy)- (**C_{12}**) and 2-(tetradecyloxy)-6-hydroxybenzoic acid (**C_{14}**) were obtained and fully characterized concerning their physicochemical properties (**Figure 2**). The purity of all products exceeded ≥ 95% as assessed by high-pressure liquid chromatography coupled uv metric (HPLC-UV) analysis (**Figure S2-6**). All solid compounds were crystalline, except for **C_8** which was liquid at room temperature. The endothermic melting point was at 32 °C (**C_6**), 20 °C (**C_8**), 33 °C (**C_{10}**), 45 °C (**C_{12}**) and 51 °C (**C_{14}**) (**Figure S12-16**). Dissociation constants (pK_a) were similar for all substances with 3.32 ± 0.07 (**C_6**), 3.24 ± 0.04 (**C_8**), 3.21 ± 0.07 (**C_{10}**), 3.21 ± 0.06 (**C_{12}**) and 3.34 ± 0.05 (**C_{14}**), respectively. The apparent solubility was assessed in phosphate buffer saline (PBS; pH 7.4) with 8.40 ± 0.18 mmol L^{-1} (**C_6**; 2 mg mL^{-1}), 3.19 ± 1.02 mmol L^{-1} (**C_8**; 0.85 mg mL^{-1}), 0.48 ± 0.07 mmol L^{-1} (**C_{10}**; 0.14 mg mL^{-1}), 0.45 ± 0.02 mmol L^{-1} (**C_{12}**; 0.15 mg mL^{-1}) and 0.18 ± 0.24 mmol L^{-1} (**C_{14}**; 0.06 mg mL^{-1}), respectively (**Figure 2A**). LogP increased linearly with increasing alkyl-chain length ($R^2 = 0.99$) with logP values of 4.42 ± 0.06 (**C_6**), 6.00 ± 0.02 (**C_8**), 7.29 ± 0.02 (**C_{10}**), 8.47 ± 0.03 (**C_{12}**) and 9.43 ± 0.02 (**C_{14}**) (**Figure 2B**). The Critical Micelle Concentration (CMC) was the lowest for **C_{10}** (6.5 ± 0.4 µmol L^{-1}), and increasing with to 31.3 ± 1.2 µmol L^{-1} and 176.3 ± 6.1 for **C_8** and **C_6** as well as 16.9 ± 1.7 µmol L^{-1} for **C_{12}** , respectively (**Figure 2C**). As of its low aqueous solubility we were unable to collect reliable data for the CMC of **C_{14}**.

