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An efficient and cost-effective approach to kahalalide F *N*-terminal modifications using a nuisance algal bloom of *Bryopsis pennata*

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ABSTRACT

Background: Kahalalide F (KF) and its isomer iso-kahalalide F (isoKF), both of which can be isolated from the mollusk *Elysia rufescens* and its diet alga *Bryopsis pennata*, are potent cytotoxic agents that have advanced through five clinical trials. Due to a short half-life, narrow spectrum of activity, and a modest response in patients, further efforts to modify the molecule are required to address its limitations. In addition, due to the high cost in producing KF analogues using solid phase peptide synthesis (SPPS), a degradation and reconstruction approach was employed using natural KF from a seasonal algal bloom to generate KF analogues.
Methods: *N*-protected KF was carefully hydrolyzed at the amide linkage between L-Thr12 and D-Val13 using dilute HCl. The synthesis of the *C*-terminal fragment began with the formation of hexanoic succinimide ester, followed by a reaction with dipeptides. The final coupling reaction was performed between the semisynthesized Fmoc–KF hydrolysis product and the *C*-terminal fragment, followed by the deprotection of the Fmoc group.
Results: Six KF analogues with an addition of an amino acid residue on the *N*-terminal chain, D-Val14–isoKF **(2)**, Val13–Val14–isoKF **(3)**, D-Leu14–isoKF **(4)**, D-Pro14–isoKF **(5)**, D-Phe14–isoKF **(6)**, and 3,4-2F-D-Phe14-isoKF **(7)** were prepared using semisynthesis at the exposed *N*-terminal chain.
Conclusions: The overall yield of the medication was 45%. This approach is economical, efficient and amendable to large-scale production while eliminated a nuisance algal bloom.
General significance: B. pennata blooms are capable of producing KF in good yields. The semisynthesis from the natural product produced *N*-terminal modifications for the construction of inexpensive semisynthetic KF libraries.

1. Introduction

The Sacoglossan sea slug, *Elysia rufescens*, is an herbivorous marine mollusk. These and many other mollusks protect themselves from predatory attacks by accumulating defensive chemicals. In this case *E. rufescens* accumulates kahalalide F (KF) in high quantities which is also found in their dietary algae *Bryopsis* spp. [1] KF occurs naturally as a mixture with its isomer, isoKF, and is a depsipeptide isolated from both *E. rufescens* and *Bryopsis pennata* (Fig. 1) [2,3]. KF and isoKF exhibit significant activity against human prostate, breast, non-small cell lung, and colon solid tumors, in particular prostate and breast cancer cell lines are highly sensitive with IC_{50} values ranging from 0.07 to 0.28 μM [2–5]. The cytotoxicity of KF and isoKF did not correlate with the expression level of MDR1 in prostate and breast cancer, indicating their potential in the treatment of multiple drug resistant cancers [5]. Additionally, in the NCI-60 DTP human tumor cell line screen, the biological response pattern of KF and isoKF was not similar to any of compounds included in the library, suggesting that they have a unique mode of action (MOA) [6]. Although the mechanism of these molecules is not completely elucidated, they were shown to effectively inhibit the receptor ErbB3, thus down-regulating PI3K/Akt signaling pathway [7]. The ErbB family including ErbB1 (EGFR, HER1), ErbB2 (HER2, Neu), ErbB3 (HER3), and ErbB4 (HER4), is part of the epidermal growth factor receptor (EGFR) family which are tyrosine kinase receptors that mediate cell interactions in human cells [8,9]. Inhibitors targeting the ErbB family exert potent anti-tumor activity. Several ErbB1/2 inhibitors, such as gefitinib, erlotinib, lapatinib, and vandetanib have been approved by the FDA as anticancer drugs. An ErbB4 inhibitor, dacomitinib, developed by Pfizer is currently in phase III clinical trials [10]. However, there are no drugs which target ErbB3 over-expressing tumor cells. Therefore KF and analogues derived using the methods reported here could yield promising ErbB3 inhibitors with clinical applications.

