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Anti-microbial and anti-mycobacterial activities of aliphatic amines derived from vanillin

Alyssa E. Patterson, Andrew J. Flewelling, Trevor N. Clark, Stephen J. Geier, Christopher M. Vogels, Jason D. Masuda, Christopher A. Gray and Stephen A. Westcott

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A.E. Patterson,* S.J. Geier, C.M. Vogels, and S.A. Westcott. Department of Chemistry and Biochemistry, Mount Allison University, Sackville, NB E4L 1G8, Canada.

A.J. Flewelling and T.N Clark. Department of Biology, University of New Brunswick, Saint John, NB E2L 4L5, Canada.

J.D. Masuda. Department of Chemistry, St. Mary's University, Halifax, NS B3H 3C3, Canada.

A.J. Flewelling,* T.N. Clark,* and C.A. Gray. Department of Chemistry, University of New Brunswick, Fredericton, NB E3B 5A3, Canada, and Department of Biology, University of New Brunswick, Saint John, NB E2L 4L5, Canada.

Corresponding author: Stephen A. Westcott (e-mail: swestcott@mta.ca). Corresponding author for anti-microbial and anti-mycobacterium studies: Christopher A. Gray (e-mail: cgray@unb.ca); corresponding author for X-ray studies: Jason Masuda (e-mail: jason.masuda@smu.ca).

*These authors contributed equally to this work.

Abstract: Ten lipophilic amines were prepared from the reductive amination of vanillin and the corresponding primary amines using sodium borohydride in methanol. All compounds have been obtained elementally pure and an X-ray diffraction study on the 4-*n*-butylaniline derivative has confirmed the molecular structure. Whilst the overall antibiotic activity of the derivatives was low, some of these compounds, particularly the boronate ester 2-methoxy-4-((2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylamino)methyl)phenol (**7**), showed a promising degree of anti-mycobacterial activity against *Mycobacterium tuberculosis* H37Ra, where activity seemed to vary by the position of the boron substitution on the aniline ring.

Key words: aliphatic amines, anti-microbial, anti-mycobacterial, boronate esters, lipophilic, tuberculosis.

Graphical Abstract:

Introduction

Although organoboron compounds, particularly boronic acids $[\text{RB}(\text{OH})_2]$ and boronate esters $[\text{RB}(\text{OR}')_2]$, are ubiquitous in organic synthesis,^{1,2} recent research has also found that these same small molecules can display remarkable biological activities.³⁻²⁵ For instance, α -aminoboronic acids are well-recognized as being a unique class of potent enzyme inhibitors.³ Indeed, Velcade® (Fig. 1a) is a boropeptide that has recently been approved by the FDA for the treatment of multiple myeloma.⁴ Interest in small-molecule boron pharmaceuticals has rapidly grown in recent years as other applications are currently being pursued. 2-Aminoethyl diphenylborinate (2-APB, Fig. 1b) is an efficient inhibitor of the epithelial calcium channel TRPV6, a member of the vanilloid (V) subfamily of transient receptor potential (TRP) ion channels.⁵ TRPV6 is overexpressed in prostate cancer, as well as in other cancers of epithelial origin (i.e. breast, thyroid, colon and ovarian), and, as such, significant research is currently focussed on designing novel TRPV6 inhibitors.

Fig. 1. A select group of bioactive boron-containing molecules.

Another important discovery in this emerging area arose when Baker and co-workers reported that 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole (AN2690, Fig. 1c) was a potent anti-fungal agent that had entered clinical trials for the treatment of onychomycosis, a fungus infecting toenails and fingernails.⁶ A structure-activity study on the benzoxaborole scaffold demonstrated that the fluoro substituent in the 5-position was necessary for optimal efficacy. More recently, Voisin-Chiret and co-workers found that simple heterocyclic boronic acid derivatives (Fig. 1d) were potent inhibitors of the *Staphylococcus aureus* NorA efflux pump.⁷ The overexpression of efflux pumps is a significant mechanism in bacterial resistance which results in the extrusion of antibiotic from the cell and lowers the overall efficacy of drug treatment. It is interesting to note that the boron-free analogues showed no activity in this study, highlighting the importance of the boron group. More relevant to this present study, however, is that observation that 2,3,1-diazaborines (Fig. 1e) have been reported to act as both anti-mycobacterial and anti-bacterial agents, particularly against *Gram*-negative organisms.⁸ Likewise, we have found that simple diamines (Fig. 1f) display moderate anti-fungal and anti-bacterial activities.⁹

Scheme 1. Interaction of nucleophiles with three-coordinate organoboronic acids.

