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Effect of the 4′-substituted phenylalanine moiety of sansalvamide A peptide on antitumor activity†

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Eight sansalvamide A peptide analogues with 4′-fluoride, 4′-chloride, 4′-bromide, 4′-iodide, and 4′-methoxyphenylalanine moieties were synthesized. The effect of these *para*-substitutions of sansalvamide A peptide on their cytotoxicity was evaluated using HCT-116, MDA-MB-231, HT-29, HCT-15, K562, HeLa, and A549 cell lines. The 4′-methoxyphenylalanine analog of sansalvamide A peptide was found to be a promising antitumor agent.

Introduction

Sansalvamide A (**1**) is a cyclic depsipeptide isolated from a marine fungus of the genus *Fusarium* (*Fusarium* sp.).[1] Since its initial discovery, sansalvamide A has received much attention due to its significant cytotoxicity toward cancer cell lines. Some preliminary mode-of-action studies showed that the cytotoxicity of sansalvamide A could be partially attributed to its inhibition of topoisomerase I. However, the exact mode-of-action remains unknown (Fig. 1).

Despite some early interest, pharmaceutical development of sansalvamide A was hampered by its susceptibility to enzymatic hydrolysis of its macrocyclic lactone and subsequent loss of activity.[2] Modification of the depsipeptide backbone to cyclic pentapeptides provided analogs with improved hydrolytic stability and sometimes improved pharmacological properties.[3–6] For example, the Silverman group synthesized sansalvamide A peptide (**2**),[7–9] and found that it is 10 times (0.98 μg mL^{-1}) more potent than the natural depsipeptide (9.8 μg mL^{-1}) against HCT-116. Twelve sansalvamide A analogs were also synthesized through introducing *N*-methylated amino acids and/or replacing phenylalanine with brominated phenylalanine.[10] Some of them exhibited greater potency than the parent peptide when screened for growth inhibition of PC-3 human prostate cancer, MDA-MB231 human breast cancer, WM-115 human melanoma cells, and S2-013 and AsPC-1 human pancreatic cancer cells. McAlpine's group reported 89 sansalvamide A derivatives that contained five modified amino acids, including the L- and D-configuration of the amino acids, and all these compounds were screened for cytotoxicity toward numerous cloned cancer cell lines.[11–18] Two consecutive D-amino acids combined with an appropriately placed *N*-methyl moiety are found to be the optimal structural motif for potency.[15–18]

Whereas *para*-bromination of phenylalanine enhances the antitumor activity of the analogs, the effects of other substitutions on phenylalanine remain unknown. Herein we describe the synthesis of eight sansalvamide A analogs (Fig. 2) with F, Cl, Br, I, OH and OMe substituted phenylalanine (Scheme 1). Their effects on the growth and apoptosis of six human pancreatic cancer cell lines were also studied.

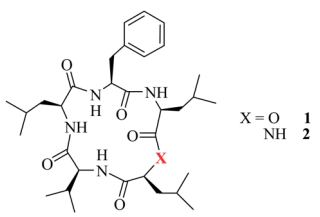
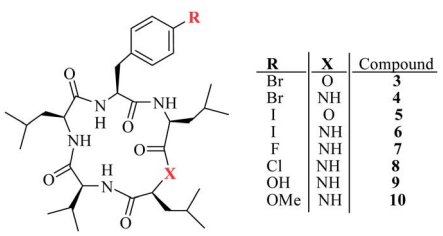


Fig. 1 Sansalvamide A (**1**) and sansalvamide A peptide (**2**).



R	X	Compound
Br	O	**3**
Br	NH	**4**
I	O	**5**
I	NH	**6**
F	NH	**7**
Cl	NH	**8**
OH	NH	**9**
OMe	NH	**10**

Fig. 2 Synthesis of sansalvamide A analogues.