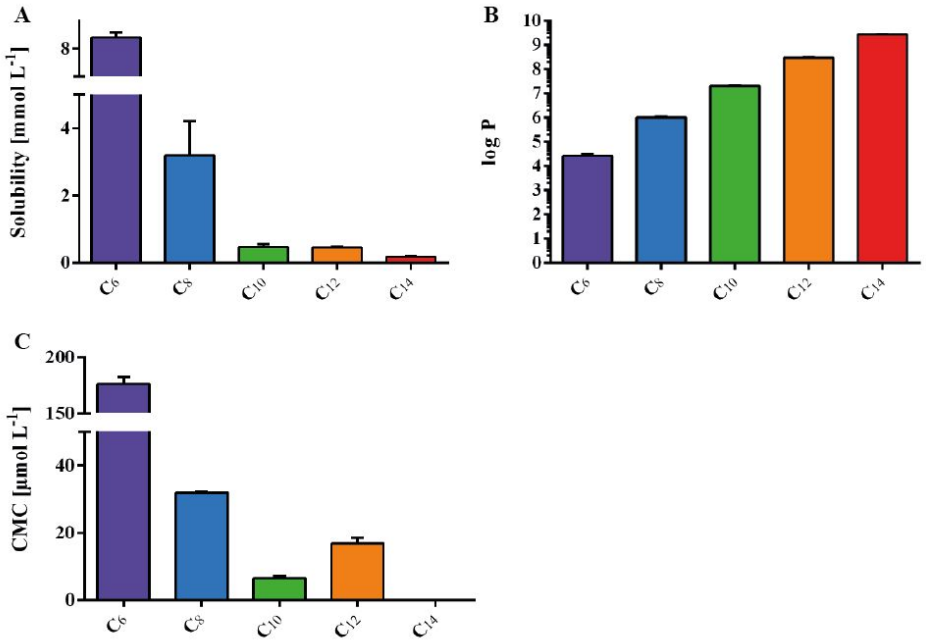


Figure 2 Physicochemical properties of the synthesized compounds. (A) Solubility in phosphate buffer saline (PBS) pH 7.4 at 25 °C, (B) Octanol/Water partition coefficient log D at pH 7.4 and (C) Critical Micelle Concentration of **C_6**, **C_8**, **C_{10}**, **C_{12}** and **C_{14}**. CMC determination of **C_{14}** was not possible, due to low water solubility. All results are displayed as mean ± standard deviation (n = 3).

Epithelial permeability was recorded across Caco-2 cell layers only after a trans epithelial electric resistance (TEER) ≥ 200 Ω cm² was reached before and maintained throughout the experiment, respectively, and an intact state was confirmed histologically. Recovery rates were 104.9 ± 2.2% (**C_6**), 78.8 ± 2.4% (**C_8**) and 5.4 ± 2.2% (**C_{10}**) (**Figure 3A**). The apparent permeation coefficients (P_{app}) was 9.84 ± 0.20 × 10^{-5} cm s^{-1} (**C_6**) and 7.95 ± 0.34 × 10^{-5} cm s^{-1} (**C_8**) (**Figure 3B**). We classified the data obtained for **C_{10}** as inconclusive (reflecting the low substance recovery rate) as we did for **C_{12}** and **C_{14}** (no compound was found in basolateral or initial apical samples). In order to characterized unspecific adsorption, the assay was repeated without cells (in a single experiment) with recovery of 110% (**C_6**), 85% (**C_8**), 79% (**C_{10}**), 69% (**C_{12}**) and 58% (**C_{14}**; **Figure S39**).

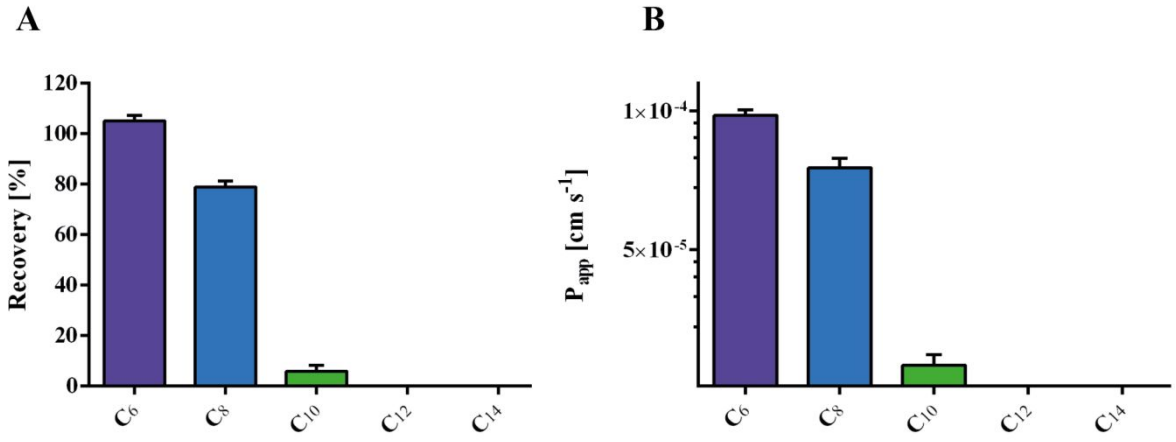


Figure 3 (A) Compound recovery and (B) apparent permeability coefficient (P_{app}) of **C_6**, **C_8** and **C_{10}** (mean ± standard deviation; n=3).

