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Chemistry and Biology of Kahalalides

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1. INTRODUCTION

With drug discovery from marine natural products hailing a renaissance over the past 5 years, the use of marine extracts in the search for biologically active natural products continues to be a powerful approach for the identification of lead compounds for chemistry programs involved in drug discovery.¹ Natural products continue to serve as valuable starting points in developing druglike candidates, and the first step in the development of therapeutic agents is the identification of lead compounds that bind to a specific target or receptor of interest.² Structure-activity relationships (SARs) of lead compounds are then studied by synthesis or semisynthesis of a number of analogues to define the key recognition elements for maximal activity.³

However, the role of natural products in drug discovery became a lower priority in terms of participation by the major pharmaceutical companies by the mid-1990s.⁴ Reasons for this decline include the perceived disadvantages of natural products, including difficulties in access and supply, structure elucidation and synthesis because of the complexity of natural products, and concerns about intellectual property rights associated with published structures and international collections. In addition, the availability of large collections of compounds prepared by combinatorial chemistry methods provides inexpensive access to large numbers of molecules for random screening and starting materials for rational design.^{5,6}

Nevertheless, the natural products chemistry field has welcomed a renaissance over the past 5 years because of new developments in analytical chemistry, spectroscopy highthroughput screening, and a disappointing number of leads generated through combinatorial chemistry.^{1,7,9} Currently, basic scientific research in chemistry and biology of marine natural products that started in the 1970s has finally borne fruit for marine-derived drug discovery. Ziconotide (Prialt; Elan Pharmaceuticals), a peptide originally discovered from a tropical cone snail, was the first marine-derived compound approved in the United States in December 2004 for the treatment of pain. Then, in October 2007, trabectedin (Yondelis; PharmaMar) was approved and became the first marine anticancer drug in the European Union. Collaborations between industrial and academic scientists continue to meet the challenges involved in discovering, understanding, and developing new anticancer drugs.¹⁰ Several other candidate compounds from marine origins are in the pipeline and are being

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evaluated in phase I-III clinical trials for the treatment of various cancers in the United States and in Europe.^{11,13} Here, we review the kahalalides, a family of structurally unrelated depsipeptides isolated from the herbivorous marine mollusk *Elysia rufescens*, *Elysia ornata*, or *Elysia grandifolia* and their algal diet of *Bryopsis pennata*.^{14,27} Two of the most active compounds of this family, kahalalide F (KF) (**6**) and isoKF (**22**), have been evaluated in phase II clinical trials in hepatocellular carcinoma, non-small-cell lung cancer (NSCLC), and melanoma. Moreover, KF (**6**) is being evaluated in phase II clinical trials in patients with severe psoriasis.^{83,93} Of greatest significance is the fact that KF (**6**) can effectively inhibit receptor tyrosine kinase ErbB3 (HER3) and the phosphatidylinositol 3-kinase-Akt signaling pathways in sensitive cell lines, which suggests that KF (**6**) and isoKF (**22**) are involved in an unknown oncosis signaling pathway, though the mechanism of action has not yet been completely characterized.^{103,107} The kahalalides would represent the first anticancer drugs that can inhibit HER3 receptors.

2. BACKGROUND

2.1. Discovery and Isolation of Naturally Occurring Kahalalides

The kahalalides (Figure 1) consist of a series of depsipeptides that were first identified from the herbivorous marine mollusk *E. rufescens*, *E. ornata*, or *E. grandifolia* and later from their algal diet of *B. pennata* or *Bryopsis plumosa* (Figure 2).¹⁶ The size and composition of this series of peptides are highly variable, ranging from a C31 tripeptide to a C77 tridecapeptide, and each peptide contains a different relatively obscure fatty acid (Table 1). Many review articles and patents on the kahalalides have appeared in the literature.^{28,60} The initial discovery of KF (**6**) and its closely related isomer, isoKF (**22**), was first reported by Hamann and Scheuer in 1993.^{14,15} In the course of the investigation of natural products from the mollusk *E. rufescens* and the alga *B. pennata*, the constituents were shown to be dominated by amino and fatty acid-derived depsipeptides. Isolation of seven compounds, kahalalides A-E, F/isoKF, and G, was accomplished using silica gel and preparative HPLC. The largest and most active peptide, KF (**6**), and its closely related isomer, isoKF (**22**), exhibited significant activity in vitro against various solid tumor cell lines.

The sea slug *E. rufescens* and the alga *B. pennata* were extracted with ethanol and subjected to silica gel flash chromatography with a stepwise gradient: *n*-hexane, *n*-hexane/EtOAc (1:1), EtOAc, EtOAc/MeOH (1:1), MeOH, and MeOH/H₂O (50:50). The EtOAc/MeOH (1:1) fraction was found to contain the depsipeptides. HPLC using an RP C18 column and a gradient from a H₂O/MeCN/TFA mixture (70:30:0.1) to a H₂O/MeCN/TFA mixture (45:55:0.1) yielded the kahalalides, and final purification was completed using isocratic conditions.^{14,15,30,32}

IsoKF (**22**) is the (4S)-methylhexanoic isomer of KF and remains inseparable from KF (**6**) under preparative scale conditions but can be resolved using LC-TOF-MS (**6**).¹⁴ This compound is currently obtained via a synthetic process by PharmaMar.³⁵ The activity of isoKF (**22**) was similar to that of KF (**6**) and showed enhanced efficacy against breast and prostate tumor cell lines. Currently, the compound has entered phase I clinical trials in advanced pretreated solid tumors.

Because KF (**6**) and isoKF (**22**) exhibit significant bioactivity against various solid tumor cell lines, isolation of new kahalalide peptides became of interest for natural products chemists. Since 1997, the structures of 16 new kahalalide derivatives have been elucidated and 14 of them isolated.^{16,23} In 1997, Scheuer et al. reported two acyclic kahalalide peptides, kahalalides H (**8**) and J (**9**) from the mollusk *E. rufescens*.¹⁶ Kahalalides H (**8**) and J (**9**) share only four amino acids (leucine, phenylalanine, serine, and valine). They have in common a 3-hydroxy-9-methyldecanoic acid, previously encountered in kahalalide E (**5**). In common with the acyclic constituent of the alga-derived kahalalide G (**7**), kahalalides H (**8**) and J (**9**) exhibited no significant activity. In 1999, a new peptide kahalalide K (**10**) was isolated from the alga *B. pennata* and kahalalide K (**10**) was determined to possess a new array of L-amino acids (Val, Tyr, and Hyp), D-amino acids (Asn, Phe, and Ala), and a 3-hydroxy-9-methyldecanoic acid previously reported in kahalalides E (**5**), H (**8**), and J (**9**).¹⁷ In 2000, one new cyclic peptide, kahalalide O (**11**), was identified from the sacoglossan *E. ornata* and the alga *B. pennata* followed by the isolation of kahalalides P (**12**) and Q (**13**), which also possessed (3R)-hydroxy-9-methyldecanoic acid determined by Mosher's ester procedure.^{18,19} In 2006, new KF analogues, kahalalides R₁ (**14**) and S₁ (**15**), were isolated from *E. grandifolia*, and kahalalide R₁ (**14**) was found to exert comparable or even higher cytotoxicity than KF (**6**) toward the MCF7 human mammary carcinoma cell line.²⁰ Kahalalides R₁ (**14**) and S₁ (**15**) differed in only the fatty acid. The fatty acid residues in kahalalides R₁ (**14**) and S₁ (**15**) were 7-methyloctanoic acid (7-Me-Oct) and 5-hydroxy-7-methyloctanoic acid (7-Me-5-Octol), respectively. Kahalalides R₁ (**14**) and S₁ (**15**) were shown to contain six units of valines and one unit each of Phe, Ile, Thr, Orn, Pro, Glu, and dehydroaminobutyric acid. In kahalalides R₁ (**14**) and S₁ (**15**), Val and Glu units replaced Thr and Ile units previously found in KF (**6**). The absolute configuration of amino acids was determined by Marfey's analysis,⁶¹ which identified one unit of D-Glu, D-Pro, L-Orn, D-*allo*-Ile, D-*allo*-Thr, and L-Pro. Absolute configuration of the individual Val units could not be unambiguously determined because Marfey's analysis suggested the presence of D- and L-isomers in these two compounds, and there are three units of Val in both the cyclic and linear fragments. In 2007, two new KF analogues, kahalalides R₂ (**16**) and S₂ (**17**) (their names are identical to the two compounds isolated by Ashour et al. in 2006), were characterized by using tandem mass spectrometry.²¹ The amino acid sequences of kahalalides R₂ (**16**) and S₂ (**17**) were proposed by collision-induced dissociation (CID) experiments with singly and doubly charged molecular ions and by comparison with the amino acid sequences of KF (**6**). The absolute configuration of kahalalides R₂ (**16**) and S₂ (**17**) could not be assessed by chemical methods because no pure kahalalide R₂ (**16**) or S₂ (**17**) was isolated from the mollusk *E. grandifolia*. In 2008, four new kahalalide peptides from the mollusk *E. rufescens* were reported.²² Kahalalide V (**18**) was identified as an acyclic derivative of kahalalide D (**4**), while kahalalide W (**19**) was determined to have a 4-hydroxy-L-Pro residue instead of the proline in kahalalide D (**4**). Kahalalide X (**20**) was an acyclic derivative of kahalalide C (**3**), and kahalalide Y (**21**) was found to have an L-proline residue instead of the hydroxyproline in kahalalide K (**10**). In 2009, our group isolated two new kahalalide peptides, NorKA (**23**) and 5-OHKF (**24**). NorKA (**23**) and KA (**1**) differ in only a methylene group.²³ The former contains an isobutyric acid as the fatty acid moiety and the latter a 2-methylbutyric acid. 5-OHKF (**24**) and KF (**6**) are different in one hydroxyl group. The fatty acid residue of **24** is 5-hydroxy-5-methylhexanoic acid.

2.2. Structure Elucidation of Kahalalides

The gross structure proposed for the kahalalide peptides was deduced from NMR methods and mass spectrometry.^{14,23} Tandem mass spectrometry is also a useful tool that can provide detailed information for characterizing the kahalalide peptides.²¹ In most cases, the absolute configuration of each amino acid in the peptide can be determined by Marfey's methods after hydrolysis.⁵⁴ L-FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide), Marfey's reagent, reacts by nucleophilic substitution of the aromatic fluorine with the free amino group on an amino acid. When a racemic mixture of amino acids is treated with this reagent, the production of analogous diastereomers occurs. These diastereomers can be separated using reverse phase HPLC. However, it is not possible to determine the sequential position of the DL-antipodal amino acids by Marfey's method.^{14,16,18,20,23}

The structure of KF (**6**) and isoKF (**22**) was initially reported to contain a cyclized macrolide region consisting of two Val residues, one D-*allo*-Ile, one Thr, one dehydroaminobutyric acid, and one L-Phe, a linear region including three Val residues, one D-*allo*-Ile, one L-Orn, one Thr, one D-Pro, and a methylhexanoic acid, conjugated with the N-terminus.¹⁴ In the ring, the carboxyl group of the Val was linked to Thr through the hydroxyl group. The proposed structure of KF (**6**) was based upon NMR methods, degradation by acid hydrolysis, mass spectrometry, and GC analysis of the individual amino acid components using Marfey's reagent. Only the Ile, Orn, Pro, and Phe amino acids have one stereoisomer. However, further work was needed to investigate the absolute configuration of the conundrum posed by multiple possible stereoisomers in the molecule including three D-Val and two L-Val isomers together with one D-*allo*-Thr isomer and one L-Thr isomer. The remaining absolute configuration was reported by Goetz and Scheuer,^{24,25} and Val-3 and Val-4 were assigned the L- and D-configurations, respectively. The group of Albericio and Giralt at the University of Barcelona²⁷ synthesized the originally proposed structure and showed the differences in chromatographic and spectroscopic behavior between the synthesized peptide and the natural peptide. Later, Rinehart et al. at the University of Illinois elected to reinvestigate the absolute configuration of KF (**6**) and finally suggested that the absolute configuration of Val-3 and Val-4 should be reversed and played an important role in the activity of KF (**6**) and isoKF (**22**) because the depsipeptide with L-Val-3 and D-Val-4 in its structure was not active, while the molecule with D-Val-3 and L-Val-4 was active.²⁶ In 2006, the group of Dmitrenok and Nagai used a carboxypeptidase hydrolysis reaction to determine the sequential positions of the DL-Phe in kahalalides P (**12**) and Q (**13**).¹⁹ The absolute configuration of amino acids in 5-OHKF (**24**) was achieved by the combination of chemical hydrolysis and Marfey's method.²³ Amino acid analysis by Marfey's method revealed 12 amino acids: L-Orn, D-*allo*-Ile (two), D-Pro, L-Thr, D-*allo*-Thr, D-Val (three), L-Val (two), and L-Phe. A single Marfey's analysis was not enough because there is more than one valine or threonine that has a different absolute configuration. We hydrolyzed 5-OHKF (**24**) and KF (**6**) partially into smaller units without the fatty acid residues and then assessed their chromatographic properties. So far, the sequential positions of DL-antipodal Leu in kahalalide E (**5**), DL-antipodal Phe in kahalalide J (**9**), L-Thr and D-*allo*-Thr in kahalalide O (**11**), and DL-antipodal valine in kahalalides R₁ (**14**) and S₁ (**15**) remain unassigned.^{16,18,20}