KF has completed its safety evaluation in phase I clinical trials in patients with various advanced solid tumors. Unfortunately, the lead

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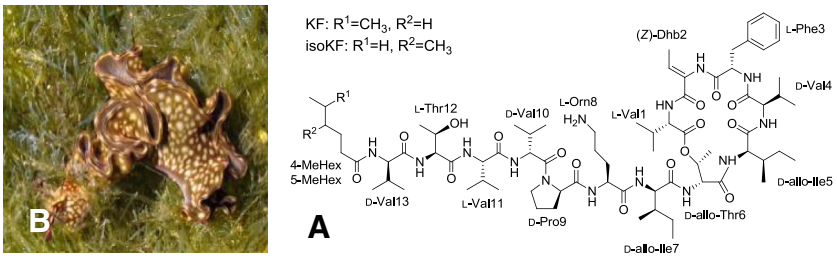
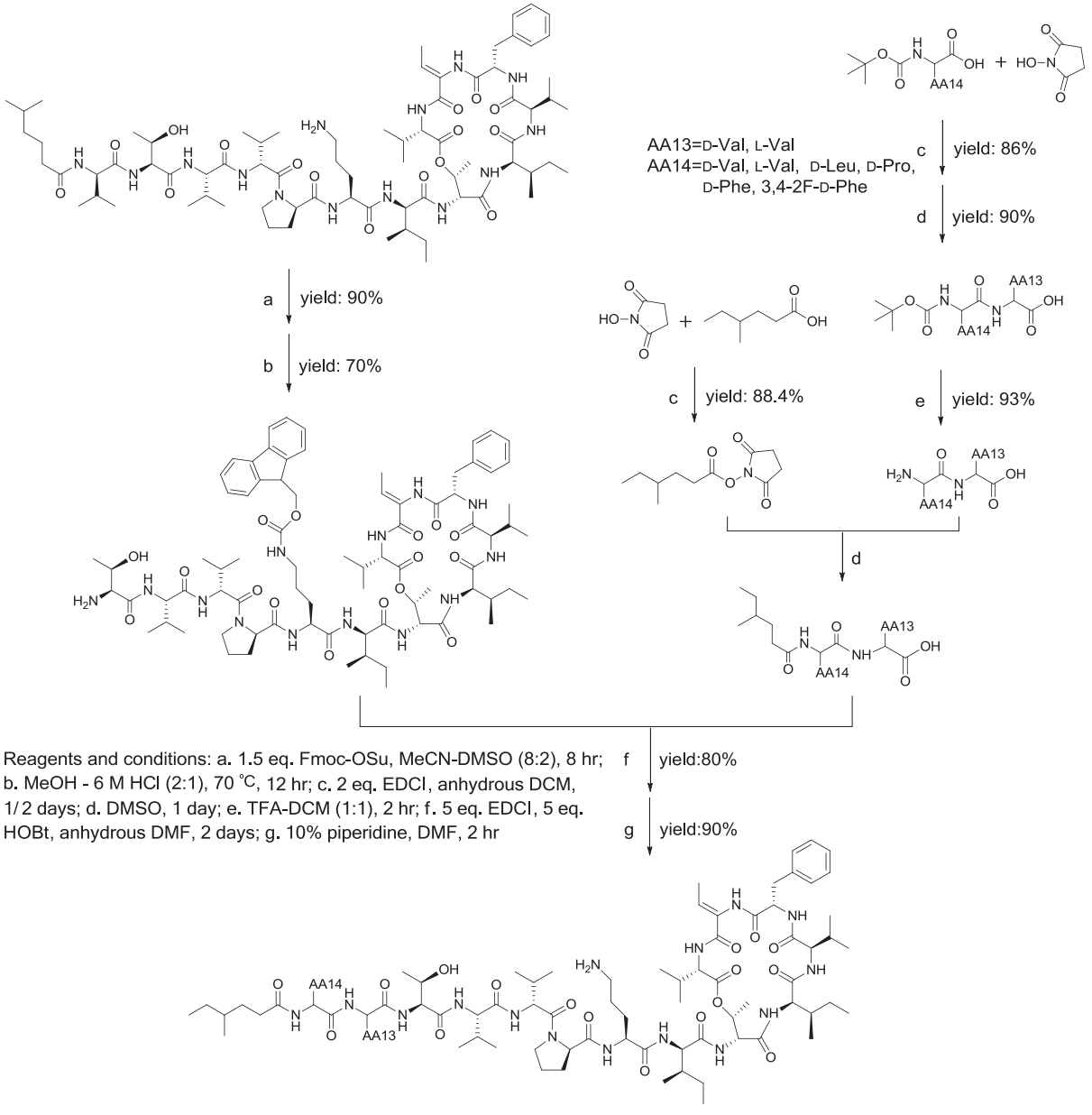


