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Efficient synthesis and biological evaluation of proximicins A, B and C

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ABSTRACT

A quick and efficient synthesis and the biological evaluation of promising antitumor-antibiotics proximicins A, B and C are reported. The characteristic repetitive unit of these molecules, the methyl 4-Boc-aminofuran-2-carboxylate **15**, was prepared in three synthetic steps in good yield using an optimised copper-catalysed amidation method. The proximicins were evaluated for their antitumor activity using cellular methods. Proximicin B induced apoptosis in both Hodgkin's lymphoma and T-cell leukemia cell lines and proximicin C exhibited significantly high cytotoxicity against glioblastoma and breast carcinoma cells. The proximicins were also screened against *Escherichia coli*, *Enterococcus faecalis* and several strains of methicillin-and multidrug-resistant *Staphylococcus aureus*. Proximicin B showed noteworthy activity against antibiotic-resistant Gram-positive cocci.

1. Introduction

Proximicins A (**1**), B (**2**) and C (**3**) are three novel aminofuran antitumor-antibiotics isolated from marine Actinomycetes of the genus *Verrucosispora*.[1,2] The distinctive features of this family of compounds are (a) the structural similarity to the DNA-binding agents distamycin[3] (**4**) and netropsin[4] (**5**), and (b) the presence in their molecular framework of an unusual γ-amino acid, 4-aminofuran-2-carboxylic acid (Fig. 1). Preliminary in vitro biological evaluation of proximicins **1**–**3** against selected carcinoma cell lines has shown that all compounds have a significantly higher cytotoxicity than netropsin and distamycin.[1] In addition, proximicins can activate cell-cycle regulatory proteins involved in the transition of cells from G1 to S phase, and proximicin C (**3**), in contrast to distamycin, can induce up-regulation of p53 and p21 in gastric adenocarcinoma cells.[1]

The antibiotics distamycin and netropsin contain two or three γ-amino-*N*-methylpyrrole rings, respectively,[5] and are well-known DNA-minor groove binding agents with strong preference for adenine/thymine (AT)-rich sequences. These oligo-heteroaromatic peptides have a crescent-like, planar molecular shape that can follow the curvature of the DNA helix and fit tightly into the minor groove.[6] Their ends, which are cationic at physiological pH, are attracted by the negatively-charged DNA phosphate backbone, thus allowing the amidic hydrogen atoms of the carboxamide groups to form bifurcated hydrogen bonds with N3 of adenine and O2 of thymine residues, which provide the correct orientation for strong van der Waals interactions with the groove walls.[6,7]

Over the past three decades, a number of sequence-specific DNA-minor groove ligands, based on the structure of distamycin and netropsin, have been synthesized aimed at targeting almost any sequence of interest with both high affinity and specificity.[8,9] In particular, higher homologues of these natural products, such as Dervan's hairpin-polyamides,[10] were prepared using a set of design rules for connecting pyrrole, imidazole, and hydroxypyrrole units to recognise the four Watson–Crick base pairs and even to compete with transcription factors both in vitro and in vivo.[11–13]

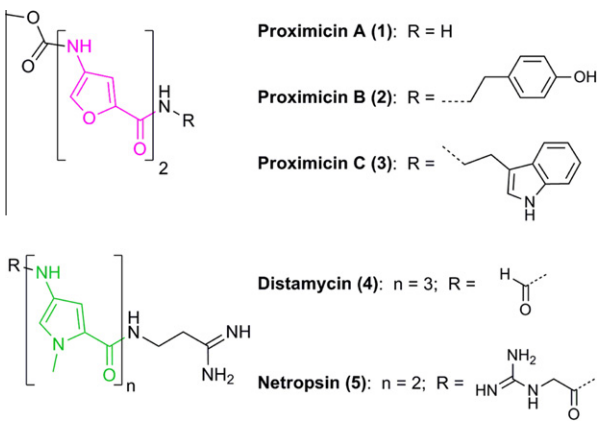

Figure 1. Structures of proximicins A (**1**), B (**2**) and C (**3**), distamycin (4) and netropsin (5).

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Proximicins are similar to distamycin and netropsin as they consist of repetitive γ-amino-heteroaromatic rings (i.e., two 4-aminofuran-2-carboxylic acids) linked through peptide bonds. However, unlike the DNA ligands **4** and **5**, these furan-based polypeptides lack ionisable tails. The C-terminus propyliamidine group of **4** and **5** is replaced by a carboxamide, a tyramine and a tryptamine residue in **1**, **2** and **3**, respectively. The N-terminus guanidine (netropsin) and formyl (distamycin) groups are substituted in **1**–**3** by methyl carbamates.