All compounds did have IC_{50} values exceeding 100 µmol L^{-1} (the upper limit of compound solubility) hence demonstrating a desirable profile as assessed in cell lines of murine fibroblasts (NIH 3T3), human kidney cells (HEK 293), human liver cells (Hep G2) or human colorectal cells (Caco-2; **Figure S18-21**) In general, the fibroblasts (**Figure 4A**) and liver cells (**Figure 4C**) responded more sensitively than the colorectal and kidney cells to the highest applied concentration for **C_{10}**, **C_{12}** and **C_{14}**. Liquid/liquid phase separation was observed for **C_{14}** during solubility and CMC experiments resulting in a high standard deviation and no observable surface activity. **C_{14}** was excluded from further investigations as poor water-solubility limit its potential as antibiotic drug candidate as well as antibiotic activity was not observed in preliminary experiments.

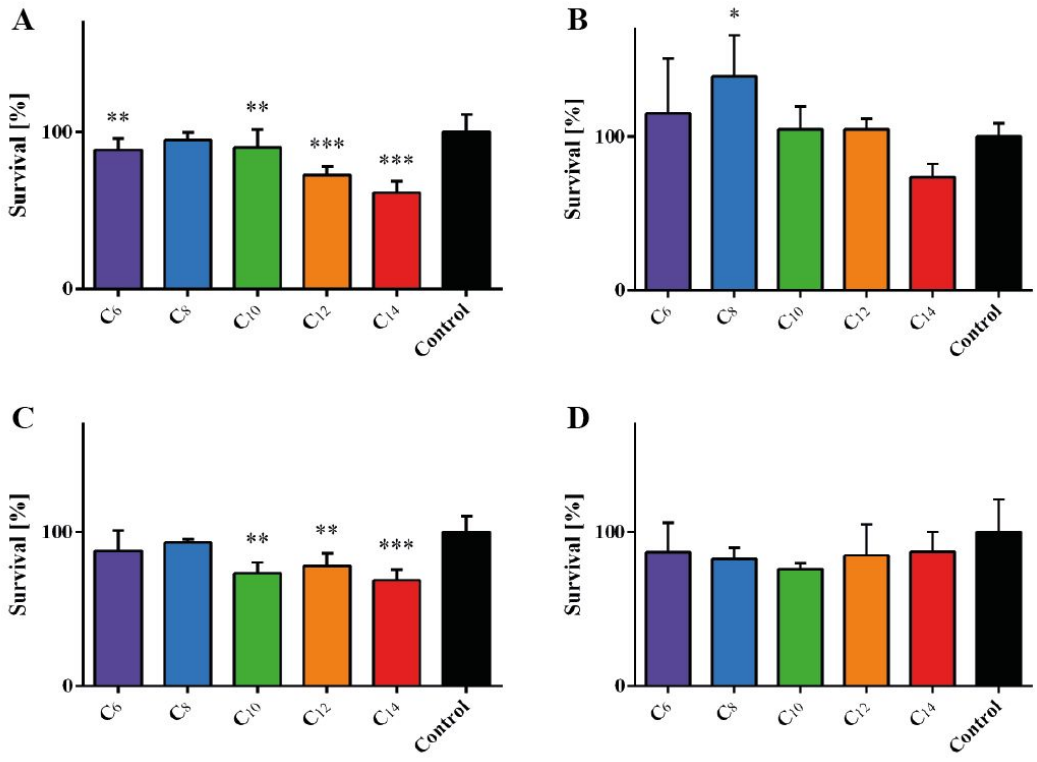


Figure 4 Cytotoxicity assay C_6, C_8, C_{10}, C_{12} and C_{14} in (A) NIH 3T3 fibroblasts (n = 12), (B) HEK 293 kidney cells (n = 3), (C) Hep G2 liver cells (n = 3) and (D) Caco-2 epithelial colorectal cell line. Data was buffer-normalized (n = 3; mean ± standard deviation; significance represented by asterisks with * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$).