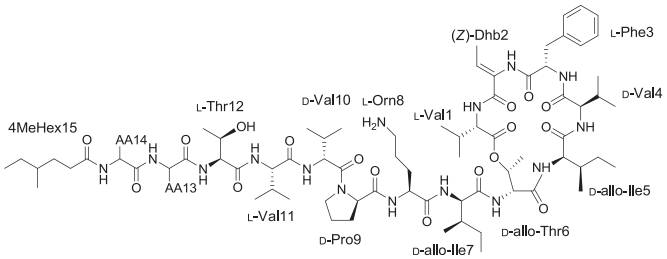
Fig. 1. (A) Structures of KF and isoKF; (B) Sources of KF: *E. rufescens* (Photo taken by Megan Huggett) and *B. pennata*.

was dropped from phase II clinical trials due to a lack of efficacy despite results indicating a limited number of patients achieved a positive response [11]. The pharmacokinetic studies suggested that KF has a short half-life (approximately 30 min) [11], which could in part be the reason for its poor efficacy. However, no further studies have provided evidence of KF degradation products in vivo. Considering the high potential for a cytotoxic drug candidate from this class, several attempts have been made to modify the molecule to produce analogues with a higher potency, better delivery, or longer half-life. The structure-activity relationship (SAR) analysis based on the analogues prepared using solid phase peptide synthesis (SPPS) revealed that the cyclic peptide system (amino acid residues 1–6) and the configurations of amino acid residues 7–13 are crucial to the activity. The substitutions with aliphatic residues, especially the modifications of the *N*-terminal lipid chain, can increase



Scheme 1. Semisynthesis of KF analogues.

Table 1
Structure of KF analogues



		isoKF (1)	D-Val14–isoKF (2)	Val13–Val14–isoKF (3)	D-Leu14–isoKF (4)	D-Pro14–isoKF (5)	D-Phe14–isoKF (6)	3,4-2F-D-Phe14–isoKF (7)
Structure	AA13	D-Val	D-Val	L-Val	D-Val	D-Val	D-Val	D-Val
	AA14	–	D-Val	L-Val	D-Leu	*trans*-D-Pro	D-Phe	3,4-2F-D-Phe

the activity [12]. Although the SPPS technique is well developed, more than 29 steps were involved [12,13]. Challenges such as the peptide cyclization by coupling L-Phe3 with D-Val4 and the formation of *Z*-didehydroaminobutyric acid residue [(*Z*)-Dhb2] decreased the overall yield. Additionally, it is costly to synthesize 1 kg of KF due in part to the high price of Fmoc-D-allo-Ile and Fmoc-D-allo-Thr. Semisynthetic approaches based on natural KF isolated from *B. pennata* blown on shore after an algal bloom could provide a less expensive method to produce KF congeners. Our group has modified natural KF at the Orn-residue which yielded two molecules with a similar or higher potency in selected cancer cell lines [14,15]. In this report we provide an approach to produce modified KF analogues with additional amino acid residues on the *N*-terminal side chain after removal of the terminal fatty acid by selective hydrolysis between threonine and valine.

2. Materials and methods

2.1. General experiment procedures

NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer. The ^{1}H and ^{13}C NMR chemical shifts were reported in ppm. IR spectra were measured on a PerkinElmer Spectrum 100 FT-IR spectrometer. HPLC–ESI-TOF was performed on Bruker MicroTOF spectrometer in line with an Agilent 1100 Series HPLC system and G1316A DAD detector.

Table 2
^{1}H and ^{13}C NMR data of additional amino acid residues of **2–7**[a] (δ in ppm, DMSO-d_6).