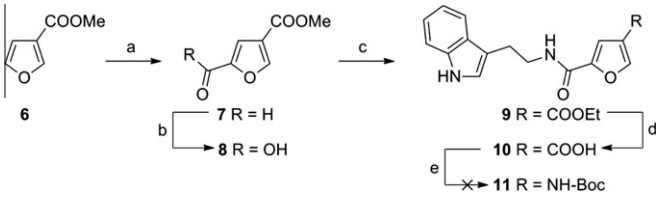
Given their interesting molecular framework, their significant anticancer activity and the ongoing studies aimed at elucidating their mechanism of action,[14] proximicins represent an attractive synthetic target. The proximicins have been previously synthesised by Süssmuth and co-workers,[14] but key intermediates were only afforded in low yield, making it difficult to produce high yield of final products. Therefore, the main focus of this work was to develop a novel synthetic route to provide sufficient pure material for extensive in vitro antitumor and antimicrobial evaluation. The MTT cell proliferation assay was used to evaluate the proximicins' cytotoxicity against selected cancer cell lines different from those previously reported. Proximicin C exhibited a strong cytostatic effect on glioblastoma and breast cancer cells. In addition, flow cytometric DNA fragmentation analysis revealed that proximicin B induced apoptosis in Hodgkin's lymphoma and T-cell leukemia cells. Finally, given the growth inhibition properties of proximicin B and, to a lesser extent, C against *Staphylococcus aureus*,[2] a thorough antimicrobial screen was carried out against a number of Gram-positive cocci, including different strains of methicillin-resistant *S. aureus* (MRSA) and *Entercoccus faecalis*, and Gram-negative bacilli such as *Escherichia coli*. Interestingly, proximicin B exhibited remarkable antimicrobial activity against all the Gram-positive bacteria, including EMRSA-15 and -16 strains, which are among the leading causes of bacterial infections in developed countries.[15]

2. Results and discussion

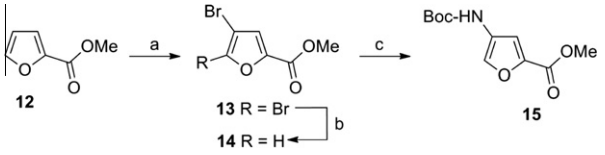
2.1. Chemistry

Proximicins A–C were previously synthesized using the methyl 4-Boc-aminofuran-2-carboxylate **15** as a building-block. This compound was in turn synthesized from 3-furaldehyde via lithiation, followed by quenching with methyl chloroformate, oxidation and Curtius rearrangement.[14] However, the metalation step of this synthetic pathway, which served to introduce the methyl ester group to the furan ring, was achieved in only low yield. This prompted us to seek alternative routes.

First, a linear approach was adopted for the preparation of **1**–**3**, and methyl 3-furoate **6** was used as starting material (Scheme 1). Vilsmeier formylation[16] of **6**, followed by Lindgren oxidation with $NaClO_2/H_2O_2$,[17] gave known intermediates ethyl 2-formyl-4-furoate **7** and acid **8**, respectively.[18] The latter was then coupled to tryptamine using a water-soluble carbodiimide (EDCI) in the presence of DMAP to furnish the ester **9**, which could serve as an



Scheme 1. Reagents: (a) $POCl_3$, DMF, CH_2Cl_2; (b) $NaClO_2$, H_2O_2, H_2O/MeCN; (c) tryptamine, EDCI-DMAP, DMF; (d) NaOH, MeOH/H_2O; (e) DPPA, Et_3N, *t*-ButOH, THF.



Scheme 2. (a) Br_2, $AlCl_3$; (b) Zn, NH_4Cl, MeOH; (c) CuI/$(CH_3NHCH_2)_2$, Boc-NH_2, K_2CO_3, toluene.

intermediate for the synthesis of proximicins C. After hydrolysis of **9** to the free acid **10**, diphenyl phosphorazidate (DPPA) was used in the Curtius rearrangement step. Unfortunately, although the acyl azide was decomposed to the isocyanate, which was detected by analytical methods (i.e., LC–MS, IR and NMR), the latter did not react with *t*-ButOH to yield the desired Boc-protected amino furan derivative **11**.

At this stage, efforts were directed towards preparation of the Boc-protected aminofuran **15**. A strategy different from the one previously reported was chosen and the synthesis of this building-block was easily achieved in three steps starting from the commercially available methyl 2-furoate **12** (Scheme 2). Bromination of **12** in the presence of $AlCl_3$ gave the methyl dibromofuroate **13**, which, in turn, was selectively debrominated with zinc to yield the methyl 4-bromo-2-furoate **14**. Direct copper-catalysed amidation of **14** with *t*-butyl carbamate, under Buchwald conditions,[19–21] successfully introduced the Boc-amino functionality to the furan ring, yielding the methyl 4-Boc-aminofuran-2-carboxylate **15**.

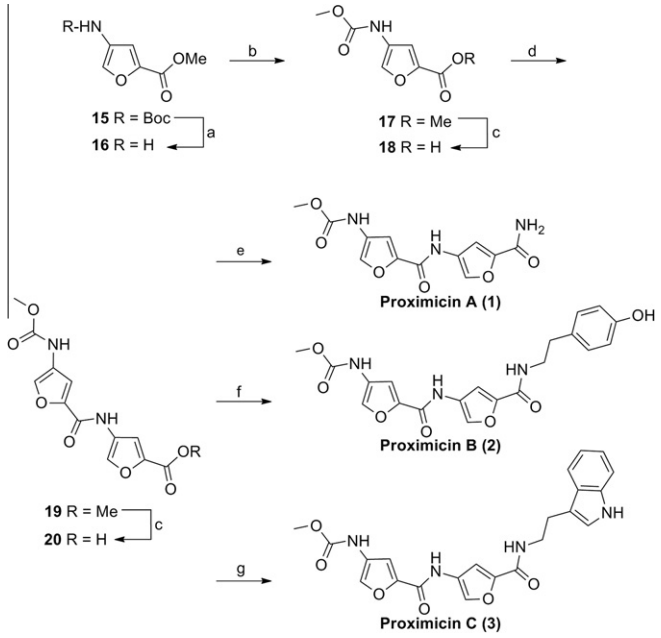
Optimisation of the amidation step was achieved by varying the concentrations of both the catalyst system (CuI/*N*,*N*′-dimethylethylenediamine) and the base (K_2CO_3), the reaction time, the solvent, and by monitoring the reaction products with analytical HPLC for a total of 25 experiments.