The majority of the bioactivity observed in these molecules arises from the electrophilic nature of the three-coordinate boron atom, which contains an empty p-type orbital,

and hence, behaves as a Lewis acid. The boron atom can readily form dative bonds with nucleophiles (i.e. hydroxyl group in serine, bases in DNA, etc) and transform from a neutral trigonal three-coordinate species to a four-coordinate tetrahedral adduct, which has an anionic resonance form, depending upon the nature of the nucleophile (Scheme 1). What sets boron-containing molecules apart from traditional organic-based pharmaceuticals is this dative bonding interaction, which can provide great stability, but can also be reversible, unlike the covalent bonds generated when using organic 'suicide inhibitors'.¹⁰ Although only a few boron-containing natural products are known, there has been recent considerable interest in developing small-molecules incorporating this remarkable element for potential therapeutic use. In this study, we have prepared three boron compounds derived from vanillin, along with a number of boron-free analogues, and examined their anti-microbial and anti-mycobacterial activities.

Experimental

General materials and methods

Reagents and solvents used were obtained from Aldrich Chemicals. Compound **10** was prepared using a modified procedure from the literature.²⁶⁻²⁸ NMR spectra were recorded on a JEOL JNM-GSX400 FT NMR (¹H: 400 MHz; ¹¹B: 128 MHz; ¹³C: 100 MHz) spectrometer. Chemical shifts (δ) are reported in ppm [relative to residual solvent peaks (¹H and ¹³C) and external BF₃·OEt₂ (¹¹B)]. Multiplicities are reported as singlet (s), doublet (d), triplet (t), quintet (quint), sextet (sext), multiplet (m), broad (br), and overlapping (ov) with coupling constants (J) reported in hertz. FT-IR spectra were obtained with a Thermo Fisher Scientific Nicolet iS5 FT-IR spectrometer in ATR mode and are reported in cm⁻¹. Melting points were measured uncorrected with a Stuart

SMP30 apparatus. Elemental analyses for carbon, hydrogen, and nitrogen were carried out at Laboratoire d'Analyse Élémentaire de l'Université de Montréal (Montréal, Québec).

General procedure for the synthesis of vanillin derivatives

To a stirred MeOH (15 mL) solution of vanillin (1.21 g, 7.95 mmol), a MeOH (5 mL) solution of the appropriate amine (7.95 mmol) was added dropwise. The reaction mixture was heated at reflux for 3 h. Upon cooling to 0 °C, NaBH₄ (0.60 g, 15.86 mmol) was added to the solution as a solid and the mixture was allowed to proceed for 18 h at room temperature. Removal of solvent under vacuum afforded an orange oil and an organic extraction using CH₂Cl₂ (3 x 10 mL) and H₂O (20 mL) was performed. The organic fraction was collected and dried with MgSO₄, filtered, and the solvent removed under vacuum to afford a viscous oil. Recrystallization from Et₂O gave compounds **1-6** and **9** as off-white solids. Compounds **7** and **8** were purified by washing with cold Et₂O to afford orange oils.

2-Methoxy-4-((octylamino)methyl)phenol (**1**)

Off-white solid; yield 1.69 g (80%), mp 68-70 °C. ¹H NMR (CDCl₃) δ: 6.85-6.75 (ov m, 3H, Ar), 3.87 (s, 3H, OCH₃), 3.69 (s, 2H, ArCH₂N), 3.50 (br s, 1H, NH), 2.60 (t, *J* = 7.2 Hz, 2H, NCH₂CH₂), 1.49 (quint, *J* = 7.2 Hz, 2H, NCH₂CH₂), 1.32-1.25 (ov m, 10H, -(CH₂)₅-), 0.86 (t, *J* = 6.9 Hz, 3H, CH₃). ¹³C{¹H} NMR (CDCl₃) δ: 147.4, 145.5, 131.2, 121.1, 115.0, 111.5, 55.6, 53.9, 49.3, 31.9, 29.7, 29.5, 29.3, 27.4, 22.7, 14.2. IR: 3284 (br, ν_{OH}), 2922 (s), 2854 (s), 1461 (m), 1377 (m), 1264 (w), 1159 (w), 1036 (w), 864 (w), 818 (w). Anal. calcd. for C₁₆H₂₇NO₂ (265.39) (%): C 72.41, H 10.25, N 5.28; found: C 72.40, H 10.34, N 5.25.

2-Methoxy-4-((nonylamino)methyl)phenol (2)

Off-white solid; yield 1.84 g (83%), mp 59-60 °C. ^1H NMR (CDCl_3) δ : 6.84-6.76 (ov m, 3H, Ar), 3.84 (s, 3H, OCH_3), 3.69 (s, 2H, ArCH_2N), 3.44 (br s, 1H, NH), 2.61 (t, $J = 7.2$ Hz, 2H, NCH_2CH_2), 1.47 (quint, $J = 7.2$ Hz, 2H, NCH_2CH_2), 1.32-1.25 (ov m, 12H, $-(\text{CH}_2)_6-$), 0.86 (t, $J = 6.9$ Hz, 3H, CH_3). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3) δ : 147.1, 145.2, 131.7, 121.1, 114.7, 111.2, 55.8, 54.0, 49.4, 32.0, 29.9, 29.6, 29.5, 29.3, 27.5, 22.7, 14.2. IR: 3284 (br, ν_{OH}), 2924 (s), 2854 (s), 1462 (m), 1377 (m), 1329 (w), 1253 (w), 1127 (w), 1034 (w), 810 (w), 724 (w). Anal. calcd. for $\text{C}_{17}\text{H}_{29}\text{NO}_2$ (279.42) (%): C 73.07, H 10.46, N 5.01; found: C 72.89, H 10.72, N 4.99.

