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Synthesis, antibacterial and cytotoxic evaluation of flavipucine and its derivatives

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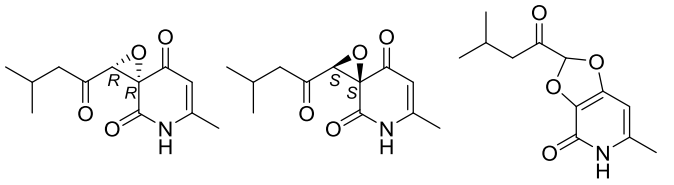
ABSTRACT

The antibacterial and cytotoxic activity of seven racemic lactams and both enantiomers of flavipucine were evaluated. Of the compounds tested in this study, flavipucine and phenylflavipucine displayed bactericidal activity against *Bacillus subtilis*. These results indicate that the pyridione epoxide moiety is a pharmacophore for antibacterial activity against *B. subtilis*. Flavipucine showed cytotoxic activity against several cancer cells. The cytotoxic activity of flavipucine against human leukemia HL-60 cells is as strong as that of SN-38, the active metabolite of irinotecan. In contrast, the cytotoxic activity of flavipucine against nonneoplastic HEK293 cells and human normal MRC-5 cells is weaker than that of SN-38. No significant differences in the biological activity of the racemates or enantiomers of flavipucine were observed.

(–)-Flavipucine [(–)-**1**, Figure 1] has been isolated from *Aspergillus flavipes*,[1,2] the fungus-caused *Macrophoma* fruit rot of the apple,[3] as well as from cultures of *Cladobotryum rubrobrunnescens*.[4] (+)-Flavipucine [(+)-**1**] was isolated from the culture extract of *Phoma sp*.[5] Both enantiomers of flavipucine reportedly show antibacterial and antifungal activity.[1,4-6] The absolute configuration of (+)-**1** was determined by comparison of experimental and calculated CD spectra.[5] Isoflavipucine (**2**) was isolated in racemic form from *Aspergillus flavipes* and *Phoma sp*.[5,7] This compound is produced via rearrangement of flavipucine.[8-11] Isoflavipucine possesses moderate inhibitory activity against *Phytophthora infestans*.[5] Berkeleyamide D (**3**) has been isolated from the acid lake fungus *Penicillium rubrum* Stoll, and was shown to inhibit matrix metalloproteinase-3 and caspase-1.[12] Rubrobramide (**4**) was isolated from *Cladobotryum rubrobrunnescens* exhibits weak cytotoxic activity against mouse lymphocytic leukemia L-1210 cells, and inhibits the germination of *Lepidium sativum*.[13] The absolute configurations of **3** and **4** were determined via the exciton chirality method in vibrational circular dichroism.[14,15]



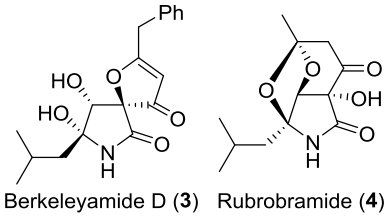


Figure 1. Structures of (–)-flavipucine [(–)-**1**], (+)-flavipucine [(+)-**1**], (±)-isoflavipucine (**2**), berkeleyamide D (**3**), and rubrobramide (**4**).

The racemic total syntheses of **1**–**4** were achieved using a regioselective Darzens reaction between isobutylglyoxal (**5**) and α-bromo-β-ketoamides **6** by our group (Scheme 1).[14-16] When *tert*-butoxycarbonyl (Boc)-protected α-bromo-β-ketoamides were

