The Journal of Antibiotics (2015) 68, 56–59

www.nature.com/ja

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In vitro antibacterial activities of a thiazolyl peptide antibiotic PM2409

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The Journal of Antibiotics (2015) **68,** 56–59; doi:10.1038/ja.2014.88; published online 2 July 2014

Thiazolyl peptide antibiotics are highly modified cysteine-containing macrocyclic peptides with several distinctive common features such as the presence of thiazole rings, dehydro amino acids and a highly substituted pyridine centerpiece at the junction of the macrocyclic ring. They are known for their potent *in vitro* antibacterial activity against a wide spectrum of pathogens.[1] Owing to their poor aqueous solubility and unfavorable pharmacokinetics, very few of them have made in to the clinic.[1,2] The chemical modifications to yield new chemical entities (NCEs), and newer formulations are some of the efforts that have been explored to overcome the unfavorable features of these antibiotics.[3,4] Recently, we have reported a potent thiazolyl peptide antibiotic PM181104 (**1**) (Figure 1), isolated from fermentation of the actinobacterium strain of the genus *Kocuria* (MTCC 5269), a microbe associated with the marine sponge species *Spirastrella inconstans var. digitata*.[5,6] The structure of this antibiotic has been revised recently and has been referred to as kocurin.[2] True to the behavior of naturally occurring thiazolyl peptide antibiotic series, kocurin too exhibited poor aqueous solubility causing hindrance in our efforts of developing this antibiotic as a clinical candidate. We embarked upon the chemical modification of kocurin to increase aqueous solubility while maintaining the antibacterial activity. Examination of the structure of kocurin suggests that its terminal chain containing dehydroalanines and proline is a region of rich hydrophobic core, and any scope to minimize (or remove) this chain through truncation has a means to improve the aqueous solubility. Following the approach that has been explored on another thiazolyl peptide antibiotic,[3] we carried out a controlled acid hydrolysis of compound **1** to obtain a new thiazolyl peptide antibiotic PM2409 (**2**). The reaction mechanism for the formation of compound **2** from **1** is believed to follow the cycle of removal of two fragments of pyruvamide, followed by prolyl-rearrangement and the final removal of pyruvoylalaninamide (Figure 1). The two-dimensional ^{1}H–^{15}N HSQC spectra of **2** displayed three NH correlations, which were assigned to the amide backbone of NH groups of tyrosine, phenylalanine and aspargine. Two pairs of NH_2 correlations additionally seen in the spectra were assigned to the aspargine side-chain NH_2 group and thiazole carboxamide NH_2 group. Complete structure assignment of **2** was achieved with the help of high-resolution NMR and mass spectral data (Supplementary Data S1).[2] When compared with the parent compound **1**, **2** exhibited much improved water solubility ($<0.001\,mg\,ml^{-1}$ vs $0.09\,mg\,ml^{-1}$).

Compound **2** was profiled against several Gram-positive bacteria, including anaerobes. It inhibited the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) with MIC at $0.12\,\mu g\,ml^{-1}$ for both. It also inhibited *Bacillus spp.* with MIC 0.06–$0.12\,\mu g\,ml^{-1}$ (Table 1). Compound **2** was also tested against 16 clinical strains of *Clostridium difficile*. Owing to concern over the possible deleterious effects of the blood component in the standard reference medium (supplemented brucella agar (SBA)), the compound was also tested in Wilkens–Chalgren agar (WCA) medium. It exhibited potent activity against the clinical *C. difficile* isolates with MIC range, MIC_{50} and MIC_{90} values of $0.25\,\mu g\,ml^{-1}$ (Table 2). As SBA contains blood and WCA does not, the data indicate that the presence of blood in the test medium has some effect on the activity of PM2409 against *C. difficile* as the MIC_{90} in SBA showed a fourfold-increased value ($1\,\mu g\,ml^{-1}$). It implies that possibly the compound binds to serum components in the blood. To rule out the contribution of the cytotoxicity (if any) of the compound **2** toward the observed *in vitro* antibacterial activities, cytotoxicity studies were carried out for the compound against different cancer and normal mammalian cell lines. The compound exhibited no cytotoxicity up to $100\,\mu g\,ml^{-1}$ in all the cell lines tested (Supplementary Data S2 and S3).