Initially, standard copper-catalysed amidation conditions[15] (10 mol % of catalyst, 2 equiv of K_2CO_3, 24 h, 110 °C) were employed and **15** was produced in only 15% yield, even with a longer reaction time (36 h). The catalyst concentration was then increased to 30 mol % and the yield gradually improved (30%), suggesting that more equivalents of CuI/1,2-diamine ligand would be required for the reaction to proceed to completion. However, when 50 mol % of the catalyst system was used, compound **15** was formed in very low yield (10%). On the other hand, higher concentrations of K_2CO_3 (i.e., 2.5 equiv) were well tolerated and resulted in a 10% increment in yield (40%) with respect to the previous experiment. Finally, it was observed that replacing dioxane with toluene led to the formation of building-block **15** in good yield (75%).

After Boc deprotection of **15**, the methyl 4-aminofurancarboxylate **16** was capped with methylchloroformate to give 4-methoxycarbonylamino-2-methyl furoate **17**, which was hydrolysed and coupled to another molecule of **16** to afford the di-furan platform **19**. Hydrolysis of the latter gave the free acid **20**, which was coupled using an HOAt–EDCI protocol, to aqueous ammonia, tyramine and tryptamine to give proximicins A, B and C, respectively, (Scheme 3). The spectroscopic data were identical to both the isolated natural products and previously synthesized materials.

2.2. Biological evaluation

The anticancer activity of proximicins was investigated using cellular methods. Apoptotic cell death was determined by flow cytometric DNA fragmentation analysis[22] against L1236 (Hodgkin's lymphoma) and Jurkat 16 (T-cell leukemia) cells. It was found that proximicin B exhibited a strong lethal effect in both L1236 and Jurkat 16 cell lines after only 24 h treatment at a concentration of 20 μg/mL and apoptosis induction was found to be dose-dependent after 48 h treatment. On the other hand, proximicin C induced apoptosis only in Jurkat 16 cells after 48 h treatment at 50 μg/mL,



Scheme 3. Reagents: (a) 4 N HCl in dioxane; (b) MeOCOCl, DIPEA, THF; (c) LiOH, H_2O/THF; (d) 16, HOAt-EDCI, CH_2Cl_2; (e) NH_4OH, HOAt-EDCI, CH_2Cl_2/DMF; (f) tyramine, HOAt-EDCI, CH_2Cl_2/DMF; (g) tryptamine, HOAt-EDCI, CH_2Cl_2/DMF.

with L1236 showing higher resistance to the drug. In addition, the MTT assay[23] was employed to determine the proximicins' cytotoxicity against selected cancer cell lines that were different from those previously reported. Proximicin C exhibited significantly higher cytotoxicity compared to proximicin B against U-87 MG (glioblastoma) and MDA-MD-231 (breast carcinoma) cells with IC_{50} values of 12.7 and 11.4 μg/mL, respectively. The variability of cytotoxic IC_{50} concentrations, in particular of Proximicins B and C, against different cancer cell lines, that were observed in our experiments suggests a specific, cell type dependent target interaction as driver of the cytotoxic effects.

Proximicins B (**2**) and C (**3**) were then tested in vitro for their antimicrobial activity against *E. coli*, *Ent. faecalis* and several strains of *S. aureus*, some of which were multidrug- and methicillin-resistant (MRSA), and minimum inhibitory concentration (MIC) values were determined (Table 1). Netropsin (**5**) and tetracycline were used as positive controls. Notably, proximicin B displayed valuable MIC values ranging from 4–8 μg/mL against the Gram-positive pathogens tested. These activities are noteworthy as they are against an effluxing strain over-expressing the NorA transporter[24] and two epidemic-MRSA strains (EMRSA-15 and -16).[25,26] The lack of activity against *E. coli* is presumably due to cell-permeability and drug efflux.

3. Conclusion

In summary, we have reported an efficient synthetic route for the preparation of proximicins A, B and C starting from the methyl 4-Boc-aminofuran-2-carboxylate **15**. The previous synthesis of this building-block involved a low yield lithiation step to attach a methyl ester residue to 3-furaldehyde ring. Alternative routes for the synthesis of **15** were sought and an efficient copper-catalysed amidation method was then chosen to introduce a Boc-amide residue at the C-4 position of the bromofuran **14**, derived in turn from the inexpensive methyl 2-furoate **12**. The amidation reaction was optimised by varying the concentration of the catalyst, the base and by changing the solvent, allowing the formation of **15** in only three steps with good yield. This synthetic pathway allowed sufficient quantities of proximicins to be prepared to investigate their biological activities in vitro.