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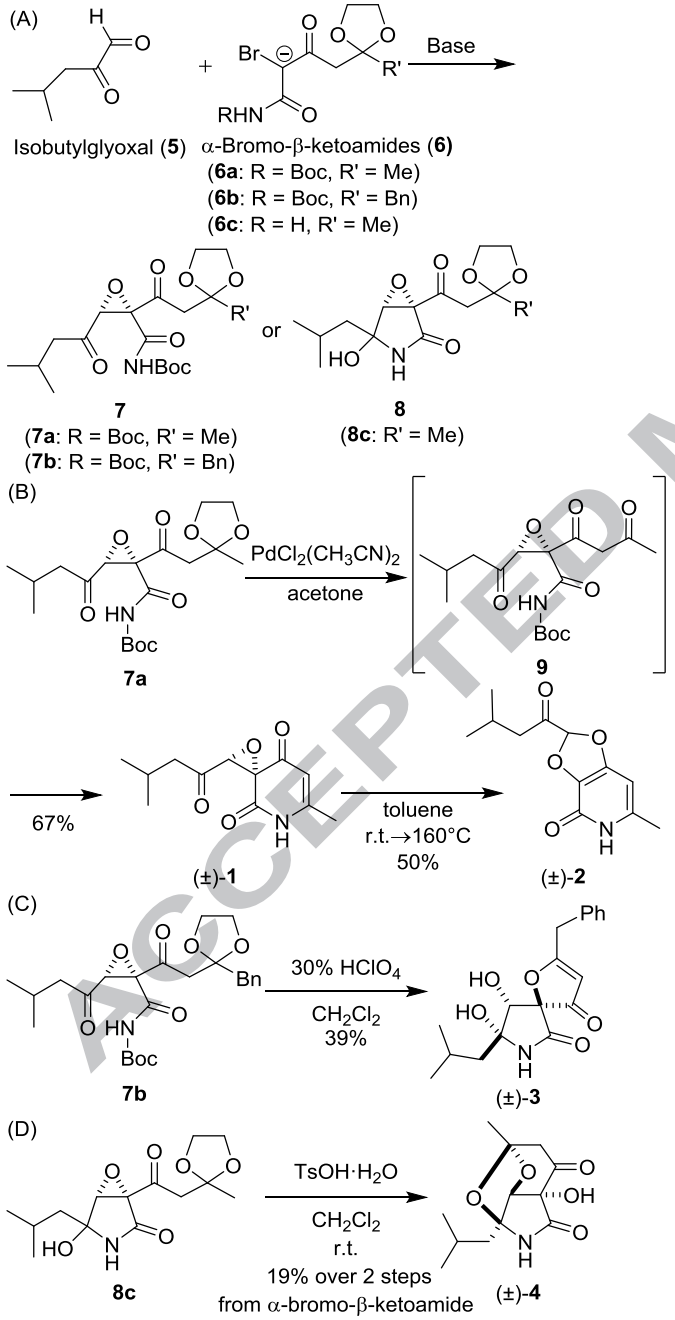
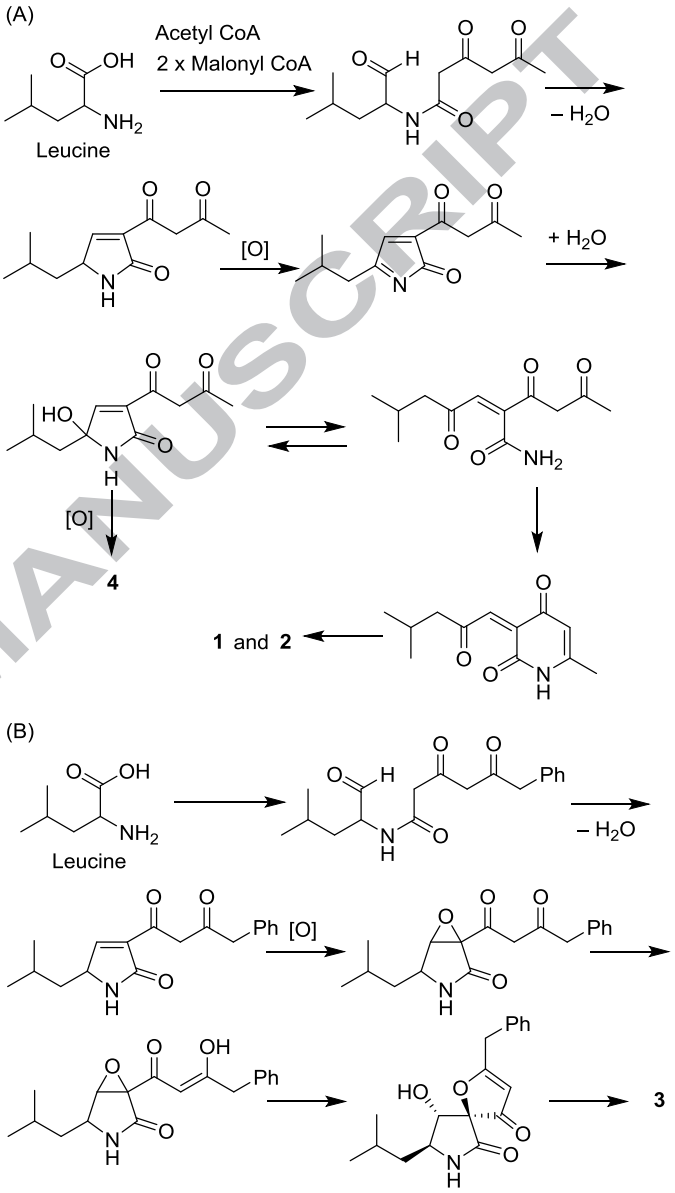
used as substrates for the Darzens reaction, epoxyamides **7** were obtained (Scheme 1A). When non-protected α-bromo-β-ketoamides were used as substrates, epoxylactams **8** were obtained. Compounds **7a**, **7b**, and **8c** were prepared using this key reaction and used for the syntheses of **1**−**4**. (±)-Flavipucine (**1**) was prepared by treatment of **7a** with a catalytic amount of $PdCl_2(CH_3CN)_2$ in acetone (Scheme 1B).[15] Formation of (±)-**1** involves removal of the ketal in **7a**, removal of the Boc group in the resultant **9**, and formation of the pyridione ring. (±)-Isoflavipucine (**2**) was synthesized via thermal isomerization of (±)-**1**.[8-11,15] (±)-Berkeleyamide D (**3**) was synthesized by removal of the protective groups in **7b** with aqueous $HClO_4$ solution in dichloromethane (Scheme 1C).[16] (±)-Rubrobramide (**4**) was prepared by treatment of **8c** with *p*-toluenesulfonic acid monohydrate ($TsOH·H_2O$).[15]