2.3. Biological Activity Profiles

2.3.1. Cytotoxicity and Antitumor Activity—Among the kahalalides, only kahalalides A (1), E (5), F (6)/isoKF (22), R₁ (14), isoKA (23), and 5-OHKF (24) exhibited significant biological activity (Table 2). In 2001, Becerro et al. studied the ecological role of KF (6).⁶² The results showed that KF (6) protected both *B. pennata* and *E. rufescens* from fish predation. *E. rufescens* is a chemically defended species. In fact, it is possible that *E. rufescens* has evolved defensive mechanisms to reduce its chances of predation. *B. pennata* is a chemically defended alga that may provide the sacoglossan an associational refuge. By feeding on *B. pennata*, *E. rufescens* sequesters algal chloroplasts and makes itself highly cryptic.^{63,67} However, the risk of predation may still be high for cryptic organisms, so the acquisition of other defensive strategies may expand the benefits of crypsis. *E. rufescens* sequesters the antipredatory compound KF (6) from *B. pennata*, accumulating it a concentration several times the concentration in the alga, and uses KF (6) to chemically defend itself. Moreover, *E. rufescens* generates KF (6) the mucus. Therefore, KF (6) is responsible for the deterrent properties of the mollusk. It is hypothesized that the KF-producing *Vibrio* sp. from the surface of *B. pennata* are acquired by *E. rufescens*, which maintains the bacteria as symbionts.^{39,43}

Early preclinical data showed that KF (6) exhibited a potent new chemical entity with significant cytotoxicity against solid tumor cell lines.¹¹⁰ Preliminary in vitro screening studies indicated micromolar activity of KF (6) against selected cell lines, in particular NSCL, colon, ovarian, and breast cancers and especially prostate cancer (Table 2). In vitro cell culture studies indicated that 10 μ M KF (6) could produce cytotoxicity to central nervous system neurons but not astrocytes or sensory and motor neurons.⁸⁰

A human tumor colony-forming unit (TCFU) assay from surgically derived tumors showed that KF (6) completely inhibits breast, colon, kidney, NSCLC, ovary, prostate, stomach, and uterine tumor specimens. An IC₅₀ of <10 nM in a limited number of specimens has been identified, and prostate and stomach tumor specimens are the most sensitive.⁷¹

In vitro antitumor activity of isoKF (22) is similar to that of KF (6). However, isoKF (22) has enhanced efficacy against breast and prostate xenografts.^{35,70}

Kahalalides R₁ (14) and KF (6) were tested and found to be comparably cytotoxic toward MCF-7 cell lines with IC₅₀ values of 0.14 and 0.22 μ M, respectively. Furthermore, kahalalide R₁ was cytotoxic toward the mouse lymphoma L1578 Y cell line with an IC₅₀ of 4.26 nM, almost identical to that of KF (6).²⁰

2.3.2. Antimicrobial Activity—In 1996, Hamann et al. reported that KA (1) was shown to inhibit 83% of the growth of *M. tuberculosis* at 12.5 μ g/mL. KF (6) exhibited antifungal activity with IC₅₀ values of 3.02 μ M against *C. albicans*, 1.53 μ M against *C. neoformans*, and 3.21 μ M against *A. fumigates*.⁶⁹ In an agar diffusion assay, KF (6) exhibited strong antifungal activity at a level of 5 μ g/disk against the plant pathogens *Cladosporium herbarum* and *Cladosporium cucumerinum* with inhibition zones of 17 and 24 mm, respectively.²⁰ Kahalalide R₁ (14) also exhibited significant antifungal activity against the two species with inhibition zones of 16 and 24 mm, respectively.²⁰

KF (6) exhibited antiviral activity at 0.5 $\mu\text{g/mL}$ (95% reduction) with herpes simplex II virus (HSV II) using mink lung cells. Furthermore, KF (6) exhibited selective activity against some of the AIDS opportunistic infections.^{14,15}

KE (5) also exhibited selective activity against herpes simplex II virus (HSV II).¹⁵

2.3.3. Antileishmanial Activity—KF (6) was tested for its activity against promastigote and amastigote stages of *Leishmania*. Their respective LC_{50} (concentration at which the proliferation of the parasites was inhibited by 50%) values are listed in Table 2.

2.3.4. Immunosuppressive Activity. KF (6) exhibited slight immunosuppressive activity in a mixed lymphocyte reaction assay (MLR) with an IC_{50} of 3 $\mu\text{g/mL}$, and with a lymphocyte viability (LcV) IC_{50} of 23 $\mu\text{g/mL}$.¹⁴

3. METABOLISM AND PHARMACOKINETICS OF KF

3.1. Metabolism of KF

An analytical method using HPLC with positive ion turbo-ion spray tandem MS has been applied to the study of KF (6) in human plasma. Ammonium acetate was chosen to replace trifluoroacetic acid to enhance sensitivity in the positive ion mode.⁷²⁻⁷⁴ A lower-limit quantitation of 1 ng/mL using a 500 μL sample volume and a linear dynamic range extending to 1000 ng/mL were obtained, and KF (6) was stable in the biomatrix for a period of 9 months at $-20\text{ }^{\circ}\text{C}$ and 24 h at room temperature. The interassay accuracy was -15.1% at the lower limit of quantitation and between -2.68 and -9.05% for quality control solutions ranging in concentration from 2.24 to 715 ng/mL.⁷² The analyte was stable in plasma for 16 h after reconstitution of plasma extracts for liquid chromatography analysis at room temperature.

High-performance liquid chromatography with ultraviolet detection was used to study the chemical degradation of KF (6) under acid, neutral, and alkaline conditions, and the results showed that the half-lives ($t_{1/2}$) of KF (6) at $80\text{ }^{\circ}\text{C}$ were 1.1, 20, and 8.6 h at pH 0, 1, and 7, respectively.⁷⁵ The half-life of KF (6) at $26\text{ }^{\circ}\text{C}$ and pH 11 was 1.65 h. Kahalalide G (7), the only product of KF (6), was produced at pH 7 and 11. In addition, metabolic conversion of KF (6) was conducted using three different enzyme systems, including pooled human microsomes, pooled human plasma, and uridine 5'-diphosphoglucuronyl transferase. Biotransformation was not observed during these in vitro studies, so KF (6) was metabolically stable.

Furthermore, infrared (IR) spectroscopy and differential scanning calorimetry (DSC) were applied to investigate stabilities of 6-lyophilized products containing crystalline (mannitol) or amorphous (sucrose) bulking agents at 5 and $30\text{ }^{\circ}\text{C}$ with or without 60% relative humidity (RH) in the dark. A stable lyophilized formulation was created, and it contained 100 μg of KF (6), 100 mg of sucrose, 2 mg of polysorbate 80, and 2.1 mg of citric acid monohydrate to be reconstituted with a vehicle composed of 5%/5%/90% (v/v/v) CEW and to be diluted further using normal saline.⁷⁶ Lyophilized products became less stable when polysorbate 80 and citric acid monohydrate concentrations were increased. Sorption to contact surfaces with

an infusion container composed of low-density polyethylene could lead to loss of KF (6).^{77,78} Therefore, KF (6) must be administered in a 3 h infusion at concentrations of 0.5–14.7 $\mu\text{g/mL}$, and an administration set consisting of a glass container and a low-extrables, DEHP-free extension set must be used. An in vitro biocompatibility study was performed, and the results showed that no significant hemolysis due to the KF (6) formulation as well as the CE vehicle was found using a static or dynamic test model.⁷⁹

IsoKF (22) was very stable in dog plasma, and no significant changes in concentration were observed after incubation for up to 4 h in a water bath at 37 °C.⁹⁴

3.2. Pharmacokinetics of KF

An HPLC-MS assay method was utilized to determine the pharmacokinetics of KF (6) (Table 3).

4. CLINICAL STATUS

Relevant preclinical experiments have shown that fractionation of a lethal or MTD dose of KF (6) by daily administration for 5 days reduces drug-induced toxicity and appears to be a viable option for the clinical evaluation of KF (6) for the treatment of cancer.^{113,114} The activity of KF (6) has been investigated in phase I clinical trials for androgen-refractory prostate cancer solid tumors and phase II clinical studies with patients having liver, non-small-lung cancer, melanoma, and psoriasis.^{83,92} Some results about clinical trials of KF (6) are listed in Table 4.

IsoKF (22) has been selected for clinical development on the basis of its in vivo activity in xenografted human tumors, as well as an acceptable nonclinical toxicology profile. The compound is in phase I clinical trials in patients with advanced malignant solid tumors.^{92,94}

5. TOTAL SYNTHESIS

As a result of the biological activity profile of the kahalalides, they, especially KF (6), have become synthetic targets in several laboratories. Some papers about total syntheses of some kahalalides have been published.^{27,95,98}

5.1. Total Synthesis of KB

Kahalalide B (2) is the first kahalalide peptide that was totally synthesized.⁹⁶ It is a cyclic depsipeptide composed of seven different amino acids (Gly, Thr, Pro, Leu, Phe, Ser, and Tyr) and the fatty acid 5-methylhexanoic acid (5-MeHex), which is also present in the structure of other members of the series. Two different strategies (A and B in Scheme 1) have been applied to accomplish the synthesis of kahalalide B (2). In strategy A, heptapeptide 175 was synthesized from the H-Gly-O-resin (174), which was produced from the commercially available chlorotrityl chloride resin (166) first by a sequential attachment of L-Thr, L-Pro, D-Leu, L-Phe, D-Ser, and L-Tyr derivative using Fmoc/t-Bu strategy and DIPCDI/HOBt as the coupling reagent. This was followed by capping with 5-methylhexanoic acid at the N-terminus. Cleavage of the resin from 175 with a TFA/DCM mixture (1:99) afforded the linear peptide 176, which was subjected to macrocyclization

using 1H-benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP)-DI-PEA. After removal of side chain protection with a TFA/H₂O mixture (95:5), kahalalide B (**2**) was produced in 16% yield. In strategy B, there were some different points in the synthesis of kahalalide B (**2**). First, the limited incorporation of the first amino acid of the sequence was performed with Fmoc-Thr(t-Bu)-OH in the presence of DIEA. In contrast, in strategy A, the first amino acid was incorporated by using Fmoc-Gly-OH. Second, in strategy A, cyclization (the step between 176 and 177) was conducted in solution through the ester bond between the carboxyl group of Gly and the side chain hydroxyl of D-Ser, but in strategy B, the ester bond was formed on the solid phase and the cyclization (the step between 181 and 2) occurred through an amide bond between the carboxyl group of Thr and the amine group of Gly (see Scheme 1). Finally, the cyclization step was performed at a concentration of 10⁻³ M with PyBOP-DIEA (3.6 equiv) in DMF for 23 h (strategy A) or 1 h (strategy B). The lower nucleophilicity of the hydroxyl compared to that of the amine probably led to the difference in time for the cyclization step. Thus, strategy B is better than strategy A. The synthesis of kahalalide B (**2**) was achieved on a solid support, and this strategy should be useful for the synthesis of other cyclodepsipeptides.

5.2. Total Synthesis of KF

Several synthetic strategies have been successfully developed for the total synthesis of KF (**6**) (Table 5). The first successful synthesis of KF (**6**) (Scheme 2) is the linear solid phase synthesis.²⁷ This methodology involves elongation of the synthetic chain on the solid phase. With the linear peptide in hand, cyclization in solution follows, and finally, deprotection allows preparation of the natural compound in a straightforward manner. Moreover, the solid phase methodology is easy to scale up and could be applied to generate a wide variety of new analogues. The Fmoc/t-Bu strategy and 2-chlorotriyl chloride resin allowed cleavage of the peptide under mild acidic conditions. Next, amino acid D-*allo*-Thr and the Thr precursor of (Z)-Dhb were both introduced without protection of the hydroxyl function. For the formation of all the amide bonds, N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU) was used. The alloc group was removed under standard conditions before the peptide was deprotected from the resin. The cyclization reaction was then performed with benzotriazol-1-yl-N-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) using DMF as a solvent. Finally, the deprotection of the Boc group afforded the natural product KF (**6**).

The original solid phase synthesis was based on an orthogonal protecting group scheme using a chlorotriyl chloride resin together with Fmoc and allyloxycarbonyl (Alloc) as temporary protecting groups, and t-Bu and Boc for the side chain protection of Thr and Orn, respectively. In 2005, p-nitrobenzyloxycarbonyl (p-NZ) was used for the permanent protection of ornithine in the synthesis of derivatives of KF (**6**), which contains acid labile residues.⁹⁵