2-Methoxy-4-((octadecylamino)methyl)phenol (3)

Off-white solid; yield 2.32 g (72%), mp 90-92 °C. ^1H NMR (CDCl_3) δ : 6.91-6.76 (ov m, 3H, Ar), 3.87 (s, 3H, OCH_3), 3.72 (s, 2H, ArCH_2N), 3.56 (br s, 1H, NH), 2.61 (t, $J = 7.2$ Hz, 2H, NCH_2CH_2), 1.50 (quint, $J = 7.2$ Hz, 2H, NCH_2CH_2), 1.38-1.23 (ov m, 30H, $-(\text{CH}_2)_{15}-$), 0.86 (t, $J = 6.9$ Hz, 3H, CH_3). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3) δ : 146.9, 144.9, 132.2, 121.1, 114.4, 111.0, 55.9, 54.0, 49.5, 32.0, 30.0, 29.8 (11C), 29.4, 27.5, 22.8, 14.2. IR: 3285 (br, ν_{OH}), 2920 (s), 2852 (s), 1463 (m), 1377 (m), 1265 (w), 1158 (w), 1035 (w), 865 (w), 720 (w). Anal. calcd. for $\text{C}_{26}\text{H}_{47}\text{NO}_2 \cdot 0.5\text{CH}_3\text{OH}$ (421.68) (%): C 75.48, H 11.71, N 3.32; found: C 75.87, H 12.20, N 3.38.

4-((4-Butylphenylamino)methyl)-2-methoxyphenol (4)

Off-white solid; yield 1.77 g (78%), mp 60-61 °C. ^1H NMR (CDCl_3) δ : 7.02 (d, $J = 8.2$ Hz, 2H, Ar), 6.92-6.88 (ov m, 3H, Ar), 6.61 (d, $J = 8.2$ Hz, 2H, Ar), 5.64 (br s, 1H, OH), 4.23 (s, 2H, ArCH_2N), 3.87 (s, 3H, OCH_3), 3.84 (br s, 1H, NH), 2.53 (t, $J = 7.8$ Hz, 2H,

ArCH₂CH₂), 1.57 (quint, $J = 7.8$ Hz, 2H, NCH₂CH₂), 1.36 (sext, $J = 7.8$ Hz, 2H, CH₂CH₃), 0.94 (t, $J = 7.8$ Hz, 3H, CH₃). ¹³C{¹H} NMR (CDCl₃) δ : 146.8, 146.3, 144.9, 132.2, 131.6, 129.3, 120.6, 114.4, 113.1, 110.4, 56.0, 48.9, 34.9, 34.2, 22.5, 14.2. IR: 3520 (br, ν_{OH}), 3410 (br, ν_{NH}), 2954 (m), 2926 (m), 2853 (m), 1614 (m), 1512 (s), 1463 (m), 1430 (m), 1268 (m), 1233 (m), 1120 (m), 814 (m), 667 (m). Anal. calcd. for C₁₈H₂₃NO₂ (285.38) (%): C 75.76, H 8.12, N 4.91; found: C 75.81, H 8.35, N 4.93.

2-Methoxy-4-((4-octylphenylamino)methyl)phenol (5)

Off-white solid; yield 1.74 g (64%), mp 43-45 °C. ¹H NMR (CDCl₃) δ : 7.00 (d, $J = 8.2$ Hz, 2H, Ar), 6.89-6.80 (ov m, 3H, Ar), 6.67 (d, $J = 8.2$ Hz, 2H, Ar), 4.36 (s, 2H, ArCH₂N), 4.00 (br s, 1H, NH), 3.88 (s, 3H, OCH₃), 2.49 (t, $J = 7.8$ Hz, 2H, ArCH₂CH₂), 1.55 (quint, $J = 7.8$ Hz, 2H, NCH₂CH₂), 1.36-1.22 (ov m, 10H, -(CH₂)₅-), 0.89 (t, $J = 7.8$ Hz, 3H, CH₃). ¹³C{¹H} NMR (CDCl₃) δ : 147.0, 145.9, 144.5, 133.2, 129.2, 124.8, 121.2, 119.6, 114.2, 110.2, 56.1, 45.3, 35.2, 32.0, 31.9, 29.6, 29.5, 29.4, 22.8, 14.2. IR: 3520 (br, ν_{OH}), 3410 (br, ν_{NH}), 2922 (m), 2851 (m), 1614 (m), 1510 (s), 1463 (m), 1269 (m), 1234 (m), 1205 (m), 1121 (m), 1033 (m), 814 (m). Anal. calcd. for C₂₂H₃₁NO₂ (341.49) (%): C 77.38, H 9.15, N 4.10; found: C 77.43, H 9.31, N 4.15.