Scheme 1. Key Darzens reactions between isobutylglyoxal and α-bromo-β-ketoamides (A), synthesis of (±)-**1** and (±)-**2** from **7a** (B), synthesis of (±)-**3** from **7b** (C), and synthesis of (±)-**4** from **8c** (D).

A common hybrid polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) system starting from leucine has been proposed for the biosyntheses of **1**, **2**, and **4** (Scheme 2A).[17,18] According to the proposed biosynthesis of a structurally related natural azaspirene,[19] a similar PKS–NRPS system can be speculated to be involved in the biosynthesis of **3** (Scheme 2B).[20]



Scheme 2. Proposed biosyntheses of **1**, **2**, and **4** (A), and **3** (B).

Inspired by the interesting biosynthesis of these natural lactams, we designed three analogues **10**−**12** (Figure 2). Both phenylflavipucine (**10**) and phenylisoflavipucine (**11**) have a benzyl group at the C-6 position. Methylberkeleyamide D (**12**) has a methyl group at the C-2 position instead of the benzyl group in berkeleyamide D (**3**). Due to the similarities in the structures and biosynthetic pathways, we speculate that compounds **10**−**12** might be isolable from natural sources in the future. Indeed, talaramide A (**13**), which possesses a benzyl group in place of the methyl group in rubrobramide (**4**), was recently isolated from the mangrove endophytic fungus *Talaromyces sp*.[21]