Furthermore, several convergent strategies (scheme 3) have been developed for the synthesis of KF (**6**).⁹⁸ Convergent strategies are defined as those in which peptide fragments are coupled to give the desired target molecule. The condensation of peptide fragments should lead to fewer problems in the isolation and purification of intermediates. The general

convergent strategy for the synthesis of KF (**6**) included dividing the peptide into fragments, the N-terminal and the fragment containing the cycle. Four strategies were applied to prepare the three distinct N-components (Scheme 3). All four strategies were based on the solid phase synthesis of a branched peptide using a tri- and tetra-orthogonal protecting scheme and subsequent cyclization and deprotection of the N-terminal function in solution. In strategy I, pentapeptide 168 was formed from the Fmoc-D-Valresin, prepared from the resin (**166**) by a sequential incorporation of D-*allo*-Ile, D-*allo*-Thr, and D-*allo*-Ile derivatives by using the Fmoc/t-Bu strategy and a DIPCDI/HOBt mixture as the coupling reagent and the esterification of the β -hydroxyl group of D-*allo*-Thr with Alloc-Val-OH using a DIPCDI/DMAP mixture. The macrocyclization of 184 by the HOBt/DIPEA/DIPCDI mixture was followed by the deprotection of Fmoc or p-NZ groups to afford the N-component 185. The corresponding C-component 5-MeHex-D-Val-Thr(t-Bu)-Val-D-Val-D-Pro-OH was synthesized from the Fmoc-D-Pro-resin by a sequential attachment of D-Val, Thr, and D-Val derivatives using the Fmoc/t-Bu method and a DIPCDI/HOBt mixture as the coupling reagent and then being capped with 5-methylhexanoic acid at the N-terminus. Finally, the condensation of the N-component 185 and the corresponding C-component using a PyAOP/DIEA mixture yielded the product KF (**6**). In strategy 2, the synthesis of 185 started with a form of 186 by the incorporation of Alloc-Phe-Z-Dhb-OH onto the resin 166. For chain elongation to the heptapeptide 187 from 186, five amino acids were sequentially attached. Ester linkage between 187 and Fmoc-Val-OH was formed by a DIPCDI/DIPEA mixture. The removal of the Fmoc group from 188 by using a TFA/CH₂Cl₂ mixture (1:99) yielded 189, which was followed by the cyclization and deprotection to afford 185. Here HOBt and DIPCDI were used for macrocyclization; Pd(PPh₃)₄ and PhSiH₃ were applied to successfully remove the Alloc group, and SnCl₂ was effective for removal of the p-NZ group. For strategies I and II, epimerization was not observed. In strategy III, the removal of the Alloc group of 168 by using Pd(PPh₃)₄ and PhSiH₃ was followed by the attachment of Alloc-Phe-Z-Dhb-OH in the presence of HOBt and DIPCDI to afford 190. The Boc group was introduced as an N^α-amino protecting group of D-*allo*-Ile in 190 after the Fmoc and Alloc groups had been removed. 191 was subjected to macrocyclization using a HOBt/DIPEA/DIPCDI mixture and removal of the Boc group using a TFA/H₂O mixture (19:1) to produce the second N-component 192, which was then condensed with the corresponding C-component in solution using a PyAOP/DIEA mixture to afford the final KF (**6**). In strategy IV, pentapeptide 193 was synthesized from 186 by a sequential attachment of D-Val, D-*allo*-Ile, and D-*allo*-Thr derivatives using the Fmoc/t-Bu method. The Boc group was used as an N^α-amino protecting group of D-*allo*-Thr in 193 to form 194, which was subjected to Fmoc-Val-OH coupling and the removal of the Fmoc group. The third N-component 196 was synthesized from 195 by macrocyclization using a HOBt/DIPEA/DIPCDI mixture and removal of the Boc group using a TFA/H₂O mixture (19:1). The condensation of 196 with the corresponding C-component produced KF (**6**). Epimerization of the C-terminal amino acids of the C-component in strategies III and IV was measured (4% for the case of Orn in strategy III and >10% for D-*allo*-Ile in strategy IV). Among these four strategies depicted in Scheme 3 showed, strategies I and II are better than strategies III and IV because the C-terminal amino acid of the C-component is D-Pro, which prevents epimerization during the coupling of the fragment in solution. The advantage of strategy II over strategy I was the fact that smaller amounts of the precious Alloc-Phe-Z-Dhb-OH were used.

5.3. Total Synthesis of KA

As Scheme 4 shows, the total synthesis of kahalalide A (**1**) began with the attachment of Fmoc-D-Phe onto the commercially available sulfonamide resin (**197**) giving **198**, which was incorporated with Fmoc-D-Leu, Fmoc-L-Thr(t-Bu), Fmoc-D-Phe, and (S)-2-methylbutyric acid to provide **199**. Deprotection of the Thr side chain in **199** was followed by ester bond formation with Fmoc-L-Ser(t-Bu). An attachment of Fmoc-L-Thr(t-Bu) and Fmoc-D-Leu afforded the key linear heptapeptides **200**. The safety-catch linker was then activated by sulfonamide alkylation with iodoacetone nitrile, and macrocyclative cleavage of depsipeptide **201** into the solution resulted from trityl deprotection of the free amine. Acidic cleavage of the t-Bu ethers is the last step of the synthesis of kahalalide A (**1**) in 15-20% overall yield.

6. STRUCTURE-ACTIVITY RELATIONSHIP

6.1. Structure-Activity Relationship Study of KA

With the total synthesis successfully accomplished, Bourel-Bonnet et al. investigated the SAR of kahalalide A (**1**) by synthesizing kahalalide A analogues. The results highlighted the importance of the free Ser and Thr side chains and the constrained depsipeptide framework for biological activity. The methylbutyrate side chain is flexible and can be replaced with other hydrophobic groups, as evidenced by increased activity with hexanoate.⁹⁷

6.2. Structure-Activity Relationship Study on KF and Its Analogues

Approximately 143 new KF analogues (Table 6 and Figure 3) have been successfully synthesized or semisynthesized by two groups to improve pharmacological properties and examine the role of each residue in the biological activity of KF (**6**) to examine the structure-activity relationship of KF (**6**).^{69,70} In 2007, our group obtained 10 new KF analogues (57-64, 104, and 105) by reaction of KF (**6**) as a starting point with diverse reactants through the amino group of Orn (acetylation with common coupling reagents) or the hydroxyl group of Thr.⁶⁹

6.2.1. Semisynthetic Analogues of KF—In 2007, our group focused on the secondary alcohol of Thr and the primary amine of Orn as the key functional groups to modify from the bacterially produced natural products because they play a crucial role in the bioactivity of this class of compounds.⁶⁹ For spectroscopic and bioassay comparison, we first converted KF (**6**) to kahalalide G (**7**) under mild basic conditions. Thus, KF (**6**) was subjected to hydrolysis in a H₂O/MeOH mixture (2:1) in the presence of potassium carbonate at room temperature, causing catalytic cleavage of macrolactone to the target ring-opened product. Further studies continued with the acetylation of KF (**6**). Treatment of free base KF (**6**) with an excess 2:1 volumetric ratio of acetic anhydride to BF₃³³·OEt₂ at room temperature after 5 min led to major product (68%), monoacetate-KF (**104**). Addition of the Dess-Martin periodinane (DMP) to a solution of KF (**6**) in acetonitrile furnished the oxidation of the secondary alcohol to the corresponding oxo-KF (**105**) in good yield (75%). Some KF analogues were obtained by modification of the primary amine group on L-Orn to the related secondary amines as potential pharmacophores. KF (**6**) was reduced to the corresponding monoalkyl- or dialkyl-amino-KF by the stepwise reductive N-alkylation of the amino groups in the presence of a carboxyaldehyde and hydride reducing agent. This stepwise one-pot

procedure includes the initial formation of the intermediate carbinol amine, which then dehydrates to form an imine. Then in situ reduction of this carbinol imine produces the alkylated amine. Sodium triacetoxyborohydride [NaBH(OAc)₃] was used as the hydride reducing agent that offers mild borohydride reduction and exhibits remarkable selectivity because the steric and electron withdrawing effects of the three acetoxy groups stabilize the boron-hydrogen bond and are responsible for its mild reducing properties. The reductive alkylation of KF was best performed under optimized conditions by the exposure of parent molecule to 5 equiv of the known aldehyde in methanol for 30 min at room temperature prior to portionwise addition of 2 equiv of triacetoxyborohydride under the same conditions. The reaction time was designed from a few hours to a couple of days and gave the desired products (57-63) in good to very good yields (Scheme 5). Furthermore, one KF analogue (**64**) was simply synthesized by treatment of DEAC-carboxylic acid and KF (**6**) in the presence of EDC and HBTU in DMF at room temperature for 1 h.

In 2008, the Albericio group reported seven new KF semisynthetic analogues (65-70 and 74) by modifying the primary amine of L-Orn in KF (**6**).⁷⁰ Compound 65 was easily obtained by adding DIPEA, tHex-OH, HOBt, and DIPCDI sequentially to a solution of KF (**6**) in a DMF/CH₂Cl₂ mixture (20:80) at 23 °C. The same procedure was applied to produce compounds 66-69 only by replacing tHex-OH with TFB-OH, cHP-OH, (p)-MTPA, or Fmoc-PEG-OH. Compound 69 was dissolved in a piperidine/DMF mixture (20:80) and stirred at 23 °C for 30 min to produce compound 70 in 75% yield. In addition, KF (**6**), D-Biotine, and HATU were dissolved in anhydrous DCM under an Ar atmosphere, and NMM was added, yielding compound 74.

6.2.2. Synthetic Analogues of KF—Giralt, Albericio, and his co-workers synthesized ~125 novel KF analogues by solid phase synthesis (Scheme 2).⁷⁰ The route is very similar to the total synthesis route of KF (**6**) with minor modifications. The whole route consists of seven steps, including the elongation of the synthetic chain on the solid phase, subsequent cyclization, and final deprotection in solution. The tetrapeptide resin 168 was synthesized from the D-Val resin 167, which was prepared from the commercially available chlorotriptyl chloride resin (**166**), by a sequential attachment of D-*allo*-Ile, D-*allo*-Thr, and D-*allo*-Ile derivatives using the Fmoc/t-Bu strategy and a DIPCDI/HOBt mixture as the coupling reagent. An ester linkage between 166 and Allo-Val-OH was produced by using diisopropylcarbodiimide (DIPCDI) in the presence of DMAP. For elongation of the chain to the decapeptide 170 from 168, six amino acids were sequentially attached and capped with 5-methylhexanoic acid at the N-terminus. Construction of (Z)-Dhb was conducted via two methods: (A) after side chain elongation from 170 to the tridecapeptide 171, stereoselective formation of the (Z)-Dhb residue on the resin by Fukase's method using EDCI and cuprous chloride and (B) direct introduction of the dipeptide, Alloc-Phe-(Z)-Dhb-OH, to 172 with a DIPCDI/HOAt mixture. The Alloc group in 172 was deprotected with Pd(PPh₃)₄ and phenylsilane, and cleavage from the resin with a TFA/CH₂Cl₂ mixture (1:99) afforded the linear depsipeptide 173, which was subjected to macrocyclization using a DIPCDI/HOBt/DIEA mixture, after removal of side chain protection with a TFA/H₂O mixture (19:1). The yield of KF (**6**) was 10-14%. In Scheme 2, two alternative routes for incorporating the amino acid Z-didehydroaminobutyric acid are listed. Method B was chosen for most analogues.

These 125 KF analogues (Figure 1 and Table 6) were synthesized following the procedure described for KF (6) described in Scheme 2, and antitumor activities of these compounds are listed in Table 7.

6.2.3. Structure-Activity Relationship of KF—KF (6) structure can be divided into three domains. Domain A includes the macrocycle formed by the six C-terminal amino acids closed by an ester bond between the carboxylic acid of Val and the hydroxyl function of D-*allo*-Thr. It is known that the ring is essential for biological activity because of the absence of activity of the acyclic natural analogue of KF, kahalalide G (7). The presence of the non-natural amino acid (Z)-Dhb and D-amino acids and the lack of a C-terminal amino acid as a result of the lactamization can contribute highly enzymatic stability to this domain and increase the rigidity of the domain, which are crucial for activity. The analogues at Phe suggested that a hydrophobic residue is required in that position. The introduction of any residues that can increase the hydrophobicity of that position can increase the activity. The L/D configuration of the carbon of the backbone is crucial. Therefore, the conformation of the ring is important for maintaining the activity. Domain B contains a peptide tail; the absolute configuration of any residue is crucial, and all analogues in which the chirality of any residue was reversed lost their activity. The domain not only can be a link between the other two domains but also can adopt or induce folding and interactions with the target protein. The amino acid Orn is important for conserving or enhancing the activity. In general, the compounds with aliphatic groups coupled to the amino group of Orn were the most active KF analogues. Several analogues exhibiting enhanced potency in several human cancer cell lines relative to KF (6) were prepared by direct reaction on the amino group of Orn in KF (6) with diverse reagents. Domain C is the N-terminal aliphatic acid that is also crucial for maintaining the activity. Any introduction of polar groups that can generate hydrogen bonds decreased the activity. Compounds with no terminal acid or a short aliphatic group had activity lower than that of KF (6). The SAR of KF (6) is summarized in Table 8. KF (6) has a more defined structure than expected and is highly sensitive to stereotopical changes that affect the chirality of R-carbon of the residue. KF (6) is not sensitive to side chain substitutions in almost every residue, and for almost every side chain, it was possible to find a distinct side chain that could preserve or even improve the activity. A more hindered replacement in each side chain was able to improve the activity, and the activity can be further increased by enhancing the hydrophobicity at any point on the molecule, solubility in water being a limiting factor.