Table 1
Antimicrobial activity of proximicins B and C[a]

Species	Strain	**2**	**3**	**5**	Tetracycline
S. aureus	ATCC29523	8	>256	64	0.5
S. aureus	EMRSA-16	8	>256	128	0.5
S. aureus	SA1199B	4	>256	16	0.25
S. aureus	EMRSA-15	8	>128	ND[b]	0.12
Ent. faecalis	NCTC 12697	4	>128	ND[b]	4
E. coli	NCTC 10418	>128	>128	ND[b]	0.12

[a] MIC values are expressed in μg/mL.
[b] Not determined.

Investigation of the proximicins' antitumor activity showed that proximicin B induced apoptosis in both Hodgkin's lymphoma and T-cell leukemia cell lines with strong lethal effect, whereas proximicin C exhibited significantly higher anti-proliferative activity against glioblastoma and breast carcinoma cells compared to proximicin B. The antimicrobial screening of proximicins B and C revealed that only proximicin B exhibited significant growth inhibition activity against all Gram-positive bacteria, including the problematic main UK epidemic methicillin-resistant *S. aureus* (EMRSA) strains. These results confirmed the proximicins' anticancer activity and showed, in particular, that proximicin B, given its significant antimicrobial activity against MRSA strains and strong ability to induce apoptosis in selected cancer cell lines, could be potentially developed into either an anticancer drug of broad utility or a chemotherapeutic agent useful for treatment of drug-resistant bacterial infections. Further in vitro and in vivo experiments are currently underway to elucidate the mechanism of action of these furan-based oligopeptides.

4. Experimental section

4.1. General information

^1H NMR and ^{13}C NMR spectra were acquired using a Bruker Avance 400 spectrometer at 400 and 100 MHz, respectively. Chemical shifts are reported in parts per million (ppm) with the solvent resonance as the internal standard and coupling constants (J) are quoted in Hertz (Hz). Spin multiplicities are described as: s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets of doublets), t (triplet), q (quadruplet) and m (multiplet). LC–MS analyses were carried out on a Phenomenex Monolithic C18 reversed-phase column (50 × 4.6 mm) with a flow rate of 3 mL min^{-1} and a linear gradient of B (5–95%) over 5 min. Eluent A: H_2O/0.1% formic acid; eluent B: CH_3CN/0.1% formic acid. TLC was performed on Merk silica gel 60 F_{254} aluminium sheets. Infrared spectra were acquired on a Perkin–Elmer FT–IR Spectrometer (Spectrum 1000) and absorbance frequencies are reported in reciprocal centimetres (cm^{-1}). Flash chromatography was performed using SuperFlash™ columns on a Varian 971-FP multi-wavelength UV Flash Chromatography. All chemicals were purchased from Fisher Scientific, Sigma–Aldrich and Merk Chemicals, and used without further purification. Preparative RP-HPLC was achieved using a Waters 600E Multi Solvent Delivery System coupled with a Waters 2847 dual λ Absorbance Detector and equipped with a Waters Atlantis Prep LC C_{18} column (250 × 19 mm) at a flow rate of 20.4 mL min^{-1}. Solvent A [0.2% (v/v) TFA in H_2O] and solvent B [0.16% (v/v) TFA in 90% MeCN]: 0% B to 100% B over 40 min

then 100% B for 10 min then 100% B to 0% B over 7 min. Separation was monitored at 214 nm by a Waters Millennium Chromatography Manager software and Empower2 Pro Chromatography software. Fractions were collected using a Waters Fraction Collector III, and data managed using Empower2 Pro Software.

4.2. Synthetic procedures

4.2.1. Methyl 5-formylfuran-3-carboxylate (7)

In a round-bottom flask fitted with a condenser and a thermometer, $POCl_3$ (2.4 mL, 26 mmol) was added dropwise over 10 min to stirred DMF (2.6 mL, 34 mmol) under nitrogen atmosphere at 0 °C. After the reaction mixture reached room temperature, methyl 2-furoate (2.1 mL, 20 mmol) was added and the temperature was gradually raised to 100 °C. (CAUTION!: at about 45 °C an exothermic reaction with evolution of HCl starts). The reaction mixture was stirred at the same temperature for 1 h and then cooled. The resulting brown tar was poured onto stirred ice-water (30 mL) and the organic phase extracted with Et_2O (6 × 25 mL), washed with H_2O (100 mL), $NaHCO_3$ (100 mL) and brine (75 mL), and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the resulting orange solid was purified by flash chromatography (hexane/EtOAc/9:1) to give **7** as a pale yellow oil (2.7 g, 87.5%). ^{1}H NMR ($CDCl_3$) (400 MHz) δ 9.67 (s, 1H), 8.19 (s, 1H), 7.51 (d, J = 0.57, 1 H), 3.86 (s, 3H). ^{13}C NMR ($CDCl_3$) (100 MHz) δ 177.8, 162.0, 153.2, 151.5, 121.5, 119.6, 52.1. MS m/z (ES+) (relative intensity) 154 ($M^{+\cdot}$). HRMS $[M+H]^+$ calculated for $C_7H_6O_4$ m/z 155.0344, found 155.0351.