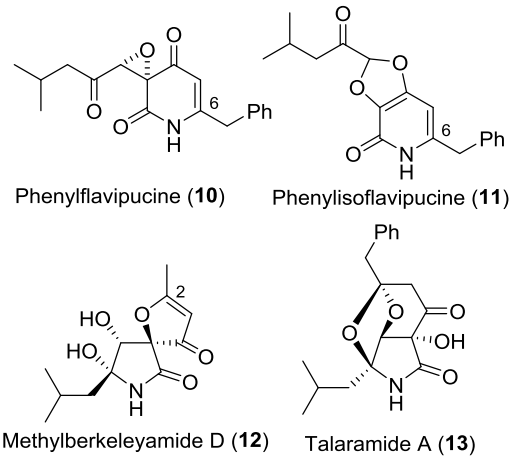
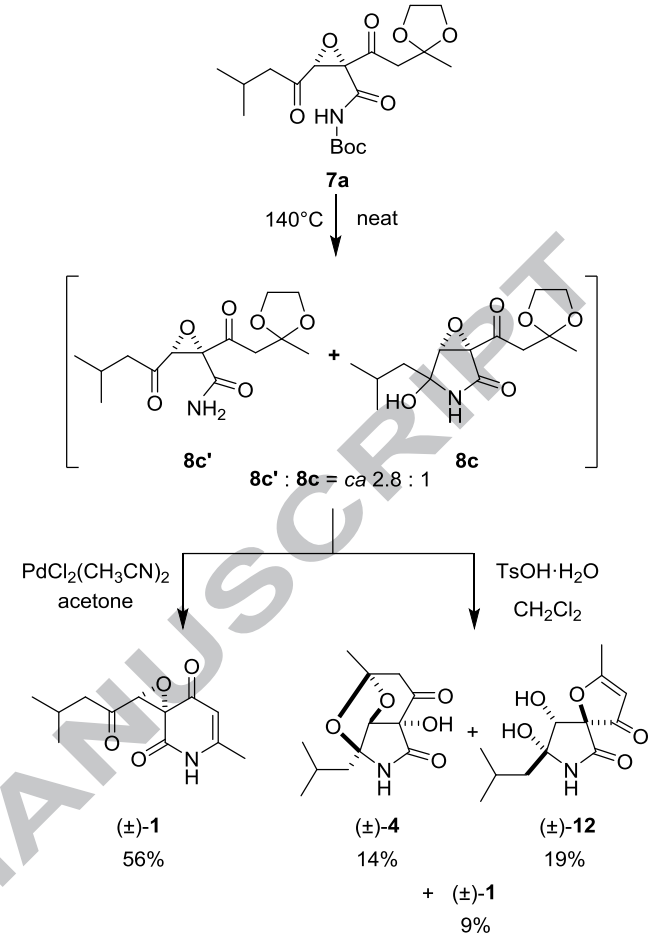


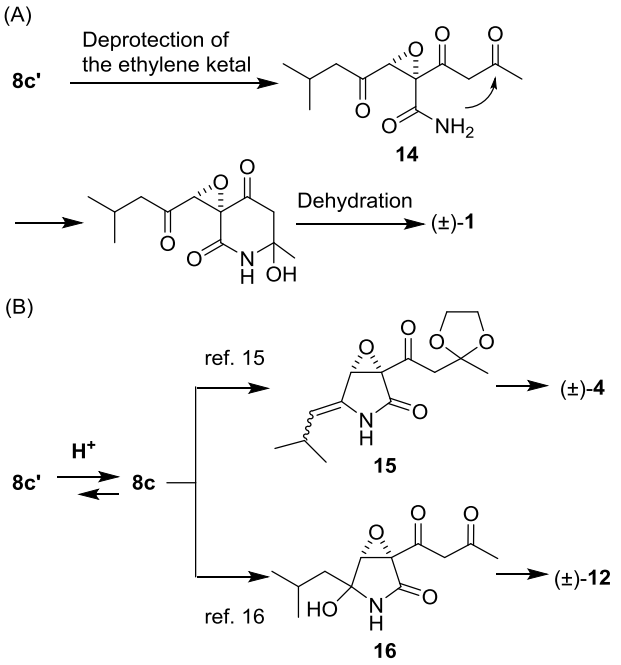
Figure 2. Structures of phenylflavipucine (**10**), phenylisoflavipucine (**11**), methylberkeleyamide D (**12**), and talaramide A (**13**).