The assessment of the minimal inhibitory concentration (MIC) values (**Table 4**, **Table S1-9**) was limited to C_8, C_{10} and C_{12} in comparison to selected antibiotics but not for C_6 and C_{14} as these compounds did not show activity in pilot experiments. C_8 was not antibacterial up to the highest concentration tested (100 µmol L^{-1}) in any bacterial strain. C_{10} and C_{12} showed antibacterial activity in all strains with MIC values ranging from 100 – 50 µmol L^{-1} (C_{10}) and 50 – 12.5 µmol L^{-1} (C_{12}; except for *C. difficile*). *S. aureus* strains ST228 and Lac* (MRSA), *S. epidermidis* RP62 as well as *E. faecalis* (VRE) and the *Streptococcus agalactiae* strain were most susceptible to C_{10} and C_{12} among all strains tested.

Table 4 Minimal inhibitory concentrations (MIC) for different bacterial strains in µmol L^{-1} (tested concentration range 100 – 0.2 µmol L^{-1}). The highest observed MIC value of three independent experiments is reported (for full data set refer to **Tables S1-9**).

	Staphylococci				***Enterococci***		**GBS**	***C. diff.***
Compound	**ST228**	**JE2**	**Lac***	**RP62a**	**Faecium**	**Faecalis**		
Octenidine	6.25	6.25	6.25	3.125	6.25	12.5	12.5	12.5

	Staphylococci				*Enterococci*		GBS	*C. diff.*
Compound	**ST228**	**JE2**	**Lac***	**RP62a**	**Faecium**	**Faecalis**		
Vancomycin	1.563	1.563	1.563	3.125	> 100	25	6.25	3.125
Ciprofloxacin	> 100	> 100	> 100	25	> 100	3.125	3.125	100
Amoxicillin	> 100	> 100	100	> 100	> 100	3.125	12.5	25
Linezolid	n.d.	n.d.	n.d.	n.d.	> 100	> 100	50	> 100
C_8	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
C_{10}	100	100	50	50	100	50	50	100
C_{12}	25	50	25	25	50	12.5	12.5	100
GA (C_{13})	25	25	25	25	6.25	6.25	6.25	n.d.
AA (C_{15})	> 100	> 100	> 100	> 100	12.5	25	6.25	n.d.

GA = Ginkgolic acid

AA = Anacardic acid

n.d. = not determined

GBS = group B *streptococci*

C. diff = Clostridium difficile

We further assessed the impact of the compounds on bacterial biofilm. While no effect on bacterial biofilm formation was observed with **C_6**, **C_8**, **C_{12}** or **C_{14}**, compound **C_{10}** inhibited biofilm formation in sub-inhibitory concentrations in *S. aureus* ST228 (**Figure 5**). **C_{10}** reduced the growth of ST228 (**Figure 5A**) and reduced the biofilm mass significantly to 74% ± 13% when exposed to 0.39 μmol L^{-1} (**Figure 5B**).