7. MECHANISM OF ACTION OF KF

7.1. KF Induces Oncosis Opposed to Apoptosis

The primary mechanism of action of KF (6) remains to be elucidated. The negative results of the NCI COMPARE analysis⁹⁹ suggested that the compound may exhibit its cytotoxicity by a unique mechanism of action. There are two forms of cell death that occur in cells: apoptosis and oncosis.¹¹⁴ Most anticancer drugs are thought to induce apoptosis, which is defined as an active and programmed process characterized by a variety of morphological changes, including cell shrinkage, cytoplasmic condensation, ladder DNA degradation, and nuclear fragmentation resulting in cell death.¹¹⁴ However, soon after exposure to KF (6),

cells start a death process, including great swelling and a series of profound morphological alterations. KF (6) induces a rapid and profound alteration of cell architecture, including extensive vesiculation of cytoplasmic organelles, dilation of the endoplasmic reticulum elements, and cytoskeletal degradation. The integrity of crucial organelles such as lysosomes, mitochondria, and endoplasmic reticulum is largely damaged. Although chromatin clump irregularly into small, condensed masses, the nuclear structure is basically preserved and no DNA degradation is found.^{100,104} These features are typical of the process named oncosis, which is a passive death process resulting from physical or chemical lethal injury.¹¹⁴ Furthermore, several biochemical results also support the induction of KF (6) by oncosis as opposed to apoptosis. First, KF (6) has no effect on the cell cycle because flow cytometry analysis revealed that KF (6) induced neither cell cycle arrest nor apoptotic hypodiploid peak.¹⁰³ Second, neither protein and nucleic acid syntheses nor topoisomerase (I or II) can be inhibited by KF (6).²⁹ Third, several markers of caspase-dependent apoptosis were negative after KF (6) exposure, including the externalization of phosphatidylserine, the release of cytochrome c from mitochondria, and cleavage of caspase-3 and PARP. Moreover, molecular or chemical inhibition of caspases by ectopic overexpression of Bcl-2 or a pan-caspase inhibitor (zVAD-fmk), respectively, failed to protect against KF (6) cytotoxicity. Specific inhibitors of cathepsin B (CA-074 Me and zFA-fmk) or D (pepstatin A) also failed to protect against cell death induced by KF (6).^{29,103} Fourth, KF (6) exhibits strong cytotoxicity against both wild-type p53 and mutated p53 tumor cells in the NCI panel.^{30,32} These results suggest that cytotoxicity induced by KF (6) is predominantly due to a process of necrotic cell death involving oncosis rather than apoptosis, which is verified by treatment of the yeast *Saccharomyces cerevisiae* with isoKF.^{105,114,115}

7.2. Multiple Targets of KF Action

Much progress has been made with respect to targets involved in the action of KF (6), and multiple targets have been identified. First, human HeLa cervical cancer cells and monkey COS-1 fibroblasts treated with KF (6) were found to become dramatically swollen and produce many large vacuoles that appeared to be a consequence of changes in lysosomal membranes that were verified by the fact that lysosomes of KF-treated cells showed a dramatic enlargement and the lysosomal pH increased.¹⁰¹ The analysis of lysosomes of human prostate cancer PC3 cells treated with KF (6) via fluorescent acidotropic probes LysoTracker Green and Acridine Orange showed that KF (6) exposure can alter the membrane permeability of lysosomes.¹⁰³ Thus, it appears that lysosomes are targets for kahalalide F action. Conjugation of KF (6) with gold nanoparticles (GNP) was found to enhance in vitro antitumor activity because there is a synergic effect between KF (6) and the GNP that can favor penetration and targeting to the lysosome of HeLa cells.¹⁰² Second, further studies of the putative effects of KF (6) on human prostate PC3 and breast cancer SKBR-3 cells by using electron microscopy showed that KF (6) can rupture the plasma membrane.¹⁰³ Recently, treatment of with isoKF (22) caused invaginations of the plasma membrane, which make the cells permeable and metabolically inactive.¹¹⁴ The plasma membrane is supposed to be the first target of KF (6) action, and the breakage of the plasma membrane causes changes in the osmotic balance of the cell, which induces alterations, including cytoplasmic swelling, vesiculation of cytoplasmic organelles, and vacuolization of mitochondria and endoplasmic reticulum. Therefore, the mechanism of action of KF (6) has

been postulated to be similar to those of other cytotoxic compounds, which induce cell death through the formation of new ion channels in the membrane and/or by changing the activity of existing channels. For example, KF (**6**) was supposed to be similar to monensin, a Na^+/H^+ ionophore produced by *Streptomyces cinnamonensis*.¹⁰¹ Although KF (**6**) could interact with the lipid bilayer, it is unlikely to form pores by itself because the molecule is too small to directly span the lipid bilayer. A minimum of 20 amino acids is required to form the pore, and KF (**6**) is composed of 14 amino acids. One possibility is that KF (**6**) forms multimers. It is also possible that the drug interferes with the membrane, which can result in the formation or modification of ion channels. 2-Hydroxylated fatty acid-containing ceramides are found to be involved in the mechanism of action of isoKF (**22**) in *S. cerevisiae* and human colon cancer line HCT116. Furthermore, overexpression of fatty acid 2-hydroxylase (FA2H) and exogenous addition of 2-hydroxylated fatty acids increase the sensitivity of mammalian cells to isoKF (**22**). Accordingly, two possibilities are proposed to explain the mechanism of action of isoKF (**22**). One is that fatty acid 2-hydroxylation allows hydrogen bonding of isoKF directly to sphingolipids to facilitate ion channel formation or permeability to the drug. Another is that 2-hydroxylation affects the formation of some kind of membrane microdomain that is important for drug membrane association.¹¹⁴ Third, the sensitivity to KF (**6**) in a panel of human tumor cell lines derived from breast (SKBR3, BT474, and MCF7), vulval (A431), NSCLC (H460, A549, SW1573, and H292), and hepatic (SKHep1, HepG2, and Hep3B) carcinoma significantly correlated with protein expression levels of ErbB3 (HER3) but not other ErbB receptors. Exposure to KF (**6**) for 4 h induced downregulation of ErbB3 expression in sensitive cell lines, as well as inhibition of the PI3K-Akt/PKB signaling pathway, which is directly linked to ErbB3.^{105,107} Moreover, ectopic expression of a constitutively active Akt mutant had a protective effect against KF (**6**) cytotoxicity. This suggests ErbB3 and the Akt pathway are major determinants of KF (**6**) action on these cell lines. Recently, reverse chemical proteomics with T7 cDNA display has been used to identify human ribosomal protein S25 (RPS 25) as a binding partner for KF (**6**). KF (**6**) binds to phage-displayed RPS25 in a dose-dependent manner with a conservative dissociation constant of $\sim 50 \mu\text{M}$.¹⁰⁸

8. CONCLUSION

In the 17 years following the discovery of the kahalalides, a large body of research (isolation, structure elucidation, synthesis, biological activities, clinical trials, and mechanisms of action) has been conducted. The mechanism of action of KF (**6**) is not completely understood, although significant advances have been made in recent years. It is likely that the PI3K/Akt signaling pathway coupled to ErbB3 receptors could be the target of KF (**6**) action. There is no report regarding ErbB3 inhibiting drugs in the clinic, so KF (**6**) would be a promising candidate for inhibitor of ErbB3 receptors in tumor cells.

KF (**6**) is currently under phase II clinical trials and isoKF (Irvalex) under phase I clinical trials. The preliminary results of the phase II clinical study of KF (**6**) in patients with advanced NSCLC, hepatocarcinoma (HC), and advanced malignant melanoma (AMM) revealed an excellent tolerability profile with no serious adverse events, a positive response, and stable disease occurring in a number of patients. KF (**6**) is a splendid example of the

important role marine natural product chemistry can play in the discovery of fundamental scientific and medical knowledge. The past 17 years of kahalalide research have challenged us and our colleagues in the field with an intricately complex puzzle.

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Biographies

Jiangtao Gao received his M.S. in Marine Biology from the Institute of Oceanology, Chinese Academy of Sciences. In 2005 he joined the graduate program in the Department of Pharmacognosy at The University of Mississippi, under the guidance of Professor Mark T. Hamann. His research interests are focused on isolation and structure elucidation of bioactive compounds from marine organisms.

Mark T. Hamann is a Professor of Pharmacy, Chemistry, and Biochemistry as well as a Research Professor with the Research Institute of Pharmaceutical Sciences at The University of Mississippi and an Adjunct Professor with the Center for Environmental Studies, University of Maryland (Baltimore, MD). He received his B.S. degree in Chemistry and Biology from Bemidji State University (Bemidji, MN) in 1985 and then started working in GMP pharmaceutical manufacturing at Solvay Pharmaceuticals (Baudette, MN). Dr. Hamann completed his Ph.D. in Marine Natural Products Chemistry in 1992 at the University of Hawaii (Manoa, HI), under the guidance of the late Professor Paul Scheuer. During his research career, he has published more than 120 scientific papers, reviews, and book chapters and currently serves as an associate editor for *Biochimica et Biophysica Acta*. Dr. Hamann's group is actively involved in the isolation, structure elucidation, and semisynthetic modification of marine natural products, with an emphasis on compounds possessing activity against infectious diseases, cancer, and neuropsychiatric disorders.

ABBREVIATIONS

(c/t)-MecHex-OH	(cis/trans)-4-methylcyclohexane-carboxylic acid
3,5-dFPhAc-OH	(3,5-difluorophenyl)acetic acid
4-GuBut-OH	4-guanidinobutyric acid
6,6-dFHep-OH	6,6-difluoroheptanoic acid
6-Ohep-OH	6-oxoheptanoic acid
Ala	alanine
Alloc	allyloxycarbonyl
AMM	advanced malignant melanoma
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Bip	2-amino-3-biphenyl-4-ylpropionic acid
Boc	tert-butyloxycarbonyl
Bza-OH	benzoic acid
cDNA	cDNA
CE	cremophor EL/ethanol
CEW	cremophor EL/ethanol/water
Cha	cyclohexylalanine or 2-amino-3-cyclohexyl-propionic acid
cHP	3-cyclohexylpropionic acid
CID	collision-induced dissociation
Cl-TrtCl-resin	2-chlorotrityl chloride resin
DEAC	(7-diethylamino)coumarin-3-carboxylic acid
DEHP	diethylhexyl phthalate
Dha	2-aminoacrylic acid
Dhb	R, β -didehydro-R-aminobutyric acid
DHB	2,5-dihydroxybenzoic acid
DIPCDI	N,N0-diisopropylcarbodiimide

DIPEA	N,N-diisopropylethylamine
DMAP	4-(N,N-dimethylamino)pyridine
DMP	Dess-Martin periodinane
DMF	N,N-dimethylformamide
DSC	differential scanning calorimetry
EDC	1-ethyl-3-[30-(dimethylamino)-propyl]carbodiimide
EDCI	1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride
Fmoc	9-fluorenylmethoxycarbonyl
GI50	growth inhibition at 50%
Glu	glutamic acid
Gly	glycine
HATU	N-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide
HBTU	N-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]- N-methyl-methanaminium hexafluorophosphate N-oxide
hCh	homocyclohexylalanine or 2-amino-4-cyclohexyl- butyric acid
Hep-OH	heptanoic acid
HOAc	acetic acid
HOAt	1-hydroxy-7-azabenzotriazole(3-hydroxy-3H-1,2,3- triazolo[4,5-b]pyridine)
HOBt	1-hydroxybenzotriazole
Ile	isoleucine
isoKF	4(S)-methylhexanoic kahalalide F
FDA	1-fluoro-2,4-dinitrophenyl-5-L-alanine amide
KF	kahalalide F
HC	hepatocellular carcinoma
HSV II	herpes simplex II virus
Hyp	4-hydroxyproline

LC50	lethal concentration at 50%
LcV	lymphocyte
Leu	leucine
Lit-OH	lithocholic acid
Lys	lysine
MCF-7	Michigan Cancer Foundation-7 (a human breast adenocarcinoma cell line)
MeHex-OH	methylhexanoic acid
MeOH	methanol
MLR	mixed lymphocyte reaction
Mst-OH	myristic acid or tetradecanoic acid
MTD	maximal tolerated dose
MTPA	R-methoxy-R-trifluoromethylphenylacetic acid
NaI	2-amino-3-naphthalen-2-ylpropionic acid
NMM	N-methylmorpholine
NSCLC	non-small-cell lung cancer
Oct-OH	octanoic acid
Oic	octahydroisoindole-1-carboxylic acid
Orn	ornithine
p-CF3Bza-OH	4-trifluoromethylbenzoic acid
PARP	poly(ADP-ribose) polymerase
PEG	polyethylene glycol
Phe	phenylalanine
Pfh-OH	perfluoroheptanoic acid
Phg	aminophenylacetic acid
PI3K	phosphoinositide 3-kinases
Pip	pipecolic acid
Pipe-OH	benzo[1,3]dioxole-5-carboxylic acid
p-NZ	p-nitrobenzyloxycarbonyl

p-MeBza-OH	4-methylbenzoic acid
p-TfIcinn-OH	3-(4-trifluoromethylbenzyl)acrylic acid
p-TfIPhAc-OH	3-(4-trifluoromethylbenzyl)acetic acid Pro proline
PyAOP	7-azabenzotriazol-1-yl-N-oxy-tris-(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yl-N-oxy-tris-(pyrrolidino)phosphonium hexafluorophosphate
RH	relative humidity
RPS	ribosomal proteins
SAR	structure-activity relationships
Ser	serine
t-Bu	tert-butyl
TCA	trichloroacetic acid
TCFU	tumor colony-forming unit
Tfa	trifluoroacetyl
TFA	trifluoroacetic acid
TFB	2,3,4,5-tetrafluorobenzoic acid
TFAA	trifluoroacetic anhydride
TGI	total growth inhibition
Thi	2-amino-3-thiophen-2-ylpropionic acid
Thr	threonine
Tic	1,2,3,4-tetraisoquinoline-3-carboxylic acid
Trp	tryptophan
Tyr	tyrosine
Und-OH	undecanoic acid
Val	valine

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Figure 1.
Structures of kahalalide peptides.

Figure 2.
Photograph of the sacoglossan mollusk *E. rufescens* and the green alga *B. pennata* collected from Hawaii (photo by M. Huggett).

Figure 3. Building blocks used for the substitution of residues in KF (6).

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.

Scheme 5.