2-Methoxy-4-((4-tetradecylphenylamino)methyl)phenol (6)

Off-white solid; yield 2.78 g (82%), mp 59-60 °C. ¹H NMR (CDCl₃) δ : 6.99 (d, $J = 8.2$ Hz, 2H, Ar), 6.89-6.80 (ov m, 3H, Ar), 6.58 (d, $J = 8.2$ Hz, 2H, Ar), 5.57 (br s, 1H, OH), 4.20 (d, $J = 5.5$ Hz, 2H, ArCH₂N), 3.85 (s, 3H, OCH₃), 3.80 (t, $J = 5.5$ Hz, 1H, NH), 2.48 (t, $J = 7.8$ Hz, 2H, ArCH₂CH₂), 1.54 (quint, $J = 7.8$ Hz, 2H, NCH₂CH₂), 1.32-1.21 (ov m, 22H, -(CH₂)₁₁-), 0.87 (t, $J = 7.8$ Hz, 3H, CH₃). ¹³C{¹H} NMR (CDCl₃) δ : 146.8, 146.3, 145.0, 132.3, 131.6, 129.2, 120.6, 114.5, 113.1, 110.3, 56.0, 48.9, 35.2, 32.1, 32.0,

29.8 (6C), 29.7, 29.5, 29.4, 22.8, 14.3. IR: 3587 (br, ν_{OH}), 3409 (br, ν_{NH}), 2916 (s), 2849 (s), 1615 (m), 1519 (m), 1467 (m), 1259 (s), 1124 (s), 1029 (s), 804 (s). Anal. calcd. for $\text{C}_{28}\text{H}_{43}\text{NO}_2$ (425.65) (%): C 79.01, H 10.18, N 3.29; found: C 78.78, H 10.45, N 3.25.

2-Methoxy-4-((2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylamino)methyl)phenol (7)

Orange oil; yield 0.79 g (28%). ^1H NMR (CDCl_3) δ : 7.64 (d, $J = 7.3$ Hz, 1H, Ar), 7.25 (t, $J = 7.3$ Hz, 1H, Ar), 6.90-6.87 (ov m, 3H, Ar), 6.63 (t, $J = 7.3$ Hz, 1H, Ar), 6.53 (d, $J = 7.3$ Hz, 1H, Ar), 6.23 (br t, $J = 4.1$ Hz, 1H, NH), 5.55 (br s, 1H, OH), 4.30 (d, $J = 4.1$ Hz, 2H, ArCH_2N), 3.86 (s, 3H, OCH_3), 1.31 (s, 12H, pin). ^{11}B NMR (CDCl_3) δ : 30 (br). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3) δ : 154.5, 146.7, 144.6, 137.1, 133.2, 131.8, 124 (br, CB), 119.9, 115.9, 114.4, 110.0, 109.7, 83.6, 56.0, 47.6, 25.0. IR: 3510 (br, ν_{OH}), 3406 (br, ν_{NH}), 2976 (m), 1603 (w), 1580 (w), 1426 (m), 1359 (m), 1269 (m), 1234 (m), 1141 (s), 1077 (m), 964 (m), 851 (m), 788 (m), 705 (s), 675 (m). Anal. calcd. for $\text{C}_{20}\text{H}_{26}\text{NBO}_4 \cdot 0.75\text{H}_2\text{O}$ (368.75) (%): C 65.14, H 7.52, N 3.80; found: C 65.07, H 7.02, N 3.86.

2-Methoxy-4-((3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylamino)methyl)phenol (8)

Orange oil; yield 1.19 g (42%). ^1H NMR (CDCl_3) δ : 7.21-7.13 (ov m, 3H, Ar), 6.88-6.81 (ov m, 3H, Ar), 6.72 (m, 1H, Ar), 5.62 (br s, 1H, OH), 4.24 (s, 2H, ArCH_2N), 3.89 (br s, NH, 1H), 3.85 (s, 3H, OCH_3), 1.33 (s, 12H, pin). ^{11}B NMR (CDCl_3) δ : 30 (br). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3) δ : 147.7, 146.7, 144.9, 131.4, 130 (br, CB), 128.8, 124.1, 120.6, 119.3, 115.7, 114.4, 110.4, 83.8, 56.0, 48.5, 25.0. IR: 3517 (br, ν_{OH}), 3400 (br, ν_{NH}), 2976 (w), 1603 (w), 1580 (w), 1512 (m), 1426 (m), 1359 (s), 1269 (s), 1141 (s), 1077 (m),

1032 (w), 964 (m), 788 (m), 705 (s). Anal. calcd. for $C_{20}H_{26}NBO_4 \cdot 0.25H_2O$ (359.74) (%): C 66.77, H 7.42, N 3.89; found: C 66.61, H 7.40, N 3.82.