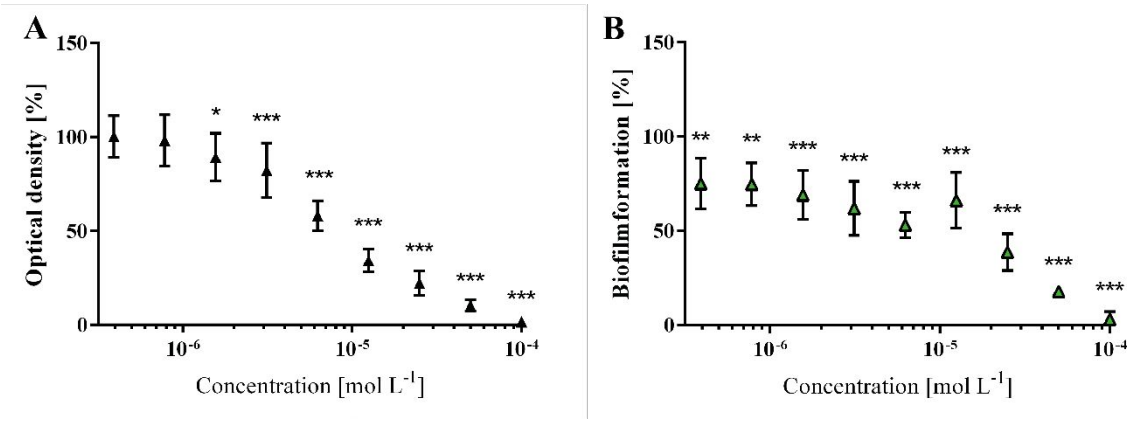


Figure 5 (A) Optical density or bacterial growth and (B) biofilm formation of *S. aureus* (ST228) after exposure to $\mathbf{C_{10}}$ for 24 hours. Data was buffer-normalized (n = 12; mean ± standard deviation, one-way ANOVA with Tukey *post hoc* comparison among groups; significance represented by asterisks with * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$).

$\mathbf{C_{10}}$ and $\mathbf{C_{12}}$ were selected for a first assessment of *in vivo* activity in a *Galleria mellonella* larvae model infected with the MRSA strain LAC* or Vancomycin-resistant *E. faecium* strain UL602570 (VRE) against the antibiotics amoxicillin, ciprofloxacin, vancomycin, and linezolid and the disinfectant octenidine as controls. Injection of 20 µL (200 µmol L^{-1}) compound solution to the VRE infected group (bodyweight 0.45 ± 0.10 g) and the MRSA infected group (bodyweight 0.31 ± 0.07 g) resulted in an applied dose of 9.10 ± 0.56 µmol kg^{-1} in the VRE group and 12.43 ± 0.77 µmol kg^{-1} in the MRSA group, respectively (**Table S9-12**).

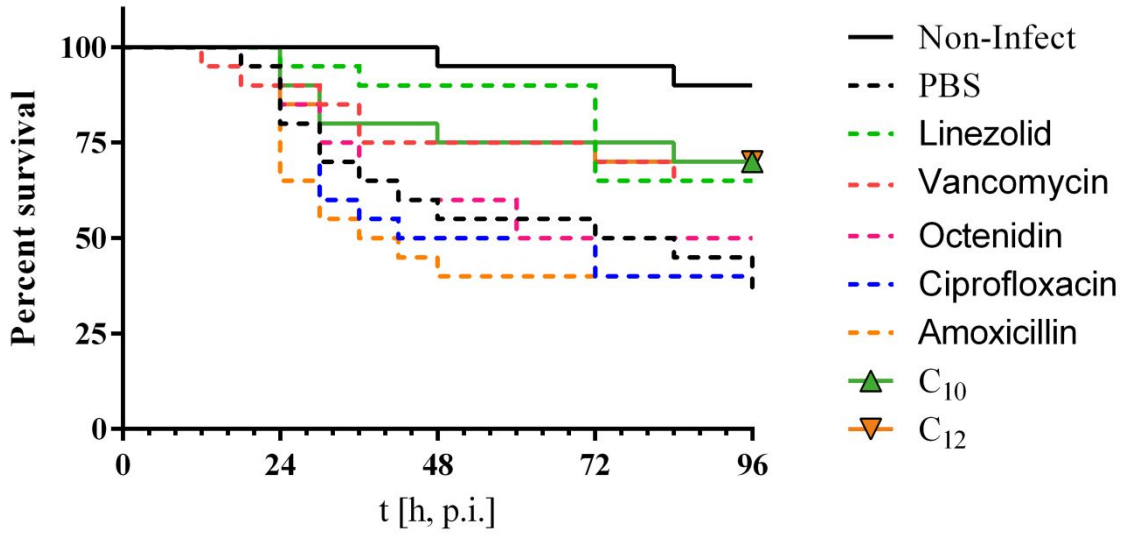


Figure 6 Survival of *Galleria mellonella* when infected with vancomycin resistant *E. faecium* UL602570 and following treatment (Kaplan-Meier plot). Survival under $\mathbf{C_{10}}$ and $\mathbf{C_{12}}$ treatment was significantly better as compared to the PBS control group and not different as compared to (Vancomycin and Linezolid. Amoxicillin, ciprofloxacin or octenidine did not significantly differ from the PBS control group (n = 20).