Table 1

Comparative Composition of Kahalalides

		amino acids																						
	kahalalide	mol formula	mol wt	Ala	Arg	Asp	Asn	Dhb	Glu	Gly	Ile	Leu	Lys	Orn	Phe	Pro	Hyp	Ser	Thr	Trp	Tyr	Val	fatty acid	refs
1	A	C ₄₆ H ₆₇ N ₇ O ₁₁	894.5									D(2)			D(2)			L(1)	L(2)				2-Me-Bu	15
2	B	C ₄₅ H ₆₃ N ₇ O ₁₁	878.5						1			D(1)			L(1)	L(1)		L(1)	L(1)		L(1)		5-Me-Hex	15
3	C	C ₄₇ H ₆₃ N ₉ O ₁₀	914.5		L(1)					L(1)					D(1)				L(1)		D(2)		Bu	15
4	D	C ₃₁ H ₄₅ N ₇ O ₅	595.4		L(1)											L(1)				D(1)			7-Me-3-Octol	15
5	E	C ₄₅ H ₆₉ N ₇ O ₈	836.5	D(2)								2				L(1)				L(1)			9-Me-3-Decol	15
6	F	C ₇₅ H ₁₂₄ N ₁₄ O ₁₆	1477.9					Z(1)		Da(2) ^a				L(1)	L(1)	D(1)			L(1)	Da(1)		D(3) L(2)	5-Me-Hex	14, 24, 27
7	G	C ₇₅ H ₁₂₆ N ₁₄ O ₁₇	1495.9					Z(1)		Da(2)					L(1)	D(1)			L(1)	Da(1)		D(3) L(2)	5-Me-Hex	15
8	H	C ₅₅ H ₈₂ N ₈ O ₁₆	1110.5			D(1)						D(1)			D(1)		L(1)	L(2)				D(1)	9-Me-3-Decol	16
9	J	C ₆₁ H ₉₄ N ₁₀ O ₁₇	1239.7			D(1)						D(1)	L(1)		2	L(1)	L(2)					D(1)	9-Me-3-Decol	16
10	K	C ₄₆ H ₆₆ N ₇ O ₁₁	892.5	D(1)			D(1)								D(1)			L(1)			L(1)	L(1)	9-Me-3-Decol	17
11	0	C ₄₈ H ₆₈ N ₈ O ₁₁	933.5						1	L(1)									2	D(1)	D(1)	L(1)	5-Me-Hex	18
12	P	C ₆₆ H ₉₉ N ₁₁ O ₁₇	1318.7									D(1)	L(1)			L(1)	L(1)				D(1)	D(1)	9-Me-3-Decol	19
13	Q	C ₆₆ H ₁₀₀ N ₁₁ O ₁₆	1302.7			D(1)						D(1)	L(1)		D(1)	L(2)						D(1)	9-Me-3-Decol	19
14	R ₁	C ₇₇ H ₁₂₇ N ₁₄ O ₁₇	1519.9					Z(1)	D(1)	Da(1)				L(1)	L(1)	D(1)			Da(1)			6	7-Me-Oct	20
15	S ₁	C ₇₇ H ₁₂₇ N ₁₄ O ₁₈	1535.9					Z(1)	D(1)	Da(1)				L(1)	L(1)	D(1)			Da(1)			6	7-Me-5-Octol	20
16	R ₂	C ₇₄ H ₁₂₁ N ₁₄ O ₁₆	1464.0					(1)		(1)	(1)			(1)	(1)	(1)			(2)			6	5-Me-Hex	21
17	S ₂	C ₇₆ H ₁₂₆ N ₁₄ O ₁₆	1492.0					(1)		(1)	(1)		1	(1)	(1)	(1)			(2)			5	5-Me-Hex	21
18	V	C ₃₁ H ₄₇ N ₇ O ₆	614.3		L(1)											L(1)				D(1)			7-Me-3-Octol	22
19	w	C ₃₁ H ₄₅ N ₇ O ₆	612.3		L(1)												L(1)			D(1)			7-Me-3-Octol	22
20	X	C ₄₇ H ₆₆ N ₉ O ₁₁	932.5		L(1)					L(1)					D(1)				L(1)		D(2)		Bu	22
21	Y	C ₄₆ H ₆₆ N ₇ O ₁₀	876.5	D(1)			D(1)								D(1)	L(1)					L(1)	L(1)	9-Me-3-Decol	22
22	isoKF	C ₇₅ H ₁₂₄ N ₁₄ O ₁₆	1477.9					Z(1)		Da(2)				L(1)		D(1)			L(1)	Da(1)		D(3) L(2)	4(S)-Me-Hex	14, 35
23	NorKA	C ₄₅ H ₆₅ N ₇ O ₁₁	880.5								D(2)				D(2)			L(1)	L(2)				IsoBu	23

	kahalalide	mol formula	mol wt	amino acids																	fatty acid	refs	
				Ala	Arg	Asp	Asn	Dhb	Glu	Gly	Ile	Leu	Lys	Orn	Phe	Pro	Hyp	Ser	Thr	Trp			Tyr
24	5-OHKF	$C_75H_{124}N_{14}O_{17}$	1493.9					Z(1)		Da(2), ^a		L(1)	L(1)	D(1)			L(1) Da(1)				D(3) L(2)	5-OH-5-MeHex	23

24	5-OHKF	$C_{75}H_{124}N_{14}O_{17}$	1493.9					Z(1)			Da(2) ^a	L(1)	L(1)	L(1)	L(1)	D(1)			L(1) Da(1)			D(3) L(2)	5-OH-5-MeHex	23
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^a *D-allo* configuration of amino acids.

Table 2

Biological Activity Profile of Kahalalides

kahalalide	biological activity	refs
A (1)	in vitro activity against <i>Mycobacterium tuberculosis</i> : 83% inhibition at 12.5 g/mL	15
E (5)	selective activity against herpes simplex II virus (HSV II)	15
F (6)	selectivity against solid tumor cell lines: IC ₅₀ values of 2.5, 0.25, and <1.0 µg/mL against A-549, HT-29, and LOVO, respectively; IC ₅₀ values of 10 and >10 µg/mL against P-388 and KB, respectively; KF is active against CV-1 cells with an IC ₅₀ of 0.25 µg/mL antiviral activity: 0.5 µg/mL (95% reduction) with HSV II using mink lung cells antifungal activity: IC ₅₀ values of 3.02 µM against <i>Candida albicans</i> , 1.53 µM against <i>Candida neoformans</i> , and 3.21 µM against <i>Aspergillus fumigatus</i> immunosuppressive activity: IC ₅₀ of 3 µg/mL in a mixed lymphocyte reaction assay, IC ₅₀ of 23 µg/mL with lymphocyte viability (LcV) antileishmanial activity: LC ₅₀ values of 6.13 µM against <i>Leishmania donovani</i> (promastigote), 8.31 µM against <i>Leishmania pifanoi</i> (promastigote), 29.53 µM against <i>L. pifanoi</i> (amastigotes); in vitro antitumor activity similar to that of KF (6)	14, 15, 68, 70
IsoKF (22)	IsoKF (22) has enhanced efficacy against breast and prostate xenografts	35, 70
R ₁ (14)	IC ₅₀ of 0.14 mmol/L against the human breast adenocarcinoma MCF-7 cell line IC ₅₀ of 4.28 mmol/L against the mouse lymphoma L1578Y cell line	20
NorKA (23)	100 µM norKA (2) inhibited 82% of the specific binding of [³ H]NPY to the Y ₁ receptor, and it showed no inhibitory activity (only 4%) for [³ H]BQ-123 binding to the ET _A receptor	23
5-OHKF (24)	in vitro antimalarial activity against D6 and W2 clones of <i>Plasmodium falciparum</i> with IC ₅₀ values of 1.5 and 1.2 µg/mL, respectively	23

Table 3

Pharmacokinetic Profile of KF

model used	results	refs
KF studied first in female mice for both intravenous and intraperitoneal administration of the drug (280 g/kg)	Upon intravenous administration to mice, there was no accumulation of the drug after repeated intravenous administration at 24 h intervals and plasma levels declined from a peak concentration of 1.0 μM with a $t_{1/2}$ of 35 min. When the drug was administered intraperitoneally at the same dose, the peak concentration was 0.3 μM approximately 1 h after dosing.	81, 82
phase I study in patients with androgen-refractory prostate cancer in which KF (20–930 mg/m ²) was administered as a daily 1 h intravenous infusion for 5 days every 3 weeks	A linear relationship between dose and AUC over a dose range of 20–560 g/m ² /day. At doses of >560 g/m ² /day, the AUC increased in a non-dose-proportional manner. On day 1, the total plasma clearance was 11.02 (4.54 L/h and the terminal $t_{1/2}$ value of intravenous KF in these patients was 0.54 $\pm$ 0.14 h.	83, 84, 111
phase I study in patients with various solid tumors in which KF was administered as a continuous weekly 1 h intravenous infusion at doses ranging from 266 to 1200 g/m ²	A linear relationship between dose and AUC over a dose range of 266–800 mg/m ² /week.	85
phase I study in patients with advanced solid tumors in which KF was administered weekly as a 1 h intravenous infusion at a starting dose of 266 g/m ² /day	This schedule was characterized by linear kinetics for C _{max} and AUC values, a short terminal half-time (0.52 h vs 0.47 h), and a narrow volume of distribution (5.5 L at the recommended dose with the once-weekly schedule vs 7.16 L at the recommended dose with the daily schedule). The volume of distribution and clearance increased with body size and were best predicted with body surface area and height, respectively.	86, 109
phase II study in patients with advanced malignant melanoma (AMM) in which KF was administered as a weekly 1 h intravenous infusion with a dose of 650 g/m ²	Means (SD) of half-life, clearance, and volume of distribution at steady state were 0.49 h (0.15), 5.60 L h ⁻¹ m ⁻² (1.26), and 4.00 L/m ² (1.00), respectively. The pharmacokinetic profile of KF in phase II studies did not differ significantly from those found in phase I studies.	87, 88

Table 4

Clinical Trials of KF

effect	model	results	refs
efficacy	phase I study in patients with androgen-refractory prostate cancer in which KF (20–930 mg/m ²) was administered as a daily 1 h intravenous infusion for 5 days every 3 weeks	Thirty-three patients were treated; one patient showed a significant decrease in PSA level (>50%) associated with clinical improvement (pain relief), while three patients exhibited stable disease for 2 (<i>n</i> = 2) or 7 (<i>n</i> = 1) months. The MTD was 560 mg/m ² /day.	84
efficacy	phase I study in patients with various solid tumors in which KF was administered as a continuous weekly 1 h intravenous infusion at doses ranging from 266 to 1200 g/m ²	Twenty-five patients were treated, and three patients achieved a clinical benefit: one hepatocarcinoma patient who received 24 infusions consisting of 400 g/m ² /week, one squamous carcinoma cavum patient who received nine infusions at the same dose, and one NSCLC patient who received 16 infusions at a dose of 530 g/m ² /week. The MTD was 1200 g/m ² /week.	85
safety	two phase I trials in which 60 cancer patients were administered KF as a 1 h intravenous infusion	Grade 4 AI was consistently the DLT and tended to coincide with LDH elevation and an ALT:AP ratio of >5.0, indicating hepatocellular damage; these effects were reversible and dose-dependent.	89
safety and efficacy	phase I study in patients with advanced solid tumors in which KF was administered weekly as a 1 h intravenous infusion at a starting dose of 266 g/m ² /day	Thirty-eight patients were enrolled and received once-weekly KF 1 h infusions at doses between 266 and 1200 g/m ² . Dose-limiting toxicities included transient grade 3/4 increases in transaminase blood levels. The maximal tolerated dose for the KF schedule was 800 g/m ² , and the recommended dose for phase II studies was 650 g/m ² . No accumulated toxicity was found. This schedule provided a favorable safety profile and hints of antitumor activity.	86
efficacy and safety	phase I study in patients with androgen or metastatic-refractory prostate cancer in which KF was administered as a 1 h intravenous infusion for 5 consecutive days every 3 weeks with a starting dose of 20 g/m ² /day	Thirty-two patients were treated at nine dose levels (20–930 g/m ² /day). The maximal tolerated dose on this schedule was 930 g/m ² /day. The recommended dose for phase II studies is 560 g/m ² /day.	83
efficacy and safety	phase II study in patients with advanced malignant melanoma (AMM) in which KF was administered weekly as a 1 h intravenous infusion with a dose of 650 g/m ²	Twenty-four patients were recruited. No objective responses were observed, but the duration of stable disease suggested some degree of antitumor activity.	87
response, safety, and tolerability	phase II study in patients with advanced non-small-cell lung cancer (NSCLC) in which KF was administered weekly as a 1 h intravenous infusion with a dose of 650 g/m ²	Thirty-one patients were enrolled in this phase II trial. The primary results for efficacy showed no complete responses. One patient had a partial response; stability occurred in eight patients and disease progression in 11 patients. Six patients had stable disease lasting for more than 3 months. The duration of stable disease suggested some antitumor activity of KF in this indication, while its toxicity was clinically negligible.	90
efficacy, safety, and tolerability	phase II study in patients with hepatocarcinoma (HC) in which KF was administered as a 1 h intravenous infusion with a dose of 650 g/m ² over 1 h per week until the disease failed to progress or unacceptable toxicity	Twenty-two patients were recruited. No objective response was observed. Stable disease occurred in nine patients with a median duration of 4.8 months. Median progression free survival was 2.4 months. KF was well tolerated in this patient population, and stable disease was the best response observed in previously untreated patients with hepatocarcinoma (HC).	91