Compound	Formula	Amino acid	Position	δ_C, mult	δ_C, mult
2	$C_{80}H_{134}N_{15}O_{17}$	D-Val 14	1	171.7	
			2	59.0, CH	4.30, m
			3	31.4, CH	2.02, m
			4	18.3, CH_3	0.86, m
			5	19.8, CH_3	0.79, m
3	$C_{80}H_{134}N_{15}O_{17}$	Val 14	1	171.6	
			2	59.0, CH	4.28, m
			3	31.5, CH	2.01, m
			4	18.3, CH_3	0.87, m
			5	19.7, CH_3	0.80, m
4	$C_{81}H_{136}N_{15}O_{17}$	D-Leu 14	1	172.9, qC	
			2	51.5, CH	4.36, m
			3	41.3, CH_2	1.41, 1.48, m
			4	25.0, CH	1.58, m
			5	23.2, CH_3	0.86, m
			6	22.2, CH_3	0.83, m
5	$C_{80}H_{131}N_{15}NaO_{17}$	D-Pro 14	1	172.2, qC	
			2	59.7, CH	4.49, m
			3	31.9, CH_2	2.00, 2.26, m
			4	26.5, CH_2	1.36, 1.51, m
			5	46.5, CH_2	3.38, 3.52, m
6	$C_{84}H_{134}N_{15}O_{17}$	D-Phe 14	1	171.4, qC	
			2	54.1, CH	4.60, m
			3	37.5, CH_2	2.75, 3.05, m
			4	138.7, qC	
			5,5′	129.6, CH	7.15, m
			6,6′	128.3, CH	7.17, m
			7	126.5, CH	7.20, m
7	$C_{81}H_{132}F_2N_{15}O_{17}$	3,4-2F-D-Phe 14	1	171.5, qC	
			2	54.1, CH	4.62, m
			3	37.1, CH_2	2.72, 2.98, m
			4	136.2, qC	
			5	117.3, CH	7.26, m
			6	149.5, qC	
			7	148.5, qC	
			8	118.6, CH	7.30, m
			9	126.5, CH	7.07, m

[a] Assignments based on ^{1}H, ^{13}C, and HSQC NMR (^{13}C 100/^{1}H 400 MHz) experiments at room temperature.

Preparative HPLC was carried out on a Waters PrepLC system with a single wavelength detector. Phenomenex Luna C_{18} 5 μm column (250 mm × 20.2 mm) was used. Optical rotations were measured on a JASCO DIP-370 polarimeter.

2.2. Isolation of KF and isoKF mixture from B. pennata

B. pennata (30 kg, wet weight) was collected on the beaches at Black Point, O'ahu, during the algal bloom season. An EtOH extract of *B. pennata* was fractionated on a silica flash column with a gradient solvent system of hexanes–EtOAc (100:0, 50:50, 0:100) and EtOAc–MeOH (50:50, 0:100). Two EtOAc–MeOH fractions were further eluted on an HP-20 column with MeOH–H_2O (50:50, 60:40, 80:20, 90:10, 100:0). The MeOH–H_2O (90:10) and 100% MeOH fractions were subjected to C_{18} glass open-tubular column with an isocratic solvent system of MeOH–H_2O (90:10), followed by preparative HPLC (Phenomenex C_{18} column 250 mm × 21.2 mm) with a gradient elution of MeCN–H_2O (70:30–100:0) at a flow rate of 8 mL/min to afford a KF and isoKF mixture (0.95 g, 0.0032% yield).

2.3. Semisynthesis of KF analogues from an isomeric mixture

The synthetic procedures are displayed in Scheme 1. The experimental details are described in the Supporting Information.

IsoKF (**1**, 6.2 mg), white amorphous powder. $[\alpha]_D^{25}$ −14 (*c* 0.1, MeOH); UV λ_{max} (MeOH) 205, 224 nm; IR neat (KBr) 3280 (s, br), 2964 (s), 2932 (s), 2877 (s), 1736 (s), 1632 (s), 1514 (s), 1465 (s), 1383 (s), 1342 (s), 1227 (s), 1025 (s) cm^{-1}; HRESIMS *m/z* calc. for $C_{75}H_{125}N_{14}O_{16}$ $[M + H]^+$ 1477.9393, detected 1477.9453.