Larvae infected with Vancomycin-resistant *E. faecium* showed an overall survival rate of 35% in contrast to 90% observed in non-infected controls, respectively (**Figure 6**). Treatment with $\mathbf{C_{10}}$ or $\mathbf{C_{12}}$ resulted in a significantly better survival of 70% as compared to the infected control group ($p \leq 0.05$). Vancomycin and Linezolid treatment insignificantly increased the survival to 65% and neither Amoxicillin, nor ciprofloxacin or octenidine improved survival statistically. The compounds were further assessed in another model using multi-drug resistant *S. aureus* (**Figure 7**). Whereas $\mathbf{C_{12}}$ (5%) failed to improve survival, $\mathbf{C_{10}}$ (25%) was significantly better as compared to the infected control (5% survival; $p \leq 0.05$) or amoxicillin (0% survival; $p \leq 0.01$) but inferior to vancomycin (45% survival; $p \leq 0.01$).

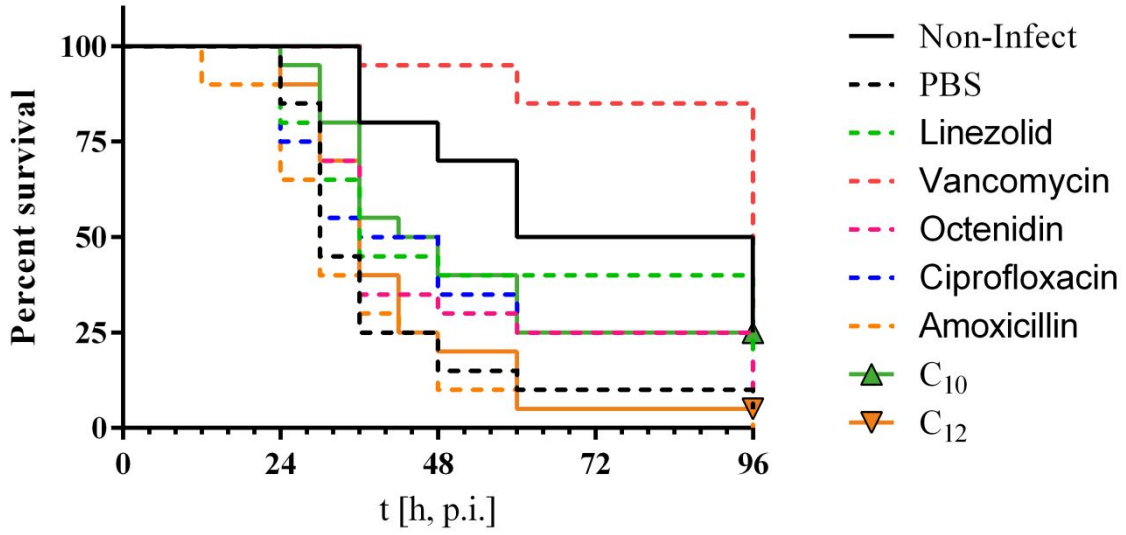


Figure 7 Survival of *Galleria mellonella* infected with multi-drug resistant *S. aureus* strain LAC* (Kaplan-Meier plot; n = 20).