In this study, alternative syntheses of (±)-**1** and (±)-**4**, starting from epoxyamide **7a**, and syntheses of new analogues (±)-**11** and (±)-**12** were accomplished. Optical resolution of racemic flavipucine (**1**) was also achieved via chiral HPLC. The antibacterial and cytotoxic activity of racemic **1**−**4**, **10**−**12**, and both enantiomers of **1** was evaluated. The structure-activity relationships of flavipucine and its related lactams were also investigated.

(±)-Flavipucine (**1**) and (±)-rubrobramide (**4**) were synthesized from epoxyamide **7a**[15] via alternative synthetic routes (Scheme 3). Heating **7a** at 140 °C gave an unstable and inseparable mixture of epoxyamide **8c′** and epoxylactam **8c**. The ratio of **8c′** to **8c** was determined to be approximately 2.8 : 1 by the ^{1}H NMR spectrum.[15] Without purification, the mixture was treated with a catalytic amount of $PdCl_2(CH_3CN)_2$ in acetone to yield (±)-**1** in 56% yield over two steps from **7a**. The formation of (±)-**1** is explained by deprotection of the ethylene ketal in **8c′** and formation of the pyridione ring (Scheme 4A). On the other hand, when the mixture of **8c′** and **8c** was treated with a catalytic amount of TsOH·H_2O, (±)-**4**, (±)-**12**, and (±)-**1** were obtained in 14%, 19%, and 9% yields, respectively, over two steps from **7a**. Under acidic conditions, the tautomeric equilibrium between **8c** and **8c′** might shift toward **8c** (Scheme 4B). Dehydration of **8c**, deprotection of the ethylene ketal in **15**, and formation of the tricyclic ring affords (±)-**4**.[15] Deprotection of the ethylene ketal in **8c**, followed by intramolecular spirocyclization of **16** affords (±)-**12**.[16]

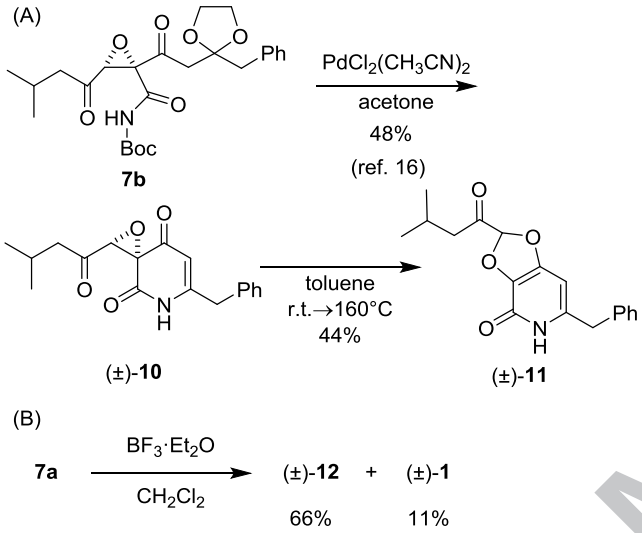


Scheme 3. Alternative syntheses of (±)-**1** and (±)-**4**



Scheme 4. Proposed mechanisms for formation of (±)-**1** (A) and (±)-**4** and (±)-**12** (B)

Following our procedure,[16] (±)-phenylflavipucine (**10**) was prepared from **7b**. Thermal isomerization of **10** in toluene at 160°C in a sealed tube gave (±)-**11** in 44% yield (Scheme 5A). Treatment of **7a** with an excess amount of $BF_3{\cdot}Et_2O$ in dichloromethane gave (±)-**12** in 66% yield, accompanying (±)-**1** in 11% yield (Scheme 5B). Compared to Scheme 3, the use of $BF_3{\cdot}Et_2O$ greatly improves the yield. Deprotection of the Boc group by $BF_3{\cdot}Et_2O$ occurs to furnish **8c**. Then, deprotection of the ketal group in **8c** and subsequent intramolecular spirocyclization produces (±)-**12**. The relative configuration of (±)-**12** was determined by NOESY correlations between H-9 and the methylene protons of the isobutyl group at C-8 in (±)-**12** (Figure 3).