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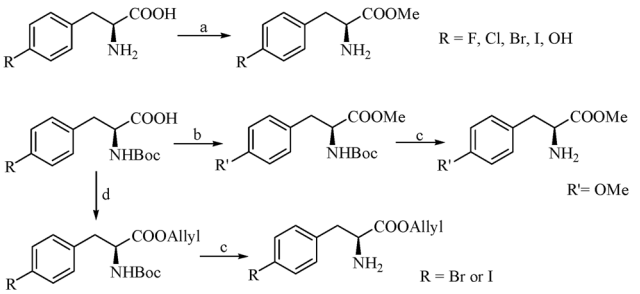


Scheme 1 Synthesis of a series of substituted phenylpropanoate derivatives. *Reagents and conditions*: (a) $SOCl_2$, MeOH; (b) MeI/K_2CO_3, acetone; (c) TFA, DCM; and (d) allyl bromide, K_2CO_3.

Results and discussion

Chemistry

The designed sansalvamide A analogues consist of five L-configuration of amino acids or (*S*)-2-hydroxy-4-methylpentanoic acid (Fig. 3). These compounds all contain a peptide bond between L-leucine and substituted L-phenylalanine, so this position was selected for macrocyclization. The target molecules would be assembled by solution phase 3 + 2 coupling of fragments **I** and **II**. A common protection strategy for peptide synthesis was used in synthesis of these compounds.[19]

Methylation or allylation of the substituted phenylalanine was carried out according to that described in Scheme 1.[20,21] The synthesis of methyl L-2-amino-3-(4-methoxyphenyl)propanoate commenced from methylation of *N*-Boc tyrosine with iodomethane in acetone in the presence of K_2CO_3 according to Reddy's method[22] to give the corresponding *N*-Boc-4-methoxylphenylalanine methyl ester in 96% yield. Subsequent treatment with TFA in DCM gave methyl 4-methoxylphenylalanine methyl ester in 93% yield. 4-Bromo- and 4-iodophenylalanine allyl esters were also prepared from the corresponding substituted *N*-Boc-phenylalanine with allyl bromide in the presence of K_2CO_3 following the Shioiri's procedure,[21] then followed by treatment with TFA in DCM[7] to give the substituted phenylalanine derivatives in 92% and 90% yields, respectively, over two steps.

As described in Scheme 2, we completed the synthesis of compound **I** through the coupling of 4-substituted phenylalanine methyl ester or allyl ester with *N*-Boc-L-leucine with HBTU, DIPEA in DCM/DMF to give dipeptides **12** and **13**. Coupling of L-valine methyl ester with *N*-Boc-L-leucine under the same conditions gave compound **14**, which was deprotected with 20–40% TFA in DCM and further coupled with *N*-Boc-L-leucine again to afford tripeptide **15**. Compound **17** was synthesized using L-valine allyl ester as a starting material, which condensed with (*S*)-2-hydroxy-4-methylpentanoic acid under the same conditions as those used in preparation of dipeptide **12** to give dipeptide **16**. Subsequent condensation of **16** with *N*-Boc-L-leucine in the presence of EDC·HCl, DMAP in DCM gave **17**.[21] The removal of the Boc protection of **12** and **13** with TFA gave fragment **I** (R_1 = H, R_2 = allyl or methyl). It was coupled with fragment **II** (R_3 = Boc, R_4 = H), prepared by saponification of **15** with LiOH in aqueous THF or from **17** through treatment with $(PPh_3)_4Pd$ in the presence of morpholine,[23] using HBTU, DIPEA to give the corresponding peptides **18** and **19**, respectively. Saponification of **18** with aqueous LiOH and hydrolysis of Boc with TFA, or removal of the allyl group of **19** with $(PPh_3)_4Pd$ and Boc with TFA, followed by macrocyclization of the linear pentapeptide with HATU, PyBOP, and DIPEA in DCM–THF–DMF gave the target compounds.