Table 5

Synthesis of KF Analogues following Scheme 2

	analogue	step	residue replaced	residue incorporated
25	[Etg ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-Etg-OH 2
26	[D-Etg ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-D-Etg-OH 2
27	[(Z)-Dhf ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-D/L-Phe-OH 2
28	[Dha ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-Ser-OH
29	[D-Thr ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-D-Thr-OH 2
30	[D- <i>allo</i> -Thr ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-D- <i>allo</i> -Thr-OH 2
31	[Gly ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-Gly-OH
32	[Aib ²]-KF	A5	Fmoc-Thr-OH 2	Fmoc-Aib-OH
33	[Trp ³]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Trp-OH
34	[hCh ³]-KF	A5	Fmoc-Phe-OH 3	Fmoc-hCh-OH
35	[Phe(3,4-Cl ₂) ³ ,4(<i>S</i>)MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Phe(3,4-Cl ₂)-OH
36	[Phe(F ₃) ³ ,4(<i>S</i>)MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Phe(F ₃)-OH
37	[Phe(4-I) ³ ,4(<i>S</i>)MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Phe(4-I)-OH
38	[Phe(4-NO ₂) ³ ,4(<i>S</i>)MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Phe(4-NO ₂)-OH
39	[Phe(4-F) ³ ,4(<i>S</i>)MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Phe(4-F)-OH
40	[Tyr(Me) ³ ,4(<i>S</i>)MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Tyr(Me)-OH
41	[Thi ³ ,4(<i>S</i>)-MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Thi-OH
42	[Tic ³ ,4(<i>S</i>)-MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Tic-OH
43	[Tyr ³ ,4(<i>S</i>)-MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Tyr(t-Bu)-OH
44	[Oic ³ ,4(<i>S</i>)-MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Oic-OH
45	[NMePhe ³ ,4(<i>S</i>)MeHex ¹⁴]-KF	A5	Fmoc-Phe-OH 3	Fmoc-NMe-Phe-OH
46	[Phe(2-Cl) ³]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Phe(2-Cl)-OH
47	[Phe(3-Cl) ³]-KF	A5	Fmoc-Phe-OH 3	Fmoc-Phe(3-Cl)-OH
48	[Phe(4-Cl) ³]-KF	A4	4(<i>S</i>)-MeHex 14	5-MeHex
		A5	Fmoc-Phe-OH 3	Fmoc-Phe(4-Cl)-OH
49	[Phe(3,4-F ₂) ³]-KF	A4	4(<i>S</i>)-MeHex 14	5-MeHex
		A5	Fmoc-Phe-OH 3	Fmoc-Phe(3,4-F ₂)-OH
50	[Nal ³]-KF	A4	4(<i>S</i>)-MeHex 14	5-MeHex
		A5	Fmoc-Phe-OH 3	Fmoc-Nal-OH
51	[Bip ³]-KF	A4	4(<i>S</i>)-MeHex 14	5-MeHex
		A5	Fmoc-Phe-OH 3	Fmoc-Bip-OH
52	[Phg ³]-KF	A4	4(<i>S</i>)-MeHex 14	5-MeHex
		A5	Fmoc-Phe-OH 3	Fmoc-Phg-OH
53	[Val ⁴]-KF	B1	Fmoc-DVal-OH 1	Fmoc-Val-OH
54	[D-Dapa ⁶]-KF	B2	Fmoc-D- <i>allo</i> -Thr-OH 6	Fmoc-D-Dapa
55	[D-Thr ⁶]-KF	B2	Fmoc-D- <i>allo</i> -Thr-OH 6	Fmoc-D-Thr-OH
56	[D-Ser ⁶]-KF	B2	Fmoc-D- <i>allo</i> -Thr-OH 6	Fmoc-D-Ser-OH

	analogue	step	residue replaced	residue incorporated
71	[N ^ε (Me)3-Lys ⁸ ,4(<i>S</i>)-MeHex ¹⁴]-KF	B3	Fmoc-Orn(Boc)-OH 8	Fmoc-Lys(Boc)-OH
		B4	5-MeHex 14	4(<i>S</i>)-MeHex
		–	additional step	DIPEA/MeI
72	[Lys ⁸]-KF	B3	Fmoc-Orn(Boc)-OH 8	Fmoc-Lys(Boc)-OH
73	[Glu ⁸]-KF	B3	Fmoc-Orn(Boc)-OH 8	Fmoc-Glu(t-Bu)-OH
		B2	Fmoc-D- <i>allo</i> -Ile-OH 7	none
75	[Orn(N ^δ Tfa) ⁸ ,4(<i>S</i>)MeHex ¹⁴]-KF	B7	5-MeHex 14	4(<i>S</i>)-MeHex
		–	additional step	TFA/DIPEA
76	[Orn(N ^δ Tfa) ⁸ ,Thr(OTfa) ¹² ,4(<i>S</i>)MeHex ¹⁴]-KF	B7	5-MeHex 14	4(<i>S</i>)-MeHex
		–	additional step	TFA/DCM (1:1), 3 days
77	[Thr(OTfa) ¹² ,4(<i>S</i>)MeHex ¹⁴]-KF	B7	5-MeHex 14	4(<i>S</i>)-MeHex
		–	additional step	TFA/DCM (1:1), 3 days
78	[noD- <i>allo</i> -Ile ⁷ ,noOrn ⁸ ,noD-Pro ⁹ ,noD-Val ¹⁰ ,noVal ¹¹ ,noThr ¹² ,noD-Val ¹³]-KF	B2	Fmoc-D- <i>allo</i> -Ile-OH 7	none
		B3	Fmoc-Orn(Boc)-OH 8	none
		B3	Fmoc-D-Pro-OH 9	none
		B3	Fmoc-D-Val-OH 10	none
		B3	Fmoc-Val-OH 11	none
		B3	Fmoc-Thr(t-Bu)-OH 12	none
		B3	Fmoc-D-Val-OH 13	none
79	[noOrn ⁸]-KF	B3	Fmoc-Orn(Boc)-OH 8	none
80	[noOrn ⁸ ,noD-Pro ⁹]-KF	B3	Fmoc-Orn(Boc)-OH 8	none
		B3	Fmoc-D-Pro-OH 9	none
81	[noOrn ⁸ ,noD-Pro ⁹ ,noD-Val ¹⁰]-KF	B3	Fmoc-Orn(Boc)-OH 8	none
		B3	Fmoc-D-Pro-OH 9	none
82	[noOrn ⁸ ,noD-Pro ⁹ ,noD-Val ¹⁰ ,noVal ¹¹]-KF	B3	Fmoc-D-Val-OH 10	none
		B3	Fmoc-Orn(Boc)-OH 8	none
		B3	Fmoc-D-Pro-OH 9	none
		B3	Fmoc-D-Val-OH 10	none
		B3	Fmoc-Val-OH 11	none
83	[noOrn ⁸ ,noD-Pro ⁹ ,noD-Val ¹⁰ ,noVal ¹¹ ,noThr ¹²]-KF	B3	Fmoc-Orn(Boc)-OH 8	none
		B3	Fmoc-D-Pro-OH 9	none
		B3	Fmoc-D-Val-OH 10	none
		B3	Fmoc-Val 11	none
		B3	Fmoc-Thr(t-Bu)-OH 12	none
84	[noOrn ⁸ ,noD-Pro ⁹ ,noD-Val ¹⁰ ,noVal ¹¹ ,noThr ¹² ,noD-Val ¹³]-KF	B3	Fmoc-Orn(Boc)-OH 8	none
		B3	Fmoc-D-Pro-OH 9	none
		B3	Fmoc-D-Val-OH 10	none
		B3	Fmoc-Val-OH 11	none
		B3	Fmoc-Thr(t-Bu)-OH 12	none
		B3	Fmoc-D-Val-OH 13	none

	analogue	step	residue replaced	residue incorporated
85	[noVal ¹¹ ,noThr ¹² ,noD-Val ¹³]-KF	B3	Fmoc-Val-OH 11	none
		B3	Fmoc-Thr(t-Bu)-OH 12	none
		B3	Fmoc-D-Val-OH 13	none
86	[Pro ⁹ ,4(<i>S</i>)MeHex ¹⁴]-KF	B3	Fmoc-D-Pro-OH 9	Fmoc-Pro-OH
87	[D-Pip ⁹ ,4(<i>S</i>)MeHex ¹⁴]-KF	B3	Fmoc-D-Pro-OH 9	Fmoc-D-Pip-OH
88	[D-Tic ⁹ ,4(<i>S</i>)MeHex ¹⁴]-KF	B3	Fmoc-D-Pro-OH 9	Fmoc-D-Tic-OH
89	[5(<i>R</i>)-Ph-Pro ⁹ ,4(<i>S</i>)-MeHex ¹⁴]-KF	B3	Fmoc-D-Pro-OH 9	Fmoc-5(<i>R</i>)-Ph-Pro-OH
90	[Val ¹⁰ ,D-Val ¹¹]-KF	B3	Fmoc-D-Val-OH 10	Fmoc-Val-OH
		B3	Fmoc-Val-OH 11	Fmoc-D-Val-OH
91	[hCh ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-hCh-OH
92	[hCh ¹¹ ,D-Cha ¹³]-KF	B3	Fmoc-Val-OH 11	Fmoc-hCh-OH
		B3	Fmoc-D-Val-OH 13	Fmoc-D-Cha-OH
93	[D-Cha ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Cha-OH
94	[Gly ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Gly-OH
95	[Phe ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Phe-OH
96	[Ala ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Ala-OH
97	[Leu ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Leu-OH
98	[D-Val ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-D-Val-OH
99	[Pro ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Pro-OH
100	[Gln ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Gln-OH
101	[Orn ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Orn(Boc)-OH
102	[Thr ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Thr(t-Bu)-OH
103	[Glu ¹¹]-KF	B3	Fmoc-Val-OH 11	Fmoc-Glu(O-t-Bu)-OH
106	[Gly ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Gly-OH
107	[Phe ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Phe-OH
108	[Ala ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Ala-OH
109	[Leu ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Leu-OH
110	[D-Thr ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-D-Thr(t-Bu)-OH
111	[Pro ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Pro-OH
112	[Gln ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Gln-OH
113	[Orn ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Orn(Boc)-OH
114	[Glu ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Glu(O-t-Bu)-OH
115	[Val ¹²]-KF	B3	Fmoc-Thr(t-Bu)-OH 12	Fmoc-Val-OH
116	[Gly ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-Gly-OH
117	[D-Phe ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Phe-OH
118	[D-Ala ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Ala-OH
119	[D-Leu ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Leu-OH
120	[Val ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-Val-OH
121	[D-Pro ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Pro-OH
122	[D-Thr ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Thr(t-Bu)-OH

	analogue	step	residue replaced	residue incorporated
123	[D-Glu ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Glu(O-t-Bu)-OH
124	[D-Gln ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Gln-OH
125	[Orn ¹³]-KF	B3	Fmoc-D-Val-OH 13	Fmoc-D-Orn(Boc)-OH
126	[no5-MeHex ¹⁴]-KF	B4	5-MeHex 14	none
127	[Ac ¹⁴]-KF	B4	5-MeHex 14	AcOH
128	[Tfa ¹⁴]-KF	B4	5-MeHex 14	TFA
129	[But ¹⁴]-KF	B4	5-MeHex 14	But-OH
130	[3-MeBut ¹⁴]-KF	B4	5-MeHex 14	3-MeBut-OH
131	[3,3-dMeBut ¹⁴]-KF	B4	5-MeHex 14	3,3-dMeBut-OH
132	[4-MePen ¹⁴]-KF	B4	5-MeHex 14	4-MePen-OH
133	[(c/t)-MecHex ¹⁴]-KF	B4	5-MeHex 14	(c/t)-MecHex-OH
134	[4(<i>R</i>)-MeHex ¹⁴]-KF	B4	5-MeHex 14	4(<i>R</i>)-MeHex-OH
135	[Hep ¹⁴]-KF	B4	5-MeHex 14	Hep-OH
136	[6,6-dFHep ¹⁴]-KF	B4	5-MeHex 14	6,6-dFHep-OH
137	[Pfh ¹⁴]-KF	B4	5-MeHex 14	Pfh-OH
138	[Oct ¹⁴]-KF	B4	5-MeHex 14	Oct-OH
139	[Und ¹⁴]-KF	B4	5-MeHex 14	Und-OH
140	[Palm ¹⁴]-KF	B4	5-MeHex 14	Palm-OH
141	[Icos ¹⁴]-KF	B4	5-MeHex 14	Icos-OH
142	[2,4-hexadie ¹⁴]-KF	B4	5-MeHex 14	2,4-hexadie-OH
143	[Bza ¹⁴]-KF	B4	5-MeHex 14	Bza-OH
144	[<i>p</i> -MeBza ¹⁴]-KF	B4	5-MeHex 14	<i>p</i> -MeBza-OH
145	[<i>p</i> -TfIBza ¹⁴]-KF	B4	5-MeHex 14	<i>p</i> -TfIBza-OH
146	[Pipe ¹⁴]-KF	B4	5-MeHex 14	Pipe-OH
147	[3,5-dFPhAc ¹⁴]-KF	B4	5-MeHex 14	3,5-dFPhAc-OH
148	[<i>p</i> -TfIPhAc ¹⁴]-KF	B4	5-MeHex 14	<i>p</i> -TfIPhAc-OH
149	[<i>p</i> -TfICinn ¹⁴]-KF	B4	5-MeHex 14	<i>p</i> -TfICinn-OH
150	[4-dMeaBut ¹⁴]-KF	B4	5-MeHex 14	4-dMeaBut-OH
151	[4-GuBut ¹⁴]-KF	B4	5-MeHex 14	4-GuBut-OH
152	[6-Ohep ¹⁴]-KF	B4	5-MeHex 14	6-Ohep-OH
153	[4-Ac-OBu ¹⁴]-KF	B4	5-MeHex 14	4-Ac-OBu
154	[4-OHBut ¹⁴]-KF	B4	5-MeHex 14	4-OHBut-OH
155	[4-(4-AcOBu)OBu ¹⁴]-KF	B4	5-MeHex 14	4-(4-Ac-OBu)OBu-OH
156	[D- <i>allo</i> -IleBut ¹⁴]-KF	B4	5-MeHex 14	Fmoc-D- <i>allo</i> -Ile-OH
			additional step	iBut-OH
157	[Lit ¹⁴]-KF	B4	5-MeHex 14	Lit-OH
158	[Lit(OTfa) ¹⁴]-KF	B4	5-MeHex 14	Lit-OH
		B7	–	trifluoroacetylion
159	[D-Val ¹ ,D-Phe ³ ,Val ⁴ , <i>allo</i> -Ile ⁵ , <i>allo</i> -Thr ⁶ , <i>allo</i> -Ile ⁷ , D-Orn ⁸ ,Pro ⁹ ,Val ¹⁰ ,D-Val ¹¹ ,D-Thr ¹² ,Val ¹³]-KF	A1	Fmoc-D-Val-OH 4	Fmoc-Val-OH