Scheme 5. Syntheses of (±)-**11** (A) and (±)-**12** (B)

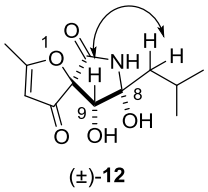


Figure 3. Selected NOESY correlations in (±)-**12**. Selected carbon atoms have been labeled using the IUPAC numbering system.

Because both enantiomers of flavipucine were isolated from microorganisms, optical resolution of (±)-**1** via high performance liquid chromatography (HPLC) using a chiral stationary phase (CSP) was examined. Optical resolution of synthetic (±)-**1** using a CHIRALPAK IA column gave both enantiomers of **1** (>99% ee, Figure S1 in the Supplementary Material). The specific rotation of (−)-**1** [$[\alpha]^{22}_D = -81.5$ (*c* 0.10, EtOH)] agrees with the literature for natural (−)-**1** [$[\alpha]^{21}_D = -71.8$ (*c* 1%, EtOH),[1] and $[\alpha]^{21}_D = -88$ (*c* 1%, EtOH)[2]]. However, the specific rotation of (+)-**1** [$[\alpha]^{23}_D = +72.5$ (*c* 0.10, EtOH)] was smaller than that reported for natural (+)-**1** [$[\alpha]_D = +132$ (EtOH)].[5]

The antibacterial activity of racemic **1**−**4**, **10**−**12**, and both enantiomers of **1** against Gram-positive *Bacillus subtilis* (*B. subtilis*) and Gram-negative *Escherichia coli* (*E. coli*) were evaluated via broth microdilution assay. The antibacterial activity was measured following the standard methods of the Clinical and Laboratory Standards Institute (CLSI).[22] The minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of these compounds are summarized in Table 1 (see also Figure S9 in the Supplementary Material). Ampicillin, a bactericidal agent,[23-25] and chloramphenicol, a bacteriostatic agent,[26] were used as positive controls for the antibacterial assay. Flavipucine (**1**) showed antibacterial activity against *B. subtilis* with both a MIC and a MBC of 8 μg/mL. Because antibacterial agents are generally regarded as bactericidal if the MBC is no more than four times the MIC,[22] the activity is bactericidal rather than bacteriostatic. Additionally, no difference in the antibacterial activity was observed between (±)-**1**, (−)-**1**, and (+)-**1**. Phenylflavipucine [(±)-**10**] exhibited weak bactericidal activity against *B. subtilis*, with a MIC and MBC of 128 μg/mL. In contrast, compounds **2**−**4**, and **11**−**12** did not show antibacterial activity against *B. subtilis*. These results clearly indicate that the pyridione epoxide moiety is important for the antibacterial activity. The substituent at the C-6 position of the pyridione ring also influences the activity (Figure 4A). None of the compounds tested in this study showed antibacterial activity against *E. coli* at concentrations below 128 μg/mL. Gram-negative bacteria have an outer cell membrane which is a lipid bilayer composed of phospholipids, lipopolysaccharides, and lipid-anchored lipoproteins. Thus, since hydrophilic compounds do not easily pass through the hydrophobic membrane through passive diffusion, it is expected that flavipucine (Clog P = 0.47) would not pass through the outer membrane of *E. coli* sufficiently to inhibit its growth.