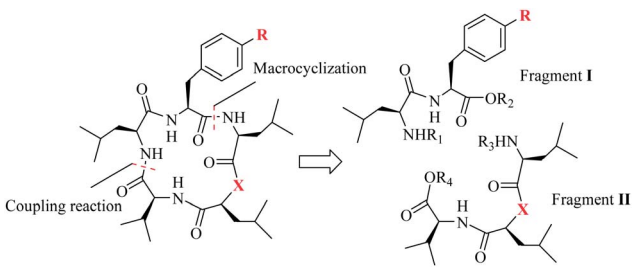
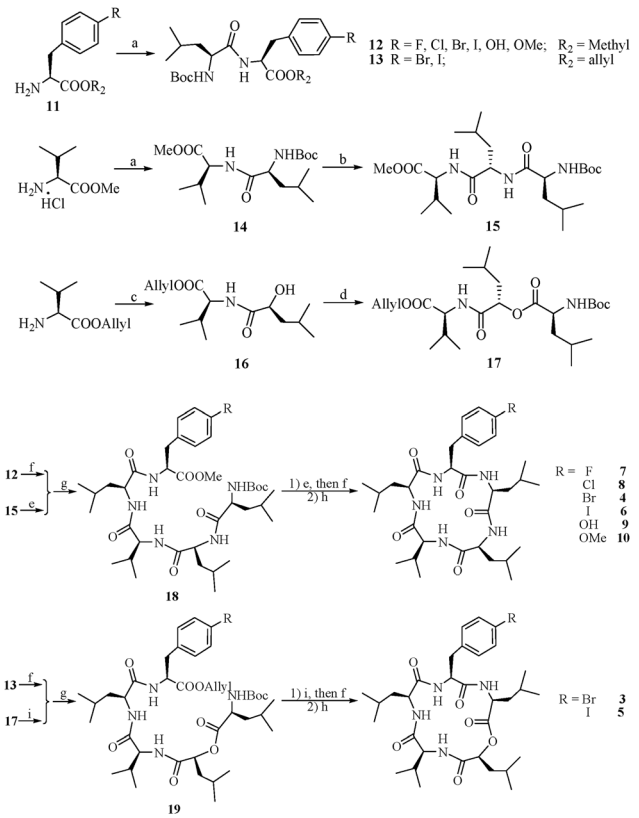


Fig. 3 Retrosynthetic analysis of sansalvamide A analogues.



Scheme 2 Synthesis of sansalvamide A analogues **3–10**. *Reagents and conditions*: (a) Boc-L-Leu-OH, HBTU, DIPEA, DCM, DMF, rt, 12–24 h; (b) TFA, DCM, rt, 1–2 h, then (a); (c) (*S*)-2-hydroxy-4-methylpentanoic acid, HBTU, DIPEA, DCM, DMF, rt, 12–24 h; (d) Boc-L-Leu-OH, EDC·HCl, DMAP, DCM, rt, 12 h; (e) LiOH, THF, rt; (f) TFA, DCM, rt; (g) HBTU, DIPEA, DCM, DMF, rt, 12–24 h; (h) PyBOP, HATU, DIPEA, DCM, THF, DMF, rt, 3–4 days; (i) $(PPh_3)_4Pd$, 4-methylmorpholine, rt.

Biological evaluation

The structure–activity relationship (SAR) of these compounds was established using sansalvamide A peptide (**2**) as a control.

[^{3}H]-Thymidine uptake assays on human colon carcinoma HCT-116 revealed **3**, **4**, **5**, and **6** to be potently cytotoxic and their IC_{50} values were determined to be 4.5 μg mL^{-1}, 0.58 μg mL^{-1}, 11.3 μg mL^{-1}, and 1.12 μg mL^{-1} respectively (Table 1). A 50% increase of potency was observed for **4** compared with **2** (IC_{50} 0.98 μg mL^{-1}),[7] demonstrating the beneficial effect of halogen *para*-substitution of the phenyl group of phenylalanine. Interestingly, *para*-iodination of the phenyl group in sansalvamide A peptide gives an analog (**6**) that is 10 times more potent than *para*-iodinated sansalvamide A depsipeptide (**5**) against HCT-116 cell lines.