Discussion

The anacardic acid derivatives $\mathbf{C_{10}}$ and $\mathbf{C_{12}}$ showed good antibacterial activity as well as promising toxicological profiles and both compounds challenged multi resistant bacterial strains *in vitro* and *in vivo*. Additionally, biofilm inhibition in *S. aureus* was found at sub-therapeutic doses for $\mathbf{C_{10}}$. Encouraging physicochemical results were obtained for solubility and lipophilicity. The found pK_a values suggested that the compounds will be charged/ionized in blood (pH 7.4) or the intestine (pH 6.8) thereby exhibiting amphiphilic properties resulting in interaction with biological barriers. Except for $\mathbf{C_{14}}$ - which was not surface active in the tested concentration range - all compounds decreased the surface tension in water with $\mathbf{C_{10}}$ resulting in the lowest observed CMC value as compared to all other tested compounds (**Figure 2C**). Permeation through human colorectal adenocarcinoma cell monolayers (Caco-2) reflected the importance of hydrophilic/lipophilic balance as the apparent permeability coefficient (P_{app}) correlated with the alkyl chain length (**Figure 3B**). While $\mathbf{C_6}$ and $\mathbf{C_8}$ had P_{app} values larger than

2-5 × 10^{-6} cm s^{-1} – suggesting complete absorption in human intestine - the assessment of P_{app} values for the more lipophilic compounds **C_{10}**, **C_{12}** and **C_{14}** suffered from a poor recovery rate and as previously reported for lipophilic compounds tested in Caco-2 permeation assays.[21, 31] The poor recovery rate was not a result of unspecific absorption as repeating the experiment without cells revealed higher recovery rates for all compounds with a trend of decreasing recovery with increasing alkyl chain length (**Figure S39**). These experiments suggested that the overall poor recovery of **C_{10}**, **C_{12}** and **C_{14}** in CaCo-2 monolayers was mainly driven by effective absorption onto/within Caco-2 cells an effect which was previously linked to prokaryotic and eukaryotic cytotoxicity of surfactants on the one hand and the reason for antimicrobial activity on the other.[32, 33] For example, octenidine displays similar surfactant like properties with a broad antiseptic activity which is why it finds application for topical treatment of skin and wound infections but prevents other administration routes as of cytotoxic effects which in fact have also been reported for skin keratinocytes.[4, 34] All tested compounds demonstrated encouraging cytotoxicity data and no IC_{50} values were obtained in the tested concentration range. Additionally, Ames test for **C_{10}** showed no increase in revenant colony numbers, neither in presence or absence of metabolic activity, indicating non-mutagenicity of this compound concerning base pair changes or frameshift in the genome (**Table S13-22**). However, decrease in proliferation was observed in higher concentrations for **C_{12}** and **C_{14}** (**Figure 4**). This observation is in accordance to recent publications linking the cytotoxic behavior of lipophilic entities with their ability to interact with cellular membranes.[32, 35] Compound **C_{10}** and **C_{12}** show similar antibacterial activity when compared to their natural precursors Anacardic acid (C_{15}) and Ginkgolic acid (C_{13}; **Table 4**). While the activity in *Staphylococci* strains is unaffected by introducing an ether function, the activity in *Enterococci*- and *Streptococcus* strains is slightly lower. However, **C_{10}** and particularly **C_{12}** reflected a broad antibacterial activity similar to octenidine and contrasting the antibiotics for which the antibacterial activity depended on the selected strain and its resistance profile. Therefore, **C_{10}** and **C_{12}** were further tested in infection models in *Galleria mellonella* (**Figure 6**, **7**), a model in which pathogen virulence compares to mice.[36-39-] Findings in this study correlated with previously measured MIC values for amoxicillin, ciprofloxacin, vancomycin, and linezolid in *E. faecium* UL602570 (**Table 4**). One surprising finding for octenidine was that in spite of having higher antibacterial activity compared to **C_{10}** and **C_{12}** *in vitro*, this did not translate into better survival *in vivo*. We hypothesize this might reflect the amphiphilic nature of **C_{10}**, **C_{12}** and octenidine [4] alike, possibly reducing the available compound *in vivo* as of strong cellular absorption hence little availability at sites of infection. Additionally, low survival was observed for the uninfected control group in the MRSA *G. mellonella* experiment (**Figure 7**). A retro perspective evaluation of our laboratory procedure revealed survival for uninfected, PBS treated groups of 88% ± 3% (n=7) and in line of what was observed for the VRE infected G. mellonella experiment presented in this study (**Figure 6**). The poor survival in the uninfected group within the MRSA infected *G. mellonella* experiment was likely linked to longer transportation of the larvae and possibly starving of this particular *G. mellonella* larvae batch. Despite the resulting overall low survival, the data indicated non-inferiority to vancomycin,