4.2.2. 4-(Methoxycarbonyl)furan-2-carboxylic acid (8)

A solution of $NaClO_2$ (1.45 g, 16.2 mmol) in H_2O (10 mL) was slowly added at 0 °C to a solution of methyl 2-formyl-4-furoate **7** (0.5 g, 3.24 mmol), NaH_2PO_4 (1 g, 22.7 mmol) and H_2O_2 (700 μL, 22.7 mmol) in MeCN/H_2O/3:2 (10 mL). The reaction mixture was allowed to stir for 2 h at room temperature (until no more oxygen evolved from the reaction). After acidification with 10% HCl to pH 3, the solution was extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated under reduced pressure to yield 0.52 g of **8** as a white crystalline solid (3.07 mmol, 95%). ^{1}H NMR ($CDCl_3$) (400 MHz) δ 13.49 (br s, 1H), 8.59 (d, J = 0.72, 1H), 7.37 (d, J = 0.73, 1H), 3.79 (s, 3H). ^{13}C NMR ($CDCl_3$) (100 MHz) δ 161.9, 158.7, 151.2, 146.1, 120.0, 115.9, 51.8. MS m/z (ES+) (relative intensity) 171 (M+1). HRMS $[M-H]^-$ calculated for $C_7H_6O_5$ m/z 169.0137, found 169.0130.

4.2.3. Methyl 4,5-dibromofuran-2-carboxylate (13)

In a 500 mL three-neck flask, fitted with overhead stirrer, dropping funnel and a trap for HBr, was placed $AlCl_3$ (58.2 g, 436 mmol). Methyl-2-furoate **12** (25 g, 198 mmol) was slowly added at 0 °C under nitrogen over 30 min (exothermic reaction). To this vigorously stirred slurry, bromine (63.4 g, 396 mmol) was added under the same condition over 1 h period (CAUTION! Evolution of HBr). The stirring was discontinued and the reaction mixture was allowed to stand overnight at room temperature. H_2O (150 mL) was then added during 40 min at 0 °C, followed by Et_2O (150 mL). The layers were separated and the aqueous phase was further extracted with Et_2O (3 × 50 mL). The combined organic fractions were washed with H_2O (3 × 50 mL), $NaHCO_3$ (3 × 50 mL) and brine, dried over $MgSO_4$ and evaporated to dryness to give 45 g of a red oil, from which a precipitate was formed. Crystallisation from hexane afforded 40 g of the dibromofuran-ester **13** as an orange solid (141 mmol, 72%) that was used without purification in the next step. ^{1}H NMR (DMSO-d_6) (400 MHz) δ 7.63 (s, 1H), 3.82 (s, 3H). ^{13}C NMR (DMSO-d_6) (100 MHz) δ 156.7, 145.5, 128.8, 121.9, 103.7, 52.3. MS m/z (ES+) (relative intensity) 284 (M+1). HRMS $[M+Na]^+$ calculated for $C_6H_4Br_2O_3$ m/z 304.8425, found 304.8427.

4.2.4. Methyl 4-bromofuran-2-carboxylate (14)

In a 250 mL round-bottom flask, to a solution of **13** (4 g, 14 mmol) in dry MeOH (20 mL) were added Zn powder (2.8 g, 42 mmol) and NH_4Cl (2.0 g, 40 mmol) under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 2.5 h, filtered through a pad of celite and the solvent evaporated under reduced pressure. The residue was treated with brine (15 mL), extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$ and concentrated to dryness to obtain a crude amber solid, which was purified by flash chromatography (1→10% EtOAc in hexane). The desired product (**14**) was obtained as 2.5 g of white crystalline solid (12 mmol, 87%). ^{1}H NMR ($CDCl_3$) (400 MHz) δ 7.57 (d, J = 0.85, 1H), 7.17 (d, J = 0.85, 1H), 3.90 (s, 3H). ^{13}C NMR ($CDCl_3$) (100 MHz) δ 158.2, 145.1, 144.5, 120.4, 101.3, 52.3. MS m/z (ES+) (relative intensity) 204 (M+1). HRMS $[M+Na]^+$ calculated for $C_6H_5BrO_3$ m/z 226.9300, found 226.9315. IR ν_{max} (cm^{-1}) 3250, 2360, 1716, 1636, 1593.

4.2.5. Methyl 4-((tert-butoxycarbonyl)amino)furan-2-carboxylate (15)

An oven-dried sealable vial (2–5 mL) was charged with CuI (177 mg, 0.93 mmol, 30 mol %), *tert*-butyl carbamate (436 mg, 3.72 mmol) and K_2CO_3 (1071 mg, 7.75 mmol), evacuated and backfilled with N_2 (×2). **14** (640 mg, 3.12 mmol) was then added, followed by dry toluene (2 mL) and *N*,*N*′-dimethylethylenediamine $(CH_3NHCH_2)_2$ (99 μL, 30 mol %, 0.93 mmol). The vial was sealed and the reaction allowed to stir at 110 °C for 23 h. After cooling to room temperature, the mixture was filtered through a pad of silica gel and eluted with EtOAc/CH_2Cl_2/1:1 (20 mL). The solvent was evaporated under reduced pressure and the yellow residue purified by flash chromatography (5→40% EtOAc in hexane) to afford 564 mg of **15** as a white crystalline solid (2.34 mmol, 75%). ^{1}H NMR ($CDCl_3$) (400 MHz) δ 7.89 (s, 1H), 7.00 (s, 1H), 6.32 (s, 1H), 3.88 (s, 3H), 1.50 (s, 9H). ^{13}C NMR ($CDCl_3$) (100 MHz) δ 158.9, 152.5, 142.8, 134.4, 126.5, 111.2, 81.2, 52.0, 28.3. MS m/z (ES+) (relative intensity) 242 (M+1). HRMS $[M+Na]^+$ calculated for $C_{11}H_{15}NO_5$ m/z 264.0848, found 264.0838. IR ν_{max} (cm^{-1}) 3330, 3140, 2970, 1730, 1720, 1700.