To determine the mechanism of action of PM2409, it was tested for its ability to inhibit the synthesis of major macromolecules that are essential for bacterial growth in a whole-cell assay using *Bacillus subtilis* strains. When *B. subtilis* were incubated with PM2409 at its MIC ($0.12\,\mu g\,ml^{-1}$), protein synthesis was inhibited selectively ($\sim$40% incorporation of the labeled amino-acid mixture during the macromolecular synthesis). There was no significant inhibition of RNA, DNA or peptidoglycan synthesis at this concentration (Figure 1c). Like other antibiotics of this class (for example, GE2270A[7] and LFF571[4]), we envisage that the compound PM2409

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Received 21 January 2014; revised 31 May 2014; accepted 6 June 2014; published online 2 July 2014

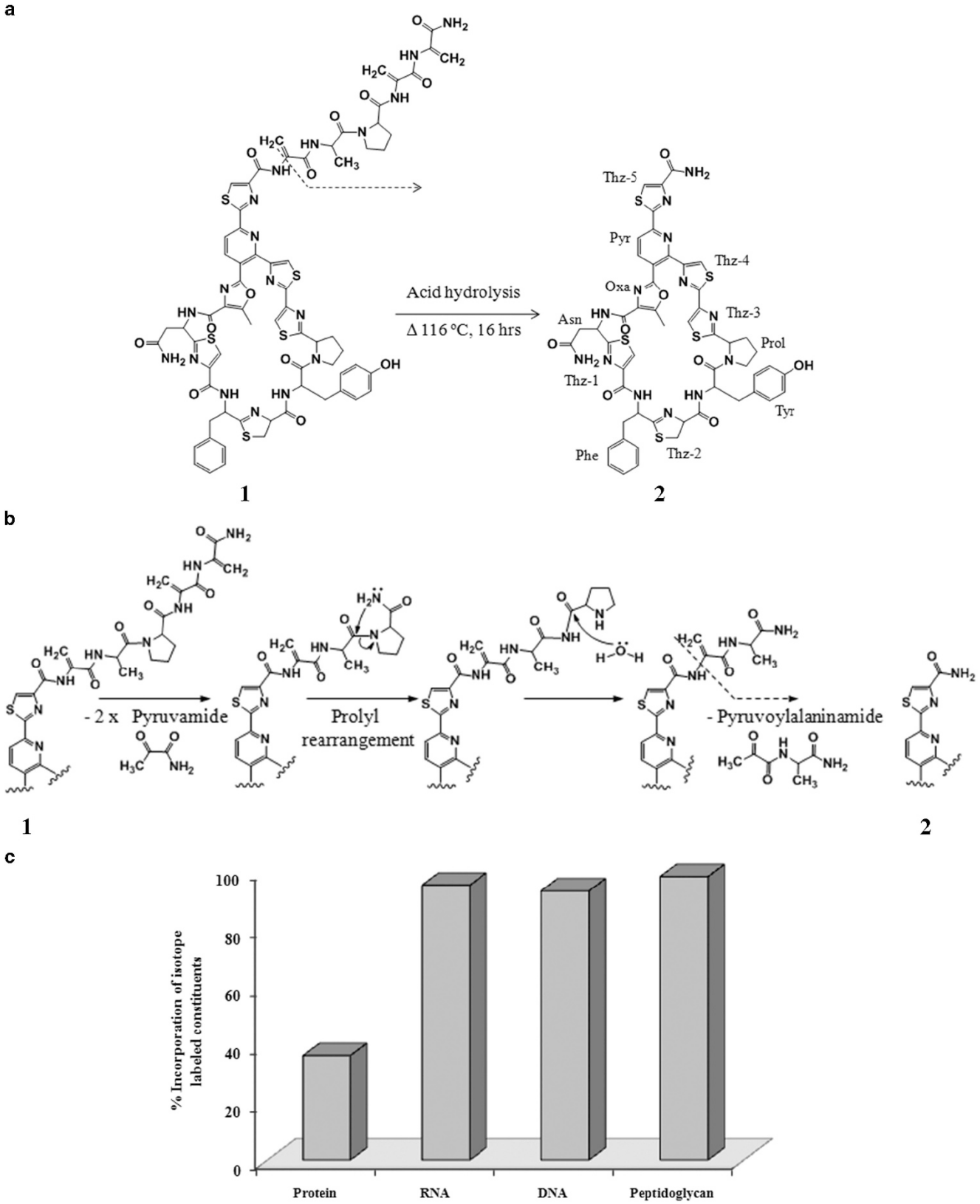

Figure 1 (**a**) Preparation of PM2409 (**2**) by the acid hydrolysis of kocurin (**1**). The constituent amino-acid residues of PM2409 are shown with their respective names on the structure. (**b**) Mechanism of formation of PM2409 (**2**) during the course of hydrolysis. (**c**) Effect of PM2409 (**2**) at its MIC ($0.125\,\mu g\,ml^{-1}$) on macromolecular synthesis of *Bacillus subtilis* HMR 122. Percentage incorporation of radiolabeled constituents during the course of macromolecular synthesis was measured by treating radiolabeled incorporation in unexposed control cultures as 100%. Results shown in the figure are representative of the mean of the duplicate studies.