D-Val14–isoKF (**2**, 6.1 mg), white amorphous powder. $[\alpha]_D^{25}$ −16 (*c* 0.1, MeOH); UV (DAD) λ_{max} (MeOH) 205, 224 nm; IR neat (KBr) 3280 (s, br), 2964 (s), 2932 (s), 2876 (s), 1736 (s), 1633 (s), 1515 (s), 1464 (s), 1383 (s), 1342 (s), 1227 (s), 1025 (s) cm^{-1}; HRESIMS *m/z* calc. for $C_{80}H_{134}N_{15}O_{17}$ $[M + H]^+$ 1577.0077, detected 1577.0082.

Val13–Val14–isoKF (**3**, 6.4 mg), white amorphous powder. $[\alpha]_D^{25}$ −14 (*c* 0.1, MeOH); UV (DAD) λ_{max} (MeOH) 205, 224 nm; IR neat (KBr) 3280 (s, br), 2964 (s), 2932 (s), 2876 (s), 1736 (s), 1633 (s), 1515 (s), 1464 (s), 1383 (s), 1342 (s), 1227 (s), 1025 (s) cm^{-1}; HRESIMS *m/z* calc. for $C_{80}H_{134}N_{15}O_{17}$ $[M + H]^+$ 1577.0077, detected 1577.0137.

D-Leu14–isoKF (**4**, 5.8 mg), white amorphous powder. $[\alpha]_D^{25}$ −30 (*c* 0.1, MeOH); UV (DAD) λ_{max} (MeOH) 205, 224 nm; IR neat (KBr) 3281 (s, br), 2964 (s), 2932 (s), 2874 (s), 1736 (s), 1633 (s), 1516 (s), 1462 (s), 1384 (s), 1343 (s), 1227 (s), 1024 (s) cm^{-1}; HRESIMS *m/z* calc. for $C_{81}H_{136}N_{15}O_{17}$ $[M + H]^+$ 1591.0233, detected 1591.0335.

D-Pro14–isoKF (**5**, 5.6 mg), white amorphous powder. $[\alpha]_D^{25}$ −16 (*c* 0.1, MeOH); UV (DAD) λ_{max} (MeOH) 205, 224 nm; IR neat (KBr) 3281 (s, br), 2965 (s), 2932 (s), 2877 (s), 1736 (s), 1633 (s), 1515 (s), 1464 (s), 1383 (s), 1342 (s), 1227 (s), 1025 (s) cm^{-1}; HRESIMS *m/z* calc. for $C_{80}H_{131}N_{15}NaO_{17}$ $[M + Na]^+$ 1596.9745, detected 1596.9690.

D-Phe14–isoKF (**6**, 6.4 mg), white amorphous powder. $[\alpha]_D^{25}$ −22 (*c* 0.1, MeOH); UV (DAD) λ_{max} (MeOH) 205, 224 nm; IR neat (KBr) 3284 (s, br), 2965 (s), 2932 (s), 2873 (s), 1736 (s), 1633 (s), 1525 (s), 1454 (s), 1389 (s), 1344 (s), 1230 (s), 1024 (s) cm^{-1}; HRESIMS *m/z* calc. for $C_{84}H_{134}N_{15}O_{17}$ $[M + H]^+$ 1625.0077, detected 1625.0131.

3,4-2F-D-Phe14–isoKF (**7**, 6.2 mg), white amorphous powder. $[\alpha]_D^{25}$ −18 (*c* 0.1, MeOH); UV (DAD) λ_{max} (MeOH) 208, 228 nm; IR neat (KBr) 3288 (s, br), 2965 (s), 2932 (s), 2877 (s), 1736 (s), 1641 (s), 1521 (s), 1455 (s), 1389 (s), 1343 (s), 1280 (s), 1233 (s), 1026 (s) cm^{-1}; HRESIMS *m/z* calc. for $C_{84}H_{132}F_2N_{15}O_{17}$ $[M + H]^+$ 1660.9888, detected 1660.9999.