4.2.6. Methyl 4-amino-furan-2-carboxylate (16)

Compound **15** (360 mg, 1.5 mmol) was dissolved in 4 N HCl/dioxane (5 mL) and the solution was allowed to stir overnight at room temperature. The volatiles were evaporated to afford 210 mg (quantitative yield) of **16** as a brown solid, which was used without purification in the next step. ^{1}H NMR (DMSO-d_6) (400 MHz) δ 8.13 (d, J = 0.88, 1H), 7.34 (d, J = 0.92, 1H), 3.80 (s, 1H). ^{13}C NMR (DMSO-d_6) (100 MHz) δ 158.3, 144.1, 141.3, 120.4, 114.8, 52.7. MS m/z (ES+) (relative intensity) 142 (M+1). HRMS $[M+H]^+$ calculated for $C_6H_7NO_3$ m/z 142.0504, found 142.0501. IR ν_{max} (cm^{-1}) 3340, 3112, 2977, 2826, 2587, 1736, 1616, 1512.

4.2.7. Methyl 4-((methoxycarbonyl)amino)furan-2-carboxylate (17)

The aminofuran acid chloride **16** (164 mg, 1.16 mmol) was suspended in dry THF (10 mL) and DIPEA (485 μL, 348 mmol) was added under nitrogen atmosphere. After stirring at room temperature for 10 min, MeOCOCl (233 μL, 3.0 mmol, dissolved in 1 mL of dry THF) was added dropwise and the reaction mixture allowed to stir at 70 °C overnight. After evaporating the volatiles, the residue was treated with EtOAc (10 mL) and then washed with H_2O (3 × 5 mL), 10% HCl (3 × 5 mL), $NaHCO_3$ (3 × 5 mL) and brine (2 × 5 mL). The solvent was evaporated under reduced pressure and the resulting brown solid was purified by flash chromatography

(5→40% EtOAc in Hexane) to afford 175 mg (0.88 mmol, 76%) of **17** as a white crystalline solid. ^{1}H NMR ($CDCl_3$) (400 MHz) δ 7.88 (s, 1H), 7.06 (s, 1H), 6.68 (s, 1H), 3.88 (s, 3H), 3.77 (s, 3H). ^{13}C NMR ($CDCl_3$) (100 MHz) δ 159.0, 154.0, 143.0, 134.7, 126.3, 111.5, 52.9, 52.1. MS *m*/*z* (ES+) (relative intensity) 200 (M+1). HRMS $[M+H]^+$ calculated for $C_8H_9NO_5$ *m*/*z* 200.0559, found 200.0554. IR ν_{max} (cm^{-1}) 3367, 3147, 2956, 1707, 1616, 1556.

4.2.8. Methyl 4-(4-((methoxycarbonyl)amino)furan-2-carboxamido)furan-2-carboxylate (19)

To a solution of **17** (80 mg, 0.4 mmol) in THF (5 mL) was added LiOH (70 mg, 3 mmol, dissolved in 2 mL of H_2O) dropwise. The reaction mixture was allowed to stir for 1.5 h at room temperature. The solvent was evaporated under reduced pressure and the residue treated with H_2O (5 mL). After acidification by 10% HCl to pH 3, the solution was extracted with EtOAc (3 × 5 mL), dried over $MgSO_4$ and concentrated in vacuo to obtain 50 mg (0.27 mmol, 70%) of the free acid (**18**) as an off-white solid. ^{1}H NMR ($CDCl_3$) (400 MHz) δ 9.70 (s, 1H), 7.85 (s, 1H), 7.05 (s, 1H), 3.77 (s, 3H). MS *m*/*z* (ES+) (relative intensity) 186 (M+1). IR ν_{max} (cm^{-1}) 3376, 3134, 2182, 1710, 1612, 1562. The acid **18** was then suspended in anhydrous DCM (10 mL) and EDCI (52 mg, 0.27 mmol) and HOAt (37 mg, 1 mmol) were added under nitrogen atmosphere. After stirring at room temperature for 1 h, a solution of the aminofuran acid chloride **16** (50 mg, 0.27 mmol) and DIPEA (47 μL, 0.27 mmol) in anhydrous DCM (2 mL) was added to the suspension and the reaction mixture was allowed to stir at room temperature for 24 h. After adding H_2O (10 mL) and EtOAc (10 mL) to the reaction mixture, the organic layer was separated, washed with H_2O (3 × 5 mL), 10% HCl (3 × 5 mL), $NaHCO_3$ (3 × 5 mL), brine (2 × 5 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to yield 75 mg (0.24 mmol, 90%) of **19** as a yellow solid, which was used without further purification in the next synthetic step. ^{1}H NMR ($CDCl_3$) (400 MHz) δ 10.78 (s, 1H), 9.78 (s, 1H), 8.26 (d, *J* = 0.69, 1H), 7.86 (s, 1H), 7.33 (d, *J* = 0.72, 1H), 7.16 (s, 1H), 3.79 (s, 3H), 3.74 (s, 3H). ^{13}C NMR ($CDCl_3$) (100 MHz) δ 158.3, 155.3, 154.0, 145.0, 141.7, 135.9, 132.3, 127.3, 126.0, 112.1, 108.4, 52.2, 52.0. MS *m*/*z* (ES+) (relative intensity) 308 (M+1). HRMS $[M+H]^+$ calculated for $C_{13}H_{12}N_2O_7$ *m*/*z* 309.0723, found 309.0732. IR ν_{max} (cm^{-1}) 3340, 3224, 2927, 1709, 1650, 1584.