To further evaluate the effect of halogen-substitution of sansalvamide A on its antitumor activities, a set of *para*-halogen-substituted analogues with fluorine, chlorine, hydroxyl, and methoxyl substitutions of the phenylalanine moiety (*i.e.* **2**, **4**, **7**, **8**, **9**, and **10**) were synthesized and their antitumor activities toward six different cancer cell lines, MDA-MB-231, HeLa, HT-29, HCT-15, K562, and A549, were determined (Fig. 4–6).

Compounds **7** and **8** were found to be not very potent against the cancer cells with **8** showing the lowest activity while **7** comparable to that of **2**. Compounds **4**, **9**, and **10** all showed activities higher than that of compound **2** at 20 μg mL^{-1} concentration. Specifically, compound **4** showed considerably improved activities against MDA-MB-231, HT-29, and HCT-15 cells relative to sansalvamide A peptide (**2**) (Fig. 4). While compound **9** is more potent than **2**.

Comparing the sansalvamide A analogues, it is clear that the 4-methoxyl analogue (**10**) is the most potential for growth inhibition of MDA-MB-231, HT-29, HCT-15, K562, and A549 cancer cells at 20 μg mL^{-1} concentration (Fig. 4 and 5).

Table 1 *In vitro* cytotoxicity data for sansalvamide A analogues toward HCT-116 cell lines

Compound	**1** (ref. 1)	**2**	**3**	**4**	**5**	**6**
IC_{50} (μg mL^{-1})	9.8	0.98	4.5	0.58	11.3	1.12

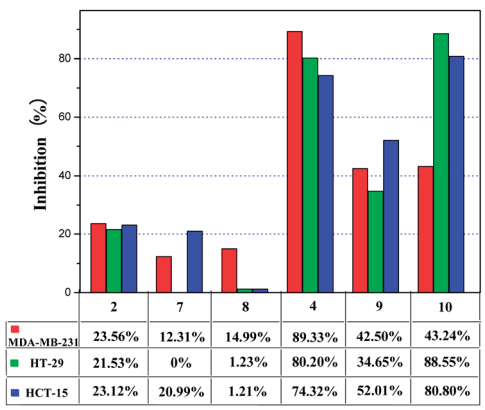


Fig. 4 Effect of selective sansalvamide A analogues on MDA-MB-231, HT-29, and HCT-15 cells. Data are represented as % growth inhibition relative to a 1% DMSO control. Each data point is an average of four wells run in three assays. All assays were run for 72 h at 20 μg mL^{-1} concentration. Error = ±5%.

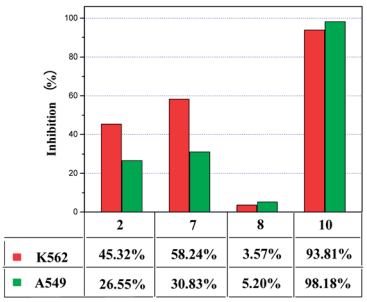


Fig. 5 Effect of selective compounds on K562 and A549 cells. Data are represented as % growth inhibition relative to a 1% DMSO control. Each data point is an average of four wells run in three assays. All assays were run for 72 h at 20 μg mL^{-1} concentration. Error = ±5%.

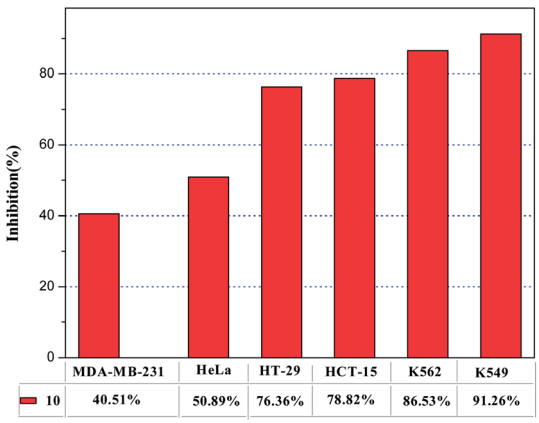


Fig. 6 Effect of compound **10** on the viability of MDA-MB-231, HeLa, HT-29, HCT-15, K562, and A549 cells. Data are represented as % growth inhibition relative to a 1% DMSO control. Each data point is an average of four wells run in three assays. All assays were run for 72 h at 2 μg mL^{-1} concentration. Error = ± 5%.