2-Methoxy-4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylamino)methyl)phenol (9)

Off-white solid; yield 1.75 g (62%), mp 111-112 °C. ^1H NMR ($CDCl_3$) δ : 7.65 (d, J = 7.8 Hz, 2H, Ar), 6.89-6.83 (ov m, 3H, Ar), 6.61 (d, J = 7.8 Hz, 2H, Ar), 5.71 (br s, 1H, OH), 4.25 (s, 2H, $ArCH_2N$), 4.20 (br s, 1H, NH), 3.84 (s, 3H, OCH_3), 1.33 (s, 12H, pin). ^{11}B NMR ($CDCl_3$) δ : 30 (br). $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ : 150.8, 146.8, 145.0, 136.5, 131.0, 120.5, 116.6 (br, CB), 114.5, 112.0, 110.3, 83.4, 56.0, 47.9, 25.0. IR: 3515 (br, ν_{OH}), 3345 (br, ν_{NH}), 1601 (s), 1512 (m), 1350 (m), 1308 (m), 1265 (m), 1140 (s), 1071 (m), 964 (m), 858 (m), 826 (m), 654 (m). Anal. calcd. for $C_{20}H_{26}NBO_4$ (355.24) (%): C 67.62, H 7.38, N 3.94; found: C 67.60, H 7.47, N 3.93.

Stability testing of vanillin derivatives

In an NMR tube, compounds were dissolved in DMSO- d_6 (1 mL) and 1 drop of D_2O was added at which point the samples were stored at 37 °C. The compounds were analyzed by multi-nuclear NMR spectroscopy after a period of 2 days.

X-ray crystallography

Crystals of **4** were grown from a saturated solution of hexanes at RT. Crystals were attached to the tip of a 400 μm MicroLoop with paratone-N oil. Measurements were made on a Bruker APEXII CCD equipped diffractometer (30 mA, 50 mV) using monochromated Mo K α radiation (λ = 0.71073 Å) at 125 K. The initial orientation and unit cell were indexed using a least-squares analysis of a random set of reflections

collected from three series of 0.5° wide scans, 10 seconds per frame and 12 frames per series that were well distributed in reciprocal space. For data collection, four ω -scan frame series were collected with 0.5° wide scans, 30 second frames and 416 frames per series at varying ϕ angles ($\phi = 0^\circ, 90^\circ, 180^\circ, 270^\circ$). The crystal to detector distance was set to 6 cm and a complete sphere of data was collected. Cell refinement and data reduction were performed with the Bruker SAINT software, which corrects for beam inhomogeneity, possible crystal decay, Lorentz and polarization effects. A multi-scan absorption correction was applied (SADABS).²⁹ The structure was solved using direct methods (SHELXS-2014)³⁰ and refined anisotropically. Hydrogen atoms bonded to carbon were included at geometrically idealized positions and were not refined. The isotropic thermal parameters of the hydrogen atoms were fixed at $1.2 U_{eq}$ of the parent carbon atom, with $1.5 U_{eq}$ used for the methyl hydrogens. Figures were made using Ortep-3 for Windows.³¹ In this molecule the *n*-butyl group was found to exhibit two-site disorder (67:33 ratio). This was modeled using a number of restraints (ISOR, RIGU and DFIX). Attempts to better model the ellipsoids of the minor component of the *n*-butyl disorder (C15b and C16b) did not result in a better model and thus they were left as they were.

General biological testing

Anti-mycobacterial susceptibility tests were performed using modified Middlebrook 7H9 broth base (BBL™ MGIT™, Becton Dickinson, Mississauga, Ontario) in non-tissue culture treated, low-binding, black 96-well microtitre plates sealed with polyester films (50 μ m). Fluorometric readings (in relative fluorescence units, RFU) were recorded using a Molecular Devices Gemini EM dual-scanning microplate spectrofluorometer with a 530 nm excitation filter and a 590 nm emission filter

operating in top-scan mode and are the mean values of 30 reads per well. Antibiotic susceptibility tests were performed using BBL™ Mueller Hinton II cation adjusted broth and Difco™ Sabouraud Dextrose broth (Becton Dickinson, Mississauga, Ontario) for bacterial and fungal cultures respectively in non-tissue culture treated clear 96-well microtitre plates. Optical densities (OD) were measured using a Molecular Devices Emax microplate reader with a 600 nm filter.