Table 1 Summary of the *in vitro* antibacterial activities of PM2409 (2) and Kocurin (1)

	MIC ($\mu g\,ml^{-1}$)[a]		
Strains	*PM2409 (**2**)*	*Kocurin (**1**)*	*Linezolid*
S. aureus ATCC 33591, MRSA	0.125	0.032	2
E. faecalis ATCC 51575, VRE	0.125	0.032	2
B. subtilis, HMR 122	0.125	0.016	1
B. megaterium FH 1127	0.063	0.008	2

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus*.
For comparison, activity for linezolid is also shown in the table.
[a]Results are representative of the mean of the duplicate results.

Table 2 Comparative *in vitro* activity of compound PM2409 (2), Kocurin (1), vancomycin and metronidazole against *C. difficile*

		Supplemented brucella agar[a]			*Wilkins–Chalgren agar*[a]		
Strain	*Compound*	*MIC range*[b]	*MIC_{50}*	*MIC_{90}*	*MIC range*[b]	*MIC_{50}*	*MIC_{90}*
C. difficile	PM2409 (**2**)	0.5–1	0.5	1	0.25	0.25	0.25
	Kocurin (**1**)	0.12–0.25	0.25	0.5	0.03–0.25	0.12	0.25
	Vancomycin	1–4	1	4	0.5–4	1	2
	Metronidazole	0.25–0.5	0.25	0.5	0.25–2	0.5	1

[a]Data shown in the plot are mean of the duplicate results. Values are shown in $\mu g\,ml^{-1}$.
[b]The values represent the range of MIC values for all 16 clinical strains of *C. difficile*.

curtails protein synthesis at translational level by binding to bacterial elongation factor thermo unstable (EF-Tu).

Considering the success of LFF571, a structurally similar semi-synthetic thiazolyl peptide (known to inhibit bacterial EF-Tu),[4,8] and the improved aqueous solubility along with the enhanced acid pH stability (data not presented), we assume that PM2409 makes a good candidate to be developed for oral nonabsorbed treatment for *C. difficile* infection. The future plan is to carry out hamster oral pharmacokinetics of PM2409, where its gut exposure would be determined using cecum sampling. The second step will be *in vivo* efficacy studies of PM2409 against *C. difficile* in a hamster model of antibiotic-associated colitis.

EXPERIMENTAL PROCEDURE

Preparation of PM2409 (2) from kocurin (1)

The preparation of **2** was carried out following the procedure that has been reported earlier for a similar reaction.[3] In a round bottom flask, 3 g kocurin was mixed with 25 ml dioxane–water–formic acid, 8:1:1 (in volume) and the mixture was kept under reflux for 16 h at $116 \pm 1\,^{\circ}C$ (Figure 1). The resultant solution was dried under reduced pressure to get a brown color solid mass. A small speck of the mixture was dissolved in chloroform–methanol, 90:10 (v/v) and analyzed on normal phase HPLC (Silica: 250×4.6 mm, 3.5 µm, chloroform–methanol 95:5 (v/v), flow rate: $1\,ml\,min^{-1}$ and UV: 309 nm). All the major products thus obtained were tested for their antibacterial activity. The peak eluting at retention time (Rt) 9.25 min displayed antibacterial activity against MRSA and VRE in the agar plate diffusion assay. Accordingly, a complete purification of the entire reaction mixture was carried out on semi-preparative normal phase HPLC (Silica: 250×10 mm, 5 µm, chloroform–methanol, 95:5 (v/v), flow rate: $6\,ml\,min^{-1}$ and UV: 309 nm), resulting in 72 mg (yield: ~24%) of white powder PM2409 (**2**) (99% purity). $[\alpha]_D^{25} + 12$ (c 0.2, $CHCl_3$–MeOH, 1:1 (v/v); UV (MeOH) λ_{max} 225, 307 and 340 (sh) nm; IR (KBr pellet) ν_{max} 3376, 2926, 2854, 1663, 1514 and 1429 cm^{-1}; HR-ESI-MS $[M+H]^+$ *m/z* 1140.2190 (calcd for $C_{52}H_{45}N_{13}O_8S_5 + H$: 1140.2118); ^1H and ^{13}C NMR (Supplementary Data S1).[2]

Solubility measurements

The solubility studies were carried out following the earlier reported method.[4] One milligram of the compound was weighed into a 5-ml glass tube, and 1 ml of Milli-Q (Millipore, Molsheim, France) (pH 5.3) water was added. After initial mixing using vortexing and sonication (15 min), the sample was equilibrated by shaking for 24 h at room temperature. After 24 h, the glass tube was visually examined. Suspensions with visible particles were filtered through a 0.22-µm polyvinylidene difluoride membrane filter. The dissolved drug concentration was analyzed using a reversed-phase (RP)-HPLC assay. The procedure was carried out in duplicate. The values reported are the mean of the duplicate studies.