4.2.9. 4-(4-((methoxycarbonyl)amino)furan-2-carboxamido)furan-2-carboxylic acid (20)

To a solution of **19** (33 mg, 0.1 mmol) in MeOH (4 mL), LiOH (14 mg, 0.47 mmol, dissolved in 2 mL of H_2O) was added dropwise. The mixture was allowed to stir at room temperature for 3 h. The solvent was evaporated under reduced pressure and the residue treated with 4 mL of H_2O. After acidification by 10% HCl to pH 3, the organic phase was extracted with EtOAc, dried over $MgSO_4$ and concentrated to dryness to afford 25 mg (0.085 mmol, 85%) of the free acid **20** as a pale yellow solid. ^{1}H NMR ($CDCl_3$) (400 MHz) δ 13.20 (br s, 1H), 10.70 (s, 1H), 9.80 (s, 1H), 8.24 (s, 1H), 7.88 (s, 1H), 7.29 (s, 1H), 7.16 (s, 1H), 3.71 (s, 3H). ^{13}C NMR (DMSO-d_6) (100 MHz) δ 159.2, 155.2, 153.9, 145.2, 142.9, 135.3, 132.1, 127.4, 126.1, 111.6, 108.6, 52.1. MS *m*/*z* (ES+) (relative intensity) 294 (M+1).

4.3. General procedure for the synthesis of proximicins A, B and C

To a solution of acid **20** in dry DMF/DCM/1:1 (5 mL) were added EDCI, HOAt and the appropriate amine, in this order. The reaction mixture was allowed to stir for 24 h at room temperature under nitrogen atmosphere. The solution was then poured on to ice-cold H_2O, extracted with EtOAc (3 × 10 mL), washed with H_2O (3 × 10 mL), 10% HCl (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (2 × 10 mL), and dried over $MgSO_4$. The solvent was then evaporated under reduced pressure and the residues purified by preparative HPLC to give the desired products.

4.3.1. Methyl (5-((5-carbamoylfuran-3-yl)carbamoyl)furan-3-yl)carbamate. Proximicin A (1)

Acid **20** (30 mg, 0.10 mmol) was treated with EDCI (21 mg, 0.11 mmol), HOAt (15 mg, 0.11 mmol) and NH_3OH (5 μL, 0.13 mol), according to the general procedure, to afford 11 mg of proximicin A (**1**) as a white solid (0.037 mmol, 37.5%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.69 (s, 1H), 9.79 (s, 1H), 8.12 (s, 1H), 7.87 (bs, 1H), 7.40 (bs, 1H), 7.18 (s, 1H), 7.17 (s, 1H), 3.67 (s, 3H). ^{13}C NMR (DMSO-d_6) (100 MHz) δ 158.9, 155.1, 154.0, 145.8, 145.1, 133.1, 131.7, 127.3, 125.8, 108.4, 107.9, 51.9. MS *m*/*z* (ES+) (relative intensity) 293 ($M^{+\bullet}$). HRMS $[M+H]^+$ calculated for $C_{12}H_{11}N_3O_6$ *m*/*z* 294.0726, found 294.0768.

4.3.2. Methyl (5-((5-((4-hydroxyphenethyl)carbamoyl)furan-3-yl)carbamoyl)furan-3-yl)carbamate. Proximicin B (2)

Acid **20** (60 mg, 0.20 mmol) was treated with EDCI (38.5 mg, 0.20 mmol), HOAt (27.5 mg, 0.20 mmol) and tyramine (27.5 mg, 0.20 mmol), according to the general procedure, to afford 38 mg of Proximicin B (**2**) as a white solid (0.092 mmol, 46%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.66 (s, 1H), 9.79 (s, 1H), 9.15 (s, 1H), 8.40 (t, *J* = 5.80, 1H), 8.12 (s, 1H), 7.87 (br s, 1H), 7.17 (br s, 1H), 7.15 (s, 1H), 7.00 (d, *J* = 8.10, 2H), 6.67 (d, *J* = 7.61, 2H), 3.68 (s, 3H), 3.44–3.40 (m, 2H), 2.71–2.67 (m, 2H). ^{13}C NMR (DMSO-d_6) (100 MHz) δ 157.5, 155.6, 155.2, 153.7, 146.1, 145.3, 133.1, 132.0, 129.4, 129.3, 127.2, 126.1, 115.1, 108.5, 107.7, 52.1, 40.1, 34.2. MS *m*/*z* (ES+) (relative intensity) 414 (M+1). HRMS $[M+H]^+$ calculated for $C_{20}H_{19}N_3O_7$ *m*/*z* 414.1301, found 414.1297.