Table 1.
Antibacterial activity of synthetic compounds against *B. subtilis* and *E. coli*.[a]

Compound	*B. subtilis*		*E. coli*	
	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)
(±)-**1**	8	8	>128	NT
(−)-**1**	8	8	NT	NT
(+)-**1**	8	8	NT	NT
(±)-**2**	>128	>128	>128	NT
(±)-**3**	>128	>128	>128	NT
(±)-**4**	>128	>128	>128	NT
(±)-**10**	128	128	>128	NT
(±)-**11**	>128	>128	>128	NT
(±)-**12**	>128	>128	>128	NT
Ampicillin[b]	<0.5	<0.5	4	NT
Chloramphenicol[b]	4	64	NT[c]	NT[c]

[a]MIC and MBC were determined according to the methods outlined by the CLSI.[22] [b]Ampicillin and chloramphenicol were used as positive controls. [c]NT means "not tested."

Table 2.
Cytotoxicity of synthetic compounds against cancer and normal cells (IC_{50}, μM)[a]

Compound	IC_{50} (μM)							
	A549	HCT116	Hela	TE8	HL-60	Jurkat	HEK293	MRC-5
(±)-**1**	32.1 ± 2.7	30.4 ± 2.0	40.5 ± 3.2	21.6 ± 2.3	1.8 ± 0.2	8.0 ± 0.2	35.9 ± 0.7	33.2 ± 2.6
(−)-**1**	NT	NT	NT	NT	1.6 ± 0.1	NT	NT	NT
(+)-**1**	NT	NT	NT	NT	1.7 ± 0.0	NT	NT	NT
(±)-**2**	> 100	> 100	> 100	> 100	19.9 ± 1.4	> 50	> 50	> 50
(±)-**3**	> 100	> 100	> 100	> 100	28.0 ± 0.6	> 50	> 50	> 50
(±)-**4**	> 100	> 100	> 100	> 100	37.7 ± 4.6	> 50	> 50	> 50
(±)-**10**	> 100	> 100	> 100	> 100	37.3 ± 4.0	> 50	> 50	> 50
(±)-**11**	> 100	> 100	> 100	> 100	> 50	> 50	> 50	> 50
(±)-**12**	> 100	> 100	> 100	> 100	> 50	> 50	> 50	> 50
SN-38[b]	6.6 ± 3.7	< 1	13.1 ± 3.7	< 1	2.4 ± 0.1	< 1	< 1	15.3 ± 5.1

[a]Fifty percent inhibitory concentrations (IC_{50}) of cell viability after treatment with various concentration of each compound for 48 h were determined using the WST-8 assay. IC_{50} values are expressed as the mean ± SD of triplicate experiments. NT means "not tested." [b]SN-38 was used as a positive control.

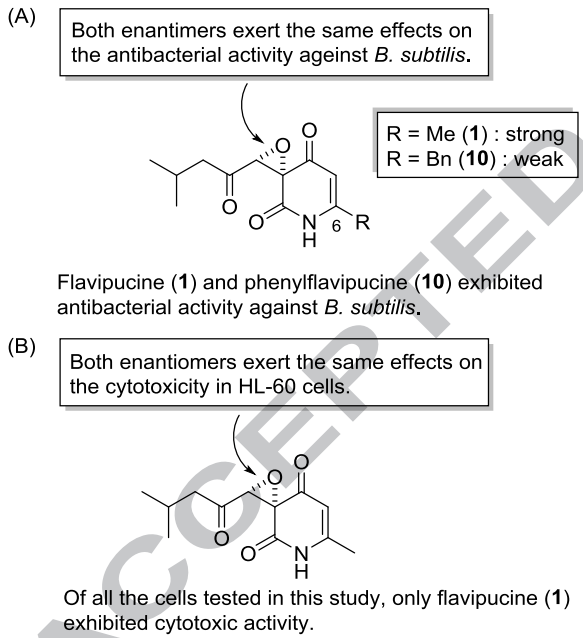


Figure 4. Structure- activity relationship of flavipucine against *B. subtilis* (A) and cytotoxic activity in human cancer cells (B)