	analogue	step	residue replaced	residue incorporated
		A2	Fmoc-D- <i>allo</i> -Ile-OH 5	Fmoc- <i>allo</i> -Ile-OH
		A2	Fmoc-D- <i>allo</i> -Thr-OH 6	Fmoc- <i>allo</i> -Thr-OH
		A2	Fmoc-D- <i>allo</i> -Ile-OH 7	Fmoc- <i>allo</i> -Ile-OH
		A3	Fmoc-Orn(Boc)-OH 8	Fmoc-D-Orn(Boc)-OH
		A3	Fmoc-D-Pro-OH 9	Fmoc-Pro-OH
		A3	Fmoc-D-Val-OH 10	Fmoc-Val-OH
		A4	Fmoc-Val-OH 11	Fmoc-D-Val-OH
		A4	Fmoc-Thr(t-Bu)-OH 12	Fmoc-D-Thr(t-Bu)-OH
		A4	Fmoc-D-Val-OH 13	Fmoc-Val-OH
160	[D-Cha ⁴ ,D-Cha ⁴ ,D-Cha ⁷]-KF	B1	Fmoc-D-Val-OH 4	Fmoc-D-Cha-OH
		B2	Fmoc-D- <i>allo</i> -Ile-OH 5	Fmoc-D-Cha-OH
		B2	Fmoc-D- <i>allo</i> -Ile-OH 7	Fmoc-D-Cha-OH
161	[D-Val ⁵ ,D-Va ⁷]-KF	B2	Fmoc-D- <i>allo</i> -Ile-OH 5	Fmoc-D-Val-OH
		B2	Fmoc-D- <i>allo</i> -Ile-OH 7	Fmoc-D-Val-OH
162	[Phe(3,4-Cl ₂) ³ , <i>p</i> CF ₃ Cinn ¹⁴]-KF	4	5-MeHex 14	<i>p</i> -CF ₃ Cinn-OH
		5	Fmoc-hCh-OH 3	Fmoc-Phe(3,4-Cl ₂)-OH
163	[noVal ¹¹ ,noThr ¹² ,noD-Val ¹³ ,Mst ¹⁴]-KF	B3	Fmoc-Val-OH 11	none
		B3	Fmoc-Thr(t-Bu)-OH 12	none
		B3	Fmoc-D-Val-OH 13	none
		B4	5-MeHex 14	Mst-OH
164	[Thr(OTfa) ¹² ,Lit(OTfa) ¹⁴]-KF	A4	5-MeHex 14	LiOH
		A7	additional step	TFA/DCM (1:1)
165	[no5-MeHex ¹⁴ , <i>N</i> -(Hep)2-D-Val ¹³]-KF	A4	additional step	heptanaldehyde/ NaBH ₃ CN/DMF and AcOH (9:1)

Table 6

KF Analogues^a

analogue	Val ¹	(Z)-Dhb ²	Phe ³	D-Val ⁴	D-allo-Ile ⁵	D-allo-Thr ⁶	D-allo-Ile ⁷	Orn ⁸	D-Pro ⁹	D-Val ¹⁰	Val ¹¹	Thr ¹²	D-Val ¹³	S-MexHex ¹⁴
25	Etg													
26	D-Etg													
27	(Z)-Dhf													
28	Dha													
29	D-Thr													
30	D-allo-Thr													
31	Gly													
32	Alb													
33		Trp												
34		hCh												
35		Phe(3,4-Cl ₂)												4
36		Phe(F ₃)												4
37		Phe(4-I)												4
38		Phe(4-NO ₂)												4
39		Phe(4-F)												4
40		Tyr(Me)												4
41		Thi												4
42		Tic												4
43		Tyr												4
44		Oic												4
45		N-MePhe												4
46		Phe(2-Cl)												
47		Phe(3-Cl)												
48		Phe(4-Cl)												
49		Phe(3,4-F ₂)												
50		Nal												
51		Bip												

analogue	Val ¹	(Z)-Dhb ²	Phe ³	D-Val ⁴	D-allo-Ile ⁵	D-allo-Thr ⁶	D-allo-Ile ⁷	Orn ⁸	D-Pro ⁹	D-Val ¹⁰	Val ¹¹	Thr ¹²	D-Val ¹³	5-MexHex ¹⁴
52			Phg											
53				Val										
54						D-Dapa								
55						D-Thr								
56						D-Ser								
57								Orn(4-FB)						
58								Orn(4-FB) ₂						
59								Orn(4-PM)						
60								Orn(2-TM)						
61								Orn(2-TM) ₂						
62								Orn(nHex)						
63								Orn(nHex) ₂						
64								Orn(DEAC)						
65								Orn(tHex)						
66								Orn(TFB)						
67								Orn(cHP)						
68								Orn(Mosh)						
69								Orn(Fmoc-PEG)						
70								Orn(PEG)						
71								N-(Me) ₃ -Lys						4
72								Lys						
73								Glu						
74								Orn(Biot)						
75								Orn(N ^δ Tfa)						4
76								Orn(N ^ε Tfa)				Thr(OTfa)		4
77												Thr(OTfa)		4
78							no		no	no	no	no	no	no
79							no							
80							no		no					
81							no		no	no				

analogue	Val ¹	(Z)-Dhb ²	Phe ³	D-Val ⁴	D-allo-Ile ⁵	D-allo-Thr ⁶	D-allo-Ile ⁷	Orn ⁸	D-Pro ⁹	D-Val ¹⁰	Val ¹¹	Thr ¹²	D-Val ¹³	5-MexHex ¹⁴
82							no		no	no	no			
83							no		no	no	no	no		
84							no		no	no	no	no	no	
85										no	no	no	no	
86								Pro						4
87								D-Pip						4
88								D-Tic						4
89								5(R)-Ph-Pro						4
90									Val		D-Val			
91											hCh			
92											hCh		D-Cha	
93													D-Cha	
94											Gly			
95											Phe			
96											Ala			
97											Leu			
98											D-Val			
99											Pro			
100											Gln			
101											Orn			
102											Thr			
103											Glu			
104												Thr(Ac)		
105												Thr(Oxo)		
106												Gly		
107												Phe		
108												Ala		
109												Leu		
110												D-Thr		
111												Pro		
112												Gln		

analogue	Val ¹	(Z)-Dhb ²	Phe ³	D-Val ⁴	D-allo-Ile ⁵	D-allo-Thr ⁶	D-allo-Ile ⁷	Orn ⁸	D-Pro ⁹	D-Val ¹⁰	Val ¹¹	Thr ¹²	D-Val ¹³	5-MexHex ¹⁴
113											Orn			
114											Glu			
115											Val			
116												Gly		
117												D-Phe		
118												D-Ala		
119												D-Leu		
120												Val		
121												D-Pro		
122												D-Thr		
123												D-Glu		
124												D-Gln		
125												D-Orn		
126													No	
127													Ac	
128													Tfa	
129													But	
130													3-MetBut	
131													3,3-dMeBut	
132													4-MePen	
133													(c/t)-MeHex	
134													4(R)-MeHex	
135													Hep	
136													6,6-dFlep	
137													Pfh	
138													Oct	
139													Und	
140													Palm	
141													Icos	
142													2,4-Hexadie	
143													Bza	

analogue	Val ¹	(Z)-Dhb ²	Phe ³	D-Val ⁴	D- <i>allo</i> -Ile ⁵	D- <i>allo</i> -Thr ⁶	D- <i>allo</i> -Ile ⁷	Orn ⁸	D-Pro ⁹	D-Val ¹⁰	Val ¹¹	Thr ¹²	D-Val ¹³	5-MeHex ¹⁴
144														<i>p</i> -MeBza
145														<i>p</i> -TfIBza
146														Pipe
147														3,5-dFPhAc
148														<i>p</i> -TfPhAc
149														<i>p</i> -TfCinn
150														4-dMeaBut
151														4-GuBut
152														6-Ohep
153														4-Ac-Obut
154														4-OHBut
155														4-(4-Ac-Obut)Obut
156														D- <i>allo</i> -Ile-IBut
157														Lit
158														Lit(OTfa)
159		D-Val	D-Phe	Val	<i>allo</i> -Ile	<i>allo</i> -Thr	<i>allo</i> -Ile	D-Orn	Pro	Val	D-ValD-Thr	Thr	Val	
160				D-Cha	D-Cha		D-Cha							
161					D-Val		D-Val							
162			Phe(3,4-Cl ₂)											<i>p</i> -TfCinn
163											no	no	no	Mst
164												Thr(OTfa)		Lit(OTfa)
165													N(Hep) ₂ -D-Val	

^aThe chemical structure of each modification can be found in Figure 3. The numeral 4 in the 5-MeHex column indicates the presence of 4(*S*)-MeHex.

Table 7

Activity Data (GI₅₀) of KF Analogues (micromolar)

	DU-145	LN-caP	IGROV	IGROV/ET	SK-BR 3	MEL-28	A-549	K-562	PANC-1	HT-29	LOVO	LOVO DOX	HELA	HELA APL
6	0.93	0.96	0.49	0.55	0.36	1.19	1.16	1.93	1.77	0.43	0.33	0.30	0.97	0.98
25	3.05	–	2.81	1.64	–	4.48	4.97	21.60	3.09	7.35	3.03	2.72	4.76	7.16
26	3.26	–	2.72	1.64	–	3.10	3.23	7.02	4.09	3.66	2.63	1.76	4.28	7.16
27	1.39	–	2.78	2.12	0.60	4.69	1.50	–	4.54	5.13	0.84	–	1.50	1.34
28	1.11	1.44	0.63	0.35	0.46	1.10	1.20	2.47	4.12	0.64	0.31	0.39	1.66	0.89
29	18.40	–	–	–	–	11.70	11.50	–	–	8.52	–	–	–	–
30	7.53	–	4.86	3.48	6.15	33.10	4.49	–	4.88	11.80	3.44	–	8.58	7.52
31	1.41	2.74	1.64	0.72	1.66	1.39	1.63	2.53	4.16	2.26	1.84	0.83	1.75	1.83
32	1.64	3.64	1.79	1.08	2.13	2.18	4.15	5.71	4.08	2.35	2.32	1.59	4.57	2.79
33	0.83	2.06	0.09	0.46	0.35	3.42	1.61	4.84	7.69	1.05	0.63	0.51	1.36	1.36
34	0.46	0.38	0.28	0.23	0.17	1.02	0.76	1.42	1.12	0.29	0.27	0.22	0.30	0.32
35	0.27	–	0.38	0.25	0.28	0.59	0.11	6.47	2.09	0.29	0.43	0.30	0.49	1.02
36	1.37	0.50	2.07	2.03	0.56	1.28	1.85	–	2.11	1.09	0.47	0.50	3.75	1.45
37	0.15	0.35	0.67	1.19	0.31	0.16	0.23	–	1.20	0.40	0.37	0.13	0.60	0.31
38	0.26	0.48	0.68	1.05	0.37	0.19	0.56	–	1.29	0.42	0.32	0.09	0.78	0.57
39	0.89	0.51	0.81	0.97	0.52	0.70	1.01	–	1.46	0.45	0.39	0.41	0.96	0.74
40	0.86	–	0.44	0.30	0.28	1.19	0.53	3.55	3.12	0.23	0.32	0.24	0.77	1.01
41	1.38	–	2.44	1.71	0.82	1.58	1.76	4.92	5.98	1.29	0.75	0.39	1.59	1.77
42	4.16	–	3.33	2.43	2.30	1.62	1.75	4.89	6.79	1.89	2.82	0.72	1.92	2.06
43	6.89	–	3.96	3.39	2.10	2.37	5.09	4.92	6.80	2.21	1.83	1.03	3.62	6.29
44	1.46	–	1.82	1.59	0.70	1.15	1.46	4.75	2.58	1.66	1.21	0.36	1.56	1.26
45	1.23	–	0.86	1.00	0.32	1.35	1.30	2.14	1.92	0.27	0.39	0.32	0.53	1.11
46	0.18	0.09	0.07	0.10	0.05	0.39	0.42	1.85	1.51	0.09	0.04	0.04	0.27	0.31
47	0.10	–	0.04	0.07	0.05	0.18	0.19	0.87	0.87	0.04	0.02	0.04	0.10	0.19
48	0.11	–	0.05	0.09	0.04	0.21	0.23	0.70	1.14	0.04	0.02	0.06	0.19	0.22
49	0.04	–	0.04	0.05	0.03	0.13	0.14	0.73	0.58	0.03	0.01	0.02	0.10	0.16
50	0.05	–	0.03	0.05	0.02	0.14	0.11	0.29	0.61	0.04	0.01	0.03	0.07	0.08
51	0.17	–	0.25	0.13	0.07	0.19	0.05	3.22	1.24	0.21	0.18	0.18	0.22	0.48