Compound **10** was also showed more potential activities through the concentration-dependent inhibition of MDA-MB-231, HeLa, HT-29, HCT-15, K562, and A549 cell growth, and the values of IC_{50} were determined (Table 2). These data demonstrate that *para*-bromination and methoxylation of the phenylalanine moiety of sansalvamide A provide a convenient means to improve its potency against MDA-MB-231, HT-29, HCT-15, K562, and A549 cell lines.

Morphological changes produced by compound **10** (24 h at 1.8 μg mL^{-1}) over HeLa and vascular smooth muscle cells (VSMC) were observed using light microscopy. As shown in Fig. 7, when HeLa cells were treated with **10**, the cells became rounded, smaller, and exhibited membrane blebbing and nuclear fragmentation. The cell density also decreased. These changes suggest an apoptosis process as the cells no longer progress through the

Table 2 *In vitro* cytotoxicity data for compound **10** toward different cell lines

Cell line	MDA-MB-231	HeLa	HT-29	HCT-15	K562	A549
IC_{50} (μM)	11.08	3.22	3.38	3.54	2.87	2.41

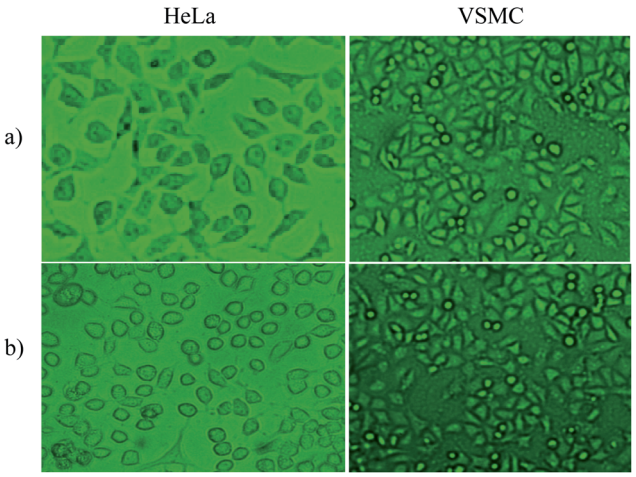


Fig. 7 Morphological changes of HeLa cells and vascular smooth muscle cells (VSMC) by compound **10**. All assays were run for 24 h with or without compound **10** at 1.8 μg mL^{-1} concentration. Error = ±5%. DMSO was used as a control. Left panels are HeLa cells, and right are VSMC. (a) No treatment control. (b) Treatment with compound **10**.

cell cycle or synthesize DNA. But **10** did not cause similar changes in VSMC under the same conditions. These results suggest that **10** is a potent and selective inhibitor of cancer cells.

Conclusions

In summary, sansalvamide A analog **10** was synthesized and was found to be potently cytotoxic toward MDA-MB-231, HeLa, HT-29, HCT-15, K562, and A549 cancer cell lines. The SAR revealed through our study is expected to be valuable in facilitating the rational design of new sansalvamide A analogs with improved pharmacological properties. Further investigation will be important to define the role of 4-methoxyl phenylalanine in sansalvamide A peptide. Additional assays of this compound on other cancer cell lines are underway, and the synthesis of the next generation of sansalvamide A analogues is also in progress. These results will be reported in due course.

Abbreviations

SAR	Structure–activity-relationship
DIPEA	*N*,*N*-Diisopropylethylamine
THF	Tetrahydrofuran
DCM	Dichloromethane
DMF	*N*,*N*-Dimethylformamide
DMAP	4-Dimethylamiopryidine
Boc	*tert*-Butyloxycarbonyl
HBTU	2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
EDC·HCl	1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide
DMSO	Dimethyl sulphoxide

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