Table 3
Differential cytotoxicity of KF and isoKF against human tumor cell lines.[a]

		H125	MCF-7	LNCAP	OVC-5	MDA	HepG2
KF	Δ CEM	300	450	250	450	250	450
	Δ CFU	450	600	450	600	500	650
isoKF	Δ CEM	350	400	150	500	350	450
	Δ CFU	500	600	300	650	500	600

[a] Values shown are differential cell killing against either leukemia, CEM (Δ CEM) or normal (Δ CFU) cells.

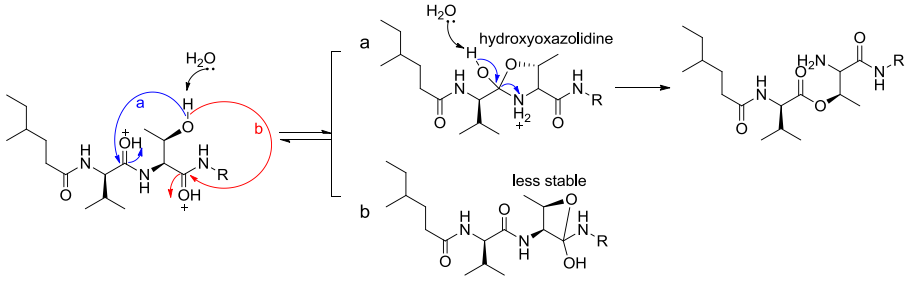
2.4. Disk diffusion assay

The cytotoxicity of natural KF and its analogues was tested using a soft-agar based disk diffusion assay [16,17]. The screen included 11 cell types: human leukemia CCRF-CEM, nine solid tumors (human colon HCT116, human lung H125, human ovarian OVCAR5, human breast cancers MCF7 and MDA-231, human prostate LNCaP, human pancreatic cancer PANC1, human liver cancer HePG2, and human brain U251N) as well as a human normal cell (hematopoietic progenitor cell, CFU-GM).

The human cancer cell lines were maintained in cell culture. They were removed from their cultures by a trypsin–collagenase–DNAase cocktail. For plating of all of the cell types other than the normal CFU, the 60 mm plates were first prepared with a hard agar bottom layer (0.6% agar in RPMI-1640 plus 15% BCS). A soft agar top layer (0.3% agar with the serum and media as above) plus 30,000 cells in 3 mL were poured into the plates and allowed to solidify.

For human CFU-GM, the cells were obtained from Poietic Technologies, Inc. (Gaithersburg, MD) overnight and washed twice with PBS. A total of 1.5×10^6 cells were plated in 3 ml of 0.3% agar with the addition of 10% L-cell conditioned media (which provides colony stimulating factor) in MEM-alpha plus 10% BCS.

Natural KF and its analogues were provided as a lyophilized powder and were dissolved in DMSO at 2–3 mg/mL and 15 μL (30–45 ug) was dropped onto a 6.5 mm filter disk. The disk was allowed to dry overnight and then placed close to the edge of the petri dish. The plates were incubated for 7–10 days (depending upon the cell type) and examined by an inverted stereo-microscope (10×) for measurement of the zone of inhibition measured from the edge of the filter disk to the beginning of normal-sized colony formation. The diameter of the filter disk, 6.5 mm, was arbitrarily taken as 200 units. A difference in zones between solid tumor cells and either normal or leukemia cells of 250 units defines solid tumor selective compounds. If the test material was excessively toxic at the first dosage, a range of dilutions



Scheme 2. Proposed mechanism of partial hydrolysis of Fmoc-KF.

of the agents (at either 1:4 or 1:10 decrements) against the same tumors were retested. At some dilution, quantifiable cytotoxicity was invariably obtained for KF.

3. Results and discussion

3.1. KF analogues

Seven semisynthetic analogues, isoKF **(1)**, D-Val14–isoKF **(2)**, Val13–Val14–isoKF **(3)**, D-Leu14–isoKF **(4)**, D-Pro14–isoKF **(5)**, D-Phe14–isoKF **(6)**, and 3,4-2F-D-Phe14–isoKF **(7)** were synthesized (Table 1). The structures were confirmed using HRMS, ^{1}H, ^{13}C, and ^{1}H-^{13}C HSQC NMR overlay experiments (Table 2, Figs. S1–S19). The overall yield (synthesis and purification) for each analogue was nearly 45%.