Anti-mycobacterial susceptibility screening assay

Anti-mycobacterial activity against *Mycobacterium tuberculosis* strain H37Ra (ATCC 25177) was evaluated using a microplate resazurin assay modified from Collins and Franzblau.³² Stock solutions (10 mM) of test compounds were prepared with sterile-filtered DMSO and stored at 4 °C. Antibiotic and test compound solutions were used within one month and one week of preparation, respectively. Immediately prior to use, stock solutions of test compounds (40 µL) were diluted with modified Middlebrook 7H9 broth (960 µL) and the resulting test compounds (100 µL) were transferred to non-peripheral wells of a 96-well microtitre plate and inoculated with suspensions of *Mycobacterium tuberculosis* H37Ra (100 µL) of cell density 2.0×10^6 cells/mL. To reduce evaporation from the plates, sterile water (200 µL) was added to perimeter wells. Positive control consisted of a triplicate value of rifampin at 0.0061 µM as well as a two-fold dilution of rifampin (0.0061 – 0.0015 µM). In addition to the rifampin positive controls, negative controls [4% DMSO in modified Middlebrook 7H9 broth (100 µL) inoculated with suspensions of *Mycobacterium tuberculosis* H37Ra (100 µL)] and blanks [2% DMSO in modified Middlebrook 7H9 broth (200 µL), and test solutions (100 µL) with modified Middlebrook 7H9 broth (100 µL)] were included in each plate. All controls and test compounds were tested in triplicate. Plates were incubated (37

°C; 5% CO₂) for 3 days in a humid environment before a solution of resazurin (0.0625 mg/mL) in 5% aqueous Tween 80 (50 µL) was added to all wells. Plates were then incubated for a further 24 h, sealed with an adhesive polyester film, and mycobacterial growth was assessed fluorometrically at 37 °C. Fluorescence values were corrected for any background fluorescence of the media and test compounds by subtracting the mean fluorescence readings of the appropriate blanks from the mean fluorescence readings of the control and test compound wells. The percentage inhibition of mycobacterial growth was then defined as: $[1 - (\text{mean test or positive control fluorescence} / \text{mean negative control fluorescence})] \times 100$.

Antibiotic susceptibility screening assay

Anti-bacterial activity against *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *S. aureus* (ATCC 33591) and *Pseudomonas aeruginosa* (ATCC 10145) and anti-fungal activity against *Candida albicans* (ATCC 14053) were evaluated using a microbroth dilution antibiotic susceptibility assay modified from McCulloch et al.³³ Stock solutions of the test compounds (10 mM) were prepared with sterile-filtered DMSO, stored at 4 °C and used within one week of preparation. Immediately prior to use, stock solutions (40 µL) were diluted with the appropriate nutrient broth (960 µL) and the resulting test solutions (100 µL) were transferred to non-peripheral wells of a 96-well microtitre plate in triplicate. Wells were then inoculated with suspensions of the appropriate microbial strain (100 µL) of cell density 5×10^5 CFU/mL and to reduce evaporation from the plates, sterile water (200 µL) was added to perimeter wells. Positive controls consisted of a triplicate two-fold serial dilution of antibiotic (2.62 – 0.66 µM, gentamicin for *S. aureus*; 10.47 – 2.62 µM, gentamicin for *P. aeruginosa*; 10.80 – 2.70 µM, nystatin for *C. albicans*; 0.6 – 0.15 µM, ciprofloxacin for MRSA; 100

μL per well) as 4% DMSO solutions in the appropriate nutrient broth inoculated with suspensions of the appropriate pathogen (100 μL). In addition to test and positive control wells, each plate contained three untreated controls [4% DMSO in the appropriate nutrient broth (100 μL) inoculated with suspensions of the appropriate pathogen (100 μL)] and three un-inoculated blanks (200 μL of 2% DMSO in the appropriate nutrient broth). The optical densities of the wells were measured before and after a 24 hour incubation period (37 °C), and the change in optical density (ΔOD) was calculated by subtracting the initial optical density from the final optical density of corresponding wells. ΔOD values were corrected for background absorbance of the media by subtracting the mean ΔOD readings of the blanks from the mean ΔOD readings of the control and test compound wells. The percentage inhibition of bacterial or fungal growth was then defined as: $[1 - (\text{mean test or positive control } \Delta\text{OD} / \text{mean negative control } \Delta\text{OD})] \times 100$.