4.3.3. Methyl (5-((5-((2-(1H-indol-3-yl)ethyl)carbamoyl)furan-3-yl)carbamoyl)furan-3-yl)carbamate. Proximicin C (3)

Acid **20** (75 mg, 0.26 mmol) was treated with EDCI (50 mg, 0.26 mmol), HOAt (35.4 mg, 0.17 mmol) and tryptamine (42 mg, 0.26 mmol), according to the general procedure, to afford 30 mg of Proximicin C (**3**) as a white solid (0.07 mmol, 27%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.79 (s, 1H), 10.69 (s, 1H), 9.79 (s, 1H), 8.53 (t, *J* = 5.72, 1H), 8.14 (d, *J* = 0.76, 1H), 7.88 (s, 1H), 7.57 (d, *J* = 7.81, 1H), 7.33 (d, *J* = 8.05, 1H), 7.18 (br s, 2H), 7.16 (d, *J* = 2.32, 1H), 7.06 (overlapped ddd, *J* = 7.70, 6.95, 1.01, 1H), 6.97 (overlapped ddd, *J* = 7.71, 7.08, 1.01, 1H), 3.68 (s, 3H), 3.49 (q, *J* = 7.48, 2H), 2.92 (t, *J* = 7.74, 2H). ^{13}C-NMR (DMSO-d_6) (100 MHz) δ 157.6, 155.2, 154.0, 146.1, 145.0, 136.2, 133.1, 132.0, 127.5, 127.2, 126.1, 122.2, 120.9, 118.4, 118.3, 111.7, 111.4, 108.6, 107.7, 52.1, 39.3, 25.2. MS *m*/*z* (ES+) (relative intensity) 437 (M+1). HRMS $[M+Na]^+$ calculated for $C_{22}H_{20}N_4O_6$ *m*/*z* 459.1281, found 459.1269.

4.4. Biological evaluation

Unless otherwise stated, all chemicals were obtained from Sigma–Aldrich Company Ltd.

4.5. Determination of apoptosis

L1236 (Hodgkin lymphoma) and Jurkat 16 (T-cell leukaemia) cell lines were cultured in RPMI medium supplemented with 10% foetal calf serum, 100 units/mL penicillin and 100 units/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were plated in duplicate and treated for 48 h with increasing concentrations of proximicins. DNA fragments released from apoptotic nuclei were detected using propidium iodide (PI). Measurement by flow cytometry of PI-emitted fluorescence allowed the quantification of degraded DNA (% DNA fragmentation).[27] Specific

apoptosis was calculated as (percentage of experimental apoptosis − percentage of spontaneous apoptosis)/(100−percentage of spontaneous apoptosis) × 100.

4.6. MTT cytotoxicity assay

Cell culture. U-87 MG glioblastoma and MDA-MB-231 cells were grown in MEM and DMEM culture medium (Invitrogen, CA), supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate, in a 5% CO_2, 95% humidity incubator. *Drug treatment*. Cells were seeded and once in logarithmic growth were incubated with increasing drug concentrations for 24 h. Then cells were grown in fresh medium for a further 48 h. *MTT assay*.[23] Cells were incubated with the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Alfa Aesar, 5 mg/mL 20 μL/well) for 4 h. After removal of the medium the insoluble formazan crystals were dissolved (200 μL DMSO) and absorbance was measured at 570 nm. Cell viability (%) was calculated as (absorbance value of treated-mean absorbance of triton treated)/mean absorbance of untreated wells × 100.

4.7. Antibacterial assay

Cation-adjusted Mueller-Hinton broth was obtained from Oxoid and was adjusted to contain 20 and 10 mg/L of Ca^{2+} and Mg^{2+}, respectively. The *S. aureus* strains used in this study included ATCC 25923, SA-1199B, EMRSA-15 and EMRSA-16. ATCC 25923 is a standard laboratory strain sensitive to antibiotics. SA-1199B over-expresses the NorA MDR efflux pump.[24] EMRSA-15[25] and EMRSA-16[26] are epidemic strains in the UK. *Ent. faecalis* NCTC 12697 and *E. coli* NCTC 10418 are antimicrobial susceptibility testing control strains and were obtained from The National Collection of Type Cultures (London, United Kingdom). *S. aureus*, *E. coli* and *Ent. faecalis* strains were cultured on nutrient agar (Oxoid) and incubated for 24 h at 37 °C prior to MIC determination. An inoculum density of 5×10^5 colony forming units (cfu) of each bacterial strain was prepared in normal saline (9 g/L) by comparison with a 0.5 MacFarland turbidity standard. The inoculum (125 μL) was added to all wells and the microtitre plate was incubated at 37 °C for the corresponding incubation time. For MIC determination, 20 μL of a 5 mg/mL methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to each of the wells and incubated for 20 min. Bacterial growth was indicated by a colour change from yellow to dark blue. The MIC was recorded as the lowest concentration at which no growth was observed.

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