3.2. Proposed mechanism of partial hydrolysis of KF

N-protected KF was hydrolyzed at the amide linkage between L-Thr12 and D-Val13 under acidic condition at a relative high yield (70%). It is hypothesized that the release of the MeHex–D-Val13 fragment is kinetically faster than any other residues, which results from the contribution of the hydroxy group of L-Thr12. The proposed mechanism of the partial hydrolysis is based on N → O acyl rearrangement [18]. The oxygens on the amide carbonyl groups would be protonated under acidic conditions. The nucleophilic β-hydroxy group interacts with the carbonyl of D-Val13, thus forming a hydroxyoxazolidine as an intermediate, followed by acyl rearrangement to produce an ester which is more easily hydrolyzed than the amide linkage (Scheme 2).

3.3. Cytotoxicity of KF analogues

Natural KF and isoKF demonstrated cytotoxicity based on the zone assay although the activity was minimal for HCT-116, U251N and PANC-1. Unfortunately, the other 6 analogues tested were inactive in the assay against all 8 solid tumor cell lines tested. Further, KF and isoKF both demonstrated selective cytotoxicity to H125, MCF-7, LNCaP, MDA-235 and HepG2 when compared to either leukemia, CEM, or normal cell, CFU-GM. The zone differential (selectivity) is presented in Table 3 and it is noted that the zone differentials were larger when compared to normal cells than when compared to the leukemia cells. Indeed, there was little or no cell killing noted against the normal cells, as has been previously commented upon Natural KF and synthetic isoKF all display selectivity against MCF-7 and OVC-5 cell lines [6].

4. Conclusions

B. pennata is a potentially invasive green algae native to Hawaii, but is also widely distributed off the coast of Australia, the Atlantic Ocean, the Mediterranean, the Caribbean, the Indian and the Pacific Oceans [19]. When conditions are favorable, these algae can grow quickly and become toxic to most herbivorous organisms [19]. During the algal bloom season, considerable quantities of *B. pennata* are washed onto the shore. A large quantity of algae producing kilograms of KF (0.003% yield) could be recovered easily. The route presented here could produce *N*-terminal modifications for the construction of improved and inexpensive KF libraries from the naturally occurring blooms and rational drug design. This approach is economical, efficient and amendable to large-scale production.

It was reported that the substitutions with aliphatic residues of the *N*-terminal lipid chain could increase the activity [12]. In this paper, we proposed a modification method of *N*-terminal elongation using one or two more low-polar amino acid residues on KF. However, the products, except for isoKF, did not display activity against selected cancer cell lines. Of the hundreds of KF analogues produced by different research groups, only two modifications on the L-Orn residue showed a similar or higher potency in selected cancer cell lines [14,15]. Perhaps it will be necessary to reconsider the KF modification approach focusing on improving the short half-life instead of increasing its potency. Much has been learned about the optimization of half-life, metabolism and bioavailability from the 15 year journey leading to the development for caspofungin (Cancidas®), the first in class antifungal derived from the echinocandins. These lipopeptides inhibit enzyme (1 → 3)-β-D-glucan synthase ultimately disrupting the fungal cell wall. This drug was based on the original natural product pneumocandin which exhibited good antifungal activity but poor water solubility, stability and pharmacokinetic profile [20]. Years of medicinal chemistry studies addressed these problems and it was found that simply adding cationic groups to the structure greatly improved not only the potency and selectivity of the molecule but also increased water solubility, stability and the pharmacokinetic profile [20]. The same challenges regarding water solubility and stability associated with the kahalalides may be addressed with the assistance of the approach presented here. Alternatively, simply exchanging the hydrogen(s) for deuterium(s) in the KF molecule could have a positive impact on prolonging half-life. The C–D bond is stronger than C–H bond due to the deuterium isotope effect, making it a more stable bond to chemical and enzymatic bond cleavages [21,22]. Several deuterated analogues, such as tramadol, linezolid, and indiplon, have higher biological half-lives than their non-deuterated drugs [22]. The next step in the KF modifications will include either the addition of more cationic groups or exchange of selective hydrogen(s) in *N*-terminal residues to deuterium(s) on the basis of our semisynthetic approach in this paper to investigate the possibility of more stable and active analogues.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2015.05.004.

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