Determination of minimum inhibitory concentrations (MIC) and median lethal concentrations (IC₅₀)

MIC and IC₅₀ values were determined for test compounds that exhibited >50% growth inhibition at a concentration of 200 μM in the initial screening assays. Assays were performed as described previously on two-fold serial dilutions of active compounds in triplicate. All compounds were tested at a minimum of 10 and maximum of 12 concentrations obtained from one dilution series (800 – 0.39 μM) for median lethal concentrations against *S. aureus* and Methicillin resistant *S. aureus*. Antimycobacterial median lethal concentrations were determined using 12 concentrations two, two-fold dilution series (200 - 6.25 μM, and 150 - 4.6785 μM). The MIC of a compound was considered to be the lowest assay concentration at which

it inhibited microbial growth by more than a mean value of 90%.³² Absolute IC₅₀s were estimated by fitting a four parameter logistic curve to the microbial growth data (percentage inhibition)³³ using Prism 6.0 (Graphpad).

Results and discussion

Vanillin is a phenolic aldehyde that is found in the extract of the vanilla bean and is primarily used as a natural flavouring agent, albeit recent research has been looking at using vanillin as a biobased monomer for making green polymers.³⁴ Vanillin also provides the structural framework for many biologically-active compounds and pharmaceutical products,³⁵ including capsaicin, the active ingredient in hot peppers. Capsaicin contains a vanillylamide head group as well as a long aliphatic tail (Fig. 2) and displays a wide range of biological activities.³⁶

Fig. 2. The structures of vanillin, capsaicin, and amines prepared in this study.

In this present study, we have prepared a number of lipophilic amines loosely based on the structural motif of vanillin and capsaicin and examined their bioactivities. Long chain amine derivatives are found in many natural products,³⁷ and while some have potent anti-microbial properties,³⁸ others are inhibitors of N-acyl ethanolamine-hydrolyzing acid amidase,³⁹ an enzyme that is encoded in the human gene NAAA

which is responsible for degrading bioactive fatty acid amides to their corresponding acids. Amines **1-10** were readily prepared and isolated in low to good yields from the reductive amination of vanillin and the corresponding primary amine (Scheme 2).^{40,41} Compounds **1-3** contain long chain aliphatic groups and **4-6** are aniline derivatives with a lipophilic aliphatic group in the para position, and both groups of compounds represent boron-free controls. We have recently found that increasing the length of the aliphatic group enhanced anti-microbial properties in related Schiff base copper complexes⁴² and were interested to see if this property translated to vanillin-derived amines. Compounds **7-9** are of singular interest to see what effect, if any, introducing a boronate ester group onto various positions of the aryl ring has on bioactivities. Compound **10** is the boron-free aniline derivative that will be investigated as a control.

Scheme 2. Synthesis of amines **1-10**.

All new amines have been characterized by a number of physical methods including multinuclear NMR and FT-IR spectroscopy and elemental analyses. The formation of amines **1-10** was monitored by the disappearance of the aldehyde peak of vanillin in the ^1H NMR spectra with the concomitant formation of a peak at ~ 4.3 ppm corresponding to the newly-formed benzylic group. Likewise, ^{13}C NMR spectroscopy

could be used to observe the formation of the benzylic carbon at approximately 48 ppm as the peak corresponding to the aldehyde carbon disappears. The ^{11}B NMR spectra for **7-9** at around δ 30 confirms the presence of the three-coordinate boronate ester groups. Although we were unable to get single crystals of **7-9** to confirm that precise structure of these boron-containing compounds, spectroscopic data and elemental analysis are fully consistent with the proposed formulation. The molecular structure of compound **4** has been studied by a single-crystal X-ray diffraction study and confirms that the reduced amines are indeed generated in these reactions. Crystallographic data are provided in Table 1 and the structure of **4** is shown in Fig. 3. Bond distances and angles are well within the expected range observed for related amines.⁴³⁻⁴⁷ Of significance is the N1-C1 bond of 1.4720(16) Å showing a true single bond for the reduced amine. For comparison, the C=N bond distance in related aldimines is around 1.28 Å.⁴⁸

Fig. 3. The molecular structure of **4** with ellipsoids shown at the 50% confidence level. Hydrogen atoms bound to carbon atoms have been omitted for clarity. Selected bond distances (Å) and angles (°): C(1)-N(1) 1.4720(16), C(1)-C(2) 1.5095(15), C(4)-O(2) 1.3707(14), C(5)-O(1) 1.3669(14), C(8)-O(2) 1.4285(14), C(9)-N(1) 1.4175(14); N(1)-C(1)-C(2) 112.59(10), C(14)-C(9)-N(1) 119.64(10), C(10)-C(9)-N(1) 121.92(11), C(9)-N(1)-C(1) 116.70(9).