linezolid and PBS treated in the MRSA infected group. It is for these aspects and general differences in bacterial virulence in different hosts why the *Galleria* data reported here should be corroborated in mammalian hosts before final conclusions are drawn. The demonstrated *in vivo* superiority of C_{10} and C_{12}, while statistically significant in the MRSA strain, has to be interpreted cautiously and is encouraging further investigations in mammals to evaluate a potential benefit. In addition and from a cytotoxicity perspective, the compounds reported here within are promising candidates for further testing and much safer than commonly used antibiotics and antiseptics including erythromycin, dicloxacillin or cefuroxime [40] as well as ciprofloxacin, povidone-iodine and octenidine dihydrochloride [34, 41, 42]. As mentioned, experimental data in other animal model systems is required before drawing final conclusions on the promising risk-benefit profile of these anacardic acid derivatives.

Supporting Information

This information is available free of charge on the ACS Publications website. Full characterization data of new compounds, plots of surface tension for CMC calculation, HPLC chromatograms for purity calculation, DSC diagrams for determination of melting point, X-Ray Powder Diffraction patters, full cytotoxicity data sets, ^{1}H and ^{13}C NMR spectra of new compounds, full Caco-2 permeation data sets, full MIC data sets including $\mu g\ mL^{-1}$ values, full Galleria mellonella data sets, full Reverse Mutation assay using Bacteria (Ames test) data sets.

Acknowledgments

We gratefully acknowledge the financial support by the Bayerische Forschungsstiftung (Grant # AZ-1204-16). Furthermore, we thank Dr. Gerd Vogg from the botanical garden in Würzburg for providing pictures of *Anacardium occidentale* fruits.

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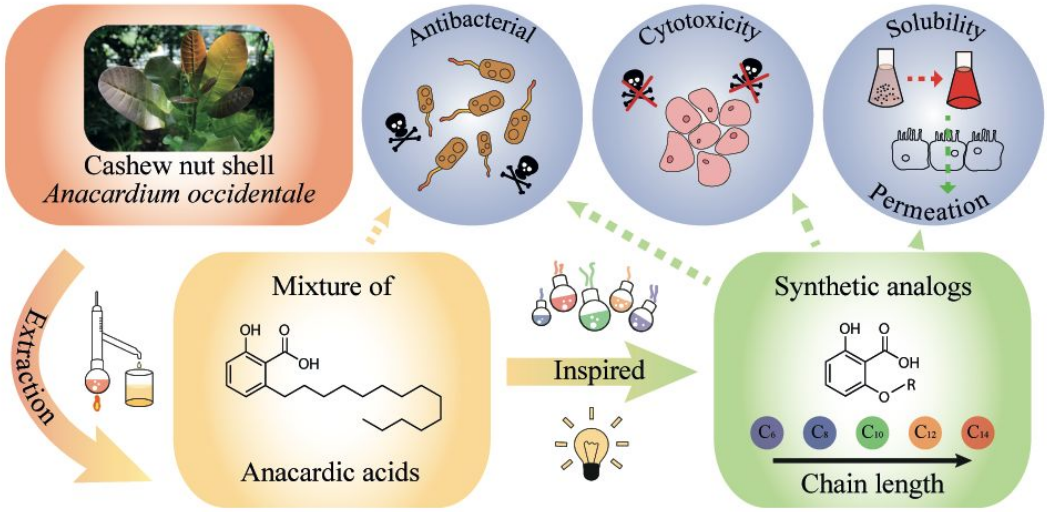
Cashew nut shell
Anacardium occidentale
Antibacterial
Cytotoxicity
Solubility
Permeation
Extraction
Mixture of
OH
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OH
Anacardic acids
Inspired
Synthetic analogs
OH
O
OH
R
C6
C8
C10
C12
C14
Chain length