	DU-145	LN-caP	IGROV	IGROV ET	SK-BR-3	MEL-28	A-549	K-562	PANC-1	HT-29	LOVO	LOVO DOX	HEL A	HEL A APL
52	0.87	–	0.50	0.81	0.44	1.11	1.09	1.81	1.66	8.20	0.42	0.41	1.73	1.62
53	4.99	–	5.65	1.22	–	7.45	4.98	37.40	1.68	16.40	5.49	15.50	3.87	7.17
54	8.80	6.88	9.55	5.68	8.66	6.40	9.48	9.66	9.71	13.20	11.50	–	7.45	7.04
55	32.00	19.30	73.20	3.76	8.20	8.20	7.16	25.30	586.00	17.60	16.40	6.03	17.70	9.52
56	5.04	3.37	16.70	0.62	0.75	1.90	1.44	19.00	267.00	0.98	0.46	0.35	3.53	1.93
65	0.66	–	0.18	0.21	0.06	0.81	0.62	1.24	1.67	0.13	0.08	0.14	0.41	0.50
66	0.73	–	0.18	0.33	–	0.98	1.04	0.98	1.58	0.12	0.08	0.15	0.29	0.49
67	0.08	–	0.05	0.10	0.04	0.09	0.11	0.06	0.21	0.07	0.04	0.09	0.08	0.17
68	0.89	–	0.25	0.34	0.09	1.04	1.01	1.11	1.55	0.15	0.09	0.17	0.54	0.55
69	0.02	–	0.04	0.07	0.04	0.07	0.04	0.01	0.15	0.09	0.04	0.20	0.06	0.05
70	0.74	1.82	0.18	0.20	0.25	0.95	0.49	0.58	1.25	0.10	0.26	0.18	0.71	0.52
71	1.02	1.06	0.61	0.64	0.37	1.11	1.39	1.84	4.16	0.79	0.36	–	0.94	1.11
72	1.29	1.33	0.56	0.65	0.39	1.47	1.44	9.35	5.11	0.52	0.43	0.30	0.91	0.51
73	21.70	1.88	1.95	3.54	–	5.89	10.90	3.32	2.50	2.67	0.79	7.95	–	–
74	0.85	0.82	0.37	0.62	0.32	0.96	0.99	4.80	1.99	0.36	0.32	0.33	0.38	0.99
75	1.33	1.46	0.31	0.49	0.11	1.91	1.14	3.75	3.80	0.27	0.31	0.28	0.50	1.21
76	1.46	1.14	0.37	0.77	0.33	17.60	1.23	3.83	2.38	0.33	0.28	0.36	0.66	0.85
77	0.62	0.61	0.29	0.23	0.20	0.72	0.77	0.85	1.43	0.24	0.21	0.19	0.29	0.50
78	5.10	–	14.00	4.40	–	14.50	9.71	73.00	4.35	32.00	14.90	30.20	0.74	14.00
79	3.67	–	–	–	–	3.67	3.67	–	–	3.67	–	–	–	–
80	–	–	–	–	–	–	23.00	–	–	9.89	–	–	–	–
81	4.30	–	–	–	–	4.30	4.30	–	–	4.30	–	–	–	–
82	9.36	–	–	–	–	9.36	9.36	–	–	9.36	–	–	–	–
83	–	–	–	–	–	–	50.10	–	–	162.00	–	–	–	–
84	11.50	–	–	–	–	11.50	11.50	–	–	11.50	–	–	–	–
85	69.40	9.85	10.70	11.60	–	8.41	36.60	3.48	2.92	25.60	19.10	25.00	–	–
86	9.38	21.30	7.57	5.00	–	6.48	3.36	8.22	7.69	7.56	11.50	13.20	6.60	4.99
87	2.28	5.79	1.39	0.89	–	1.59	0.80	6.45	7.62	1.16	2.25	1.22	3.08	1.51
88	2.39	4.33	1.52	1.06	–	3.63	1.43	7.90	7.38	1.32	2.62	2.29	3.40	3.64
89	8.93	10.30	7.20	3.05	–	6.16	3.19	7.82	5.17	7.19	7.33	12.60	6.28	4.75
90	3.39	–	–	–	–	3.39	3.39	–	–	3.39	–	–	–	–

	DU-145	LN-caP	IGROV	IGROV ET	SK-BR 3	MEL-28	A-549	K-562	PANC-1	HT-29	LOVO	LOVO DOX	HEL A	HEL A APL
91	0.35	0.64	0.28	0.38	0.23	0.33	0.19	0.49	1.04	0.24	0.24	0.11	0.39	0.46
92	3.30	3.38	2.39	3.03	2.06	3.18	3.60	1.79	2.90	2.90	2.81	1.21	2.95	4.34
93	1.41	2.98	1.27	1.09	0.79	1.73	1.97	2.10	7.15	2.01	2.09	0.83	1.30	1.45
94	8.95	3.05	6.44	5.78	19.00	6.51	9.65	2.57	7.21	13.50	10.70	–	7.58	7.16
95	0.61	0.86	0.34	0.19	0.33	0.73	0.72	1.74	2.54	0.27	0.29	–	0.43	0.82
96	3.90	2.80	2.00	1.25	3.25	3.45	3.73	4.32	9.79	2.59	2.68	–	3.10	3.27
97	0.94	1.03	0.46	0.35	0.45	0.96	1.28	2.10	3.29	0.61	0.31	–	1.02	0.94
98	3.13	2.44	2.09	1.39	3.69	6.34	5.09	4.29	4.43	4.84	3.31	–	5.02	3.40
99	8.72	3.47	9.47	5.63	18.50	6.34	9.40	9.57	7.99	13.10	16.10	–	7.39	6.98
100	8.54	3.12	9.27	5.51	18.10	6.21	8.07	9.38	7.24	12.90	14.30	–	7.23	6.83
101	1.23	1.76	1.75	1.35	4.46	1.52	2.92	0.22	1.73	4.61	3.61	7.59	1.28	1.11
102	7.56	5.41	5.18	1.91	2.07	7.72	7.56	9.83	5.51	11.00	2.97	2.99	6.47	7.00
103	7.48	5.30	9.13	4.69	15.20	7.58	7.41	9.64	8.78	19.30	16.30	15.60	7.69	6.87
106	0.57	0.23	0.08	0.20	0.63	0.21	0.52	0.13	0.52	0.89	0.60	2.39	0.25	0.23
107	0.19	0.06	0.18	0.25	0.30	0.15	0.07	0.02	0.14	0.23	0.33	0.38	0.13	0.15
108	1.42	1.38	2.01	1.23	5.82	1.24	0.64	0.82	1.16	1.67	2.05	1.08	1.27	1.28
109	0.96	1.05	0.64	0.75	0.43	1.27	0.64	0.54	1.11	1.00	0.42	0.28	1.06	1.17
110	5.21	4.17	8.12	9.19	10.70	6.94	6.84	1.93	3.14	18.50	17.00	11.60	7.48	8.00
111	4.84	6.62	4.52	7.41	3.08	2.44	1.61	1.48	5.14	4.63	4.66	2.86	2.10	2.95
112	0.91	–	0.36	0.48	0.15	1.17	1.14	1.90	1.74	0.45	0.26	0.30	1.81	1.70
113	6.67	6.75	4.70	7.62	2.88	5.27	3.27	1.62	8.05	5.90	4.94	2.88	3.42	6.67
114	2.48	1.07	0.86	1.13	1.92	4.83	1.63	8.13	4.12	1.31	1.71	9.65	2.55	2.06
115	0.18	0.33	0.18	0.26	0.17	0.21	0.26	0.72	1.12	0.30	0.28	0.13	0.13	0.32
116	5.69	–	10.50	5.36	–	6.95	0.72	38.50	0.50	10.90	8.76	11.10	0.67	7.37
117	3.90	2.28	2.01	2.24	2.34	2.64	2.65	3.40	5.44	2.34	2.62	–	2.21	1.79
118	1.81	–	2.13	1.61	–	1.99	1.46	7.41	1.66	3.03	1.98	0.80	1.03	1.68
119	1.34	1.79	1.05	1.31	–	1.09	1.92	2.72	2.91	0.66	0.61	0.33	2.09	1.00
120	0.55	0.85	1.53	2.89	–	1.00	1.33	0.31	1.51	0.64	1.30	1.76	2.31	1.04
121	2.06	1.16	2.49	4.70	–	1.05	2.83	1.31	2.75	5.16	4.57	3.51	2.89	1.18
122	1.47	1.95	1.17	1.00	–	1.22	1.55	8.51	3.91	1.06	0.37	0.33	2.03	1.04
123	2.84	–	2.01	1.31	–	3.73	3.25	7.02	5.31	2.91	1.95	2.03	2.94	2.91

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	DU-145	LN-caP	IGROV	IGROV ET	SK-BR 3	MEL-28	A-549	K-562	PANC-1	HT-29	LOVO	LOVO DOX	HEL A	HEL A APL
124	1.46	-	1.57	1.33	-	1.34	0.23	6.80	0.17	2.30	1.46	0.61	0.45	2.42
125	1.16	0.85	1.13	1.03	1.72	1.14	0.75	0.88	1.42	0.77	2.30	2.04	1.37	1.10
126	5.98	-	11.10	4.01	-	8.05	5.39	40.50	5.49	17.80	9.46	16.70	5.16	7.75
127	7.11	-	-	-	-	7.11	7.11	-	-	7.11	-	-	-	-
128	3.42	-	-	-	-	6.84	6.84	-	-	3.42	-	-	-	-
129	1.74	-	-	-	-	3.48	3.48	-	-	1.74	-	-	-	-
130	2.07	-	2.25	1.36	-	1.98	1.68	7.79	8.47	2.98	1.63	0.88	2.09	2.33
131	0.34	-	-	-	-	3.42	1.71	-	-	0.34	-	-	-	-
132	0.34	-	-	-	-	3.42	0.34	-	-	0.34	-	-	-	-
133	0.69	1.07	0.53	0.60	0.35	1.01	1.04	2.94	2.66	0.55	0.24	0.34	0.90	0.63
134	1.69	2.98	0.83	0.55	0.54	2.01	1.69	2.07	8.01	0.73	0.40	0.25	2.33	1.28
135	0.85	1.09	0.35	0.29	0.23	0.81	1.18	2.74	2.36	0.50	0.32	0.26	0.97	0.78
136	4.52	1.84	1.33	0.99	-	2.94	2.03	2.96	2.04	1.96	0.44	1.81	-	-
137	0.53	0.76	0.85	0.70	0.29	0.73	0.64	0.83	2.32	0.51	1.16	0.29	0.96	0.60
138	0.58	0.78	0.68	0.53	0.34	0.93	1.05	1.00	2.83	0.58	0.32	0.30	0.95	0.53
139	0.18	0.42	0.22	0.17	0.06	0.19	0.16	1.67	1.63	0.37	0.29	0.10	0.14	0.24
140	0.15	0.13	0.19	0.35	0.04	0.14	0.14	0.12	0.20	0.25	0.30	0.26	0.28	0.33
141	15.70	1.65	5.17	4.69	3.40	1.89	1.88	9.14	1.79	3.95	3.81	-	5.44	1.52
142*	6.85	-	-	-	-	6.85	3.43	-	-	0.69	-	-	-	-
143	1.21	1.19	1.00	0.63	0.87	1.12	1.32	0.43	5.38	1.34	0.35	-	1.21	1.04
144	1.00	1.03	0.48	0.37	0.46	1.09	1.07	1.08	2.92	0.61	0.33	-	0.93	0.95
145	0.31	0.34	0.25	0.14	0.30	0.84	0.20	9.18	1.84	0.22	0.25	-	0.35	0.52
146	-	-	-	-	-	-	0.64	-	-	0.41	-	-	-	-
147	1.84	1.65	1.37	0.89	1.27	2.34	1.62	9.29	7.73	1.72	0.79	-	1.41	2.02
148	5.28	1.63	1.00	0.87	-	2.80	1.84	2.23	2.17	1.68	0.46	1.85	-	-
149	1.00	0.50	0.40	0.14	-	0.38	0.74	1.39	1.82	0.42	0.25	0.60	-	-
150*	6.77	-	-	-	-	6.77	6.77	-	-	6.77	-	-	-	-
151	8.62	3.93	9.36	5.56	18.30	6.27	9.28	9.46	9.51	13.00	15.00	-	7.30	6.90
152	7.56	5.36	9.22	4.74	7.27	7.66	7.49	9.75	8.87	19.50	8.59	5.05	7.77	6.94
153*	6.70	-	-	-	-	6.70	6.70	-	-	6.70	-	-	-	-
154*	6.89	-	-	-	-	6.89	6.89	-	-	6.89	-	-	-	-

	DU-145	LN-caP	IGROV	IGROV ET	SK-BR 3	MEL-28	A-549	K-562	PANC-1	HT-29	LOVO	LOVO DOX	HELA	HELA APL
155*	0.63	–	–	–	–	0.63	0.63	–	–	0.63	–	–	–	–
156*	6.46	–	–	–	–	6.46	6.46	–	–	6.46	–	–	–	–
157	5.10	–	14.00	4.40	–	14.50	9.71	73.00	4.35	32.00	14.90	30.20	0.74	14.00
158	0.39	0.85	0.38	0.35	0.12	0.21	1.06	0.97	1.64	0.39	0.34	0.23	1.07	1.04
159	40.90	21.30	161.00	15.90	12.30	8.20	7.16	25.30	586.00	17.60	16.40	13.30	17.70	9.52
160	0.15	0.30	0.21	0.26	0.25	0.17	0.16	0.36	1.32	0.36	0.28	0.14	0.26	0.23
161	1.36	2.34	1.83	2.17	2.12	1.64	2.40	22.00	4.71	2.50	2.46	1.43	1.40	2.73
162	0.11	–	0.17	0.12	0.21	0.13	0.05	4.19	1.36	0.19	0.21	0.23	0.18	0.31
163	44.60	7.21	9.10	8.92	–	4.15	32.10	3.00	2.45	23.10	17.70	22.60	–	–
164	0.70	0.73	0.41	0.64	0.08	0.20	0.95	1.07	1.66	0.46	0.29	0.34	1.66	1.33
165	24.90	3.72	8.39	19.50	14.10	7.86	6.64	6.16	6.84	14.20	14.00	11.00	2.37	1.81

Table 8

Structure and Activity of KF

	amino acids		SAR	domain	SAR
1	L-Val	L configuration is important		A	ring is essential
2	(Z)-Dhb	double bond is crucial			L/D configuration of the α -carbon of the backbone that maintains the conformation of the ring is crucial
3	L-Phe	A hydrophobic residue can increase the activity			
4	D-Val	D configuration is important			
5	D- <i>allo</i> -Ile	D configuration is important			
6	D- <i>allo</i> -Thr	configurations of two chiral centers are very important			
7	D- <i>allo</i> -Ile	D configuration is important		B	configuration of the α -carbons of the amino acids is crucial; domain adopts or induces some sort of folding and/or interactions with other molecules
8	L-Orn	aliphatic groups coupled to the amino group of Orn can increase the activity			
9	D-Pro	D configuration and the ring are important			
10	D-Val	D configuration is important			
11	L-Val	L configuration is important			
12	L-Thr	L configuration is important, and aliphatic substitutions can increase the activity			
13	D-Val	D configuration is important			
14	5-MeHex	long aliphatic group is essential		C	domain does not interact selectively with any other molecule, and its main function is as an aliphatic buoy