With elementally-pure samples in hand, we decided to examine all new amines for their potential anti-fungal, anti-bacterial, and anti-mycobacterial properties, using capsaicin as a natural product control. Unfortunately, the bioactivities exhibited by capsaicin and our synthetic derivatives were largely disappointing; none of the compounds were significantly active (i.e. inhibited growth by more than 50% when tested at 200 μM) against the fungus *Candida albicans* or the Gram-negative bacterium *Pseudomonas aeruginosa*, and most were also inactive against the drug-susceptible and drug resistant strains of the Gram-positive bacterium *Staphylococcus aureus*. However, compounds **2** and **3**, the most lipophilic of the aliphatic derivatives, did inhibit the growth of both *S. aureus* ($\text{IC}_{50}\text{s} \pm \text{SE}$: $167 \pm 1 \mu\text{M}$ and $7.98 \pm 1.11 \mu\text{M}$ respectively; MICs 400 μM and 12.5 μM respectively) and methicillin-resistant *S. aureus* ($\text{IC}_{50}\text{s} \pm \text{SE}$: $193 \pm 3 \mu\text{M}$ and $9.59 \pm 1.09 \mu\text{M}$ respectively; MICs 400 μM and 12.5 μM respectively) probably because of an increased ability to permeate or disrupt the membrane of the Gram-positive cell envelope. The greatest bioactivity observed in our assays was against *Mycobacterium tuberculosis* H37Ra, where capsaicin and six of the synthetic derivatives significantly inhibited the growth of the mycobacterium (Table 2). As we have observed before,^{42,49-51} the more lipophilic derivatives caused enhanced, albeit weak to moderate, growth inhibition of *M. tuberculosis* H37Ra. Of particular interest, however, was the superior and specific activity exhibited by a

single boronate ester derivative (**7**). This may indicate that regiochemistry could be of extreme importance in determining the anti-mycobacterial activity of the boronate compounds, and a more detailed study investigating this is certainly warranted.

Conclusions

We have prepared ten lipophilic amines derived from vanillin and examined their initial bioactivities. Three of these compounds are of singular interest as they contain boronate ester functionalities as recent research has focused on expanding the breadth of known families of boron-containing pharmaceuticals. Although most of the compounds showed little or no appreciable antimicrobial activities, the significant and highly specific activity displayed by the boronate ester **7** is extremely encouraging. Future work in this area will build on this study in an effort to design a more potent series of aminoboron compounds with enhanced antimicrobial activity, the results of which will be disclosed in due course.

Supplemental material

Supplementary material is available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/x/cjc-y>. Crystallographic information has also been deposited with the Cambridge Crystallographic Data Centre (CCDC 1415779). Copies of the data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: + 44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

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Table 1. Crystallographic data collection parameters.

Complex	4
Formula	C ₁₈ H ₂₃ NO ₂
Molecular weight	285.37
Crystal system	Monoclinic
Space group	P2 ₁ /n
<i>a</i> (Å)	9.3946(9)
<i>b</i> (Å)	13.0944(13)
<i>c</i> (Å)	13.4513(13)
α (°)	90
β (°)	110.2810(10)
γ (°)	90
<i>V</i> (Å ³)	1552.1(3)
<i>Z</i>	4
$\rho_{\text{calc.}}$ (Mg/m ³)	1.221
Crystal size (mm ³)	0.426 x 0.278 x 0.236
Temperature (K)	125(2)
Radiation	Mo- <i>K</i> _α (λ = 0.71073 Å)
μ (mm ⁻¹)	0.079
Total reflections	11680
Total unique reflections	3380
No. of variables	238
θ Range (°)	2.242 to 27.000
Largest difference peak/hole (e/Å ³)	0.395 and -0.200
S (goodness-of-fit) on F ²	1.036
<i>R</i> ₁ ^a (<i>I</i> > 2 <i>s</i> (<i>I</i>))	0.0406
<i>wR</i> ₂ ^b (all data)	0.1125

^a*R*₁ = $\sum ||F_o| - |F_c|| / \sum |F_o|$.
^b*wR*₂ = $(\sum [w(F_o^2 - F_c^2)^2] / \sum [wF_o^4])^{1/2}$, where $w = 1 / [\sigma^2(F_o^2) + (0.0555P)^2 + (0.4636P)]$, where $P = (\max(F_o^2, 0) + 2 \cdot F_c^2) / 3$.

Table 2. Anti-mycobacterial activities of capsaicin and compounds **1-10** against *Mycobacterium tuberculosis* H37Ra.

Compound	IC ₅₀ [±SEM] (μM) ¹	MIC (μM) ²
Capsaicin	110 [1.05]	200
1	144 [1.03]	200
2	31.7 [1.07]	150
3	45.3 [1.03]	75
4	111 [1.04]	>200
5	70.5 [1.04]	150
6	inactive ³	inactive
7	46.2 [1.01]	200
8	inactive	inactive
9	inactive	inactive
10	inactive	inactive

¹Absolute median inhibitory concentrations estimated by four parameter logistic analysis.

²Minimum inhibitory concentration is defined as the lowest concentration that caused more than 90% inhibition of mycobacterial growth.

³Compounds were considered inactive if they caused less than 50% growth inhibition when tested at 200 μM in the initial bioactivity screening assay.