In vitro activity against MRSA, VRE and *Bacillus* spp.

The MIC of PM2409 was evaluated following the procedure set by the National Committee for Clinical Laboratory Standards (NCCLS, now called the Clinical Laboratory Standards Institute). The procedure follows the twofold dilution approach (document no. M7-A5, NCCLS) in Mueller-Hinton broth (MHB).[9] The culture tubes were incubated at $37\,^{\circ}C$ for 24 h before the activity was recorded. The MIC is defined as the lowest concentration of antibiotic that inhibited visible growth.

In vitro activity against *C. difficile*

All experiments related to *in vitro* potency studies against *C. difficile* were carried out at Micromyx, Kalamazoo, MI, USA. The solvent used was dimethyl sulfoxide and the stock concentration was $2.56\,mg\,ml^{-1}$. The test organisms for the assays were recent clinical isolates or reference strains acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). The quality control organisms included in the assay were: *Bacteroides fragilis* 0123 (ATCC 25285) and *C. difficile* 4381 (ATCC 700057). The growth and test media were those recommended by the Clinical and Laboratory Standards Institute (CLSI) for growth and susceptibility testing of anaerobes.[10] The test organisms were maintained frozen at $-80\,^{\circ}C$. The isolates were subcultured on SBA plates (Remel; Lenexa, KS, USA) in a Bactron II anaerobic chamber and incubated for 48 h at $35\,^{\circ}C$ in the Bactron II anaerobe chamber (Sheldon Manufacturing Inc., Cornelius, OR, USA). The reference medium[10] employed for the agar dilution MIC assay of anaerobic bacteria was Brucella Agar (Becton Dickinson; Sparks, MD, USA; Lot No. 9271330) supplemented with hemin (Sigma, St Louis, MO, USA; Lot No. 027K2055), Vitamin K1 (Sigma; Lot No. 106K1523) and 5% lysed sheep blood (Cleveland Scientific; Bath, OH, USA; Lot No. 130145). In addition, the compound was assayed using WCA(Becton Dickinson; Franklin Lakes, NJ, USA; Lot No. 9110652).

Anaerobic bacteria were assayed using a reference agar dilution method.[10] Drug dilutions and drug-supplemented agar plates were prepared manually. Organisms were grown in a Bactron II Anaerobic Chamber (Sheldon Manufacturing, Cornelius, OR, USA) for 48 h before assay. Following inoculation, the drug-supplemented plates were incubated at $35\,^{\circ}C$ for 48 h in the anaerobic environment (5% hydrogen, 5% carbon dioxide and 90% nitrogen) of the Bactron II. The MIC was read as per CLSI guidelines.[10]

Macromolecular synthesis inhibition in *B. subtilis* HMR 122

The macromolecular synthesis inhibition studies were performed as described previously.[11] In brief, *B. subtilis* was grown at $37\,^{\circ}C$ at 200 r.p.m. in a MHB to early log phase (optical density at 600 nm (OD_{600}) of 0.3). $1.0\,\mu Ci\,ml^{-1}$ of glycine [2-3H], $0.1\,\mu Ci\,ml^{-1}$ of thymidine [6-3H], $1.0\,\mu Ci\,ml^{-1}$ of uridine [5, 6-3H] and $1.0\,\mu Ci\,ml^{-1}$ of amino-acid mixture [U-3H] were used as precursors for peptidoglycan, DNA, RNA and protein synthesis, respectively. Radioactive precursors were added during the early logarithmic phase and 3 min before the addition of the test compound PM2409 (**2**) at the MIC level ($0.12\,\mu g\,ml^{-1}$) of the test strain. After 30 min, ice-cold trichloroacetic acid (TCA), 10% (w/v) was added to the wells to achieve a 5.0% (w/v) final concentration. The TCA-insoluble fraction was collected on a glass microfiber

filter (Whatman International Ltd., Maidstone, UK) (GF/C, 25 mm; Whatman cat 1822025) by using a Millipore (Bedford, MA, USA) 12 sample manifold (XX27 025 50). Each filter was first washed with 5 ml of ice-cold 10% TCA, followed by 5 ml of cold distilled water. The filters were dried at 40 °C for 1 h. The dried filters were counted in a liquid scintillation counter (TRI-CARB, model 1600CA by Packard, Perkin Elmer, Waltham, MA, USA) using scintillation fluid. The percentage incorporation of the isotope-labeled precursors was plotted against the test compound.

ACKNOWLEDGEMENTS

We thank Dr Somesh Sharma, Managing Director, Piramal Life Sciences for his support and encouragement that we received during the course of this work. We also thank Dr K. Nagarajan, Hikal Ltd., Bangalore for discussions and suggestions regarding this work.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)