The cytotoxic activity against six cancer cells (A549, HCT116, Hela, TE8, HL-60, and Jurkat cells), non-neoplastic cells (HEK293 cells) and normal cells (MRC-5 cells) was evaluated via the WST-8 method after treatment with test compounds for 48 h (Table 2, Figure S10 in the Supplementary Material).[27] SN-38, the active metabolite of irinotecan, was used as a positive control in this study.[28] A549 (human lung carcinoma), HCT116 (human colon carcinoma), Hela (human cervix epitheloid carcinoma), TE8 (human esophageal squamous cell carcinoma), HEK293 {human embryonic kidney cells transformed with sheared human adenovirus (Ad)5 DNA}, and MRC (human normal diploid fibroblast) cells are all adherent cells. HL-60 (human promyelocytic leukemia), and Jurkat (human T-cell acute lymphoblastic leukemia) cells are suspension cells. Interestingly, (±)-**1** inhibited proliferation moderately in A549, HCT116, Hela, and TE8 cells. The other compounds did not influence proliferation of adherent cancer cells at concentrations under 100 μM. Compound (±)-**1** has strong cytotoxic activity against HL-60 cells. No difference between (−)-**1** and (+)-**1** in the cytotoxicity against HL-60 cells was observed (Figure 4B). Although compound (±)-**1** also exhibited cytotoxicity against non-neoplastic HEK293 cells and normal MRC-5 cells, the cytotoxicity of (±)-**1** is weaker than that of SN-38. Compounds (±)-**2**, (±)-**3**, (±)-**4**, and (±)-**10** possess moderate cytotoxic activity against HL-60. DNA fragmentation was not observed in the HL-60 cells after treatment with (±)-**1** for 6 h (Figure S11 in the Supplementary Material). The cell death induced by (±)-**1** was not suppressed by pretreatment with Z-Asp-CH_2-DCB, an inhibitor of caspases (Figure S12 in the Supplementary Material). These results suggest the possibility that the cell death caused by (±)-**1** is mediated by non-apoptotic death mechanisms.

In this study, the syntheses and biological activities of flavipucine and its derivatives were reported. The target derivatives were designed based on the biosynthesis of flavipucine and its related natural products. The compounds were prepared via a Darzens reaction between isobutylglyoxal and α-bromo-β-ketoamides. Of the compounds tested, flavipucine (**1**) was the most potent bactericide against *B. subtilis* and showed the greatest cytotoxic activity against several human cancer cells. No significant differences in the activity of the enantiomers of flavipucine were observed. On the other hand, significant differences in the biological activity of flavipucine and its derivatives were observed. Phenylflavipucine (**10**) demonstrated weak bactericidal activity against *B. subtilis*. The other derivatives had no antimicrobial activity. Racemic isoflavipucine (**2**), berkeleyamide D (**3**), and rubrobramide (**4**) exhibited weak cytotoxic activity against HL-60 cells. The present findings may provide valuable information for the design and development of novel lactam drugs. Further investigations of the mechanism of

action of flavipucine are currently underway and will be reported in due course.

Acknowledgments

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Conflicts of interest

The authors report no conflicts of interest associated with this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at

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Graphical Abstract

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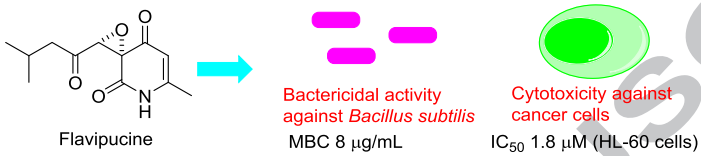

Highlights

· Biological activity of flavipucine and its derivatives was evaluated.

· Flavipucine displayed bactericidal activity against *Bacillus subtilis*.

· Flavipucine exhibited cytotoxic activity against several cancer cells.

· Flavipucine showed weak cytotoxic activity against non-neoplastic and normal cells.

· No differences in biological activity between enantiomers of flavipucine were observed.