Structure−Activity Relationship of Kahalalide F Synthetic Analogues

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Kahalalide F (KF) is a natural product currently under phase II clinical trials. Here, we report the solid phase synthesis of 132 novel analogues of kahalalide F and their in vitro activity on a panel of up to 14 cancer cell lines. The structure−activity relationship of these analogues revealed that KF is highly sensitive to backbone stereotopical modification but not to side chain size modification. These observations suggest that this compound has a defined conformational structure and also that it interacts with chiral compounds through its backbone and not through its side chains. The N-terminal aliphatic acid appears to be a hydrophobic buoy in a membrane-like environment. Moreover, significant improvement of the in vitro activity was achieved.

Introduction

Kahalalide F (KF,[a] Figure 1) is a depsipetide that was first isolated in 1993 by Hamman and Scheuer from a marine mollusk, *Elysia rufescens*, and from the algae that the mollusk feeds on, *Bryopsis pennata*.[1] It has also been reported in another mollusk, *Elysia grandifolia*.[2,3] KF shows in vitro activity against prostate and colon cancer cells and to a lesser extent, antiviral, antifungal, and bactericide activity.[4] Two clinical phase I trials in adult patients with advanced prostate cancer and pretreated solid tumors have established the recommended dose for two distinct treatment regimes (once a week and 5 consecutive days every 3 weeks).[5,6] KF phase II clinical trials in hepatocellular carcinoma, non-small-cell lung cancer (NSCLC) and melanoma finished in 2006. Moreover, KF is being tested in phase II clinical trials in patients with severe psoriasis.

Phase I trials showed that the dose-limiting toxicity was a reversible elevation of liver transaminases. The mechanism of action of KF is mostly unknown. KF is a National Cancer Institute COMPARE-negative compound, which indicates that its cytotoxicity might be related to a unique mode of action. A first study indicated that cells treated with KF became swollen and developed large vacuoles, which appeared to be a consequence of changes in lysosomal membranes.[7] Recently, a further study has revealed that KF induces oncosis[8] and that it has a 5- to 40-fold greater effect on cancer cells than healthy cells.[6,9] The mechanism of action of KF does not affect the nuclear structure of the cell, while the integrity of crucial organelles such as mitochondria, endoplasmic reticulum, and lysosomes is severely compromised. The alterations found indicate that the osmotic balance of the cell may be altered, possibly as a result of cellular membrane damage. Thus, it appears that KF does not induce apoptosis, the main mechanism by which tumor cells are killed by anticancer drugs or radiotherapy.[10] Moreover, the mechanism of action of KF is caspase-independent and not affected by DNA expression.[8]

With the aim to identify new KF analogues with improved pharmacological properties and examine the role of each residue on the biological activity of this compound, our group developed a structure−activity relationship (SAR) program. In order to understand this study, it is noted that the KF structure is divided into three domains: domain A includes the macrocycle; domain B contains the peptide tail; domain C is the N-terminal aliphatic acid (Figure 1). Here, we report the synthesis and in vitro cytotoxicity of 132 KF analogues on several human cancer cell lines.

Results and Discussion

Synthesis of KF Analogues. The analogues were synthesized following the scheme used for the first KF synthesis with minor modifications (Scheme 1).[11,24] These modifications were mainly (i) the use of DIPCDI/HOBt as coupling reagents in the solid-

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[a] Abbreviations: Alloc, allyloxycarbonyl; Bip, 2-amino-3-biphenyl-4-ylpropionic acid; Boc, *tert*-butyloxycarbonyl; Bza-OH, benzoic acid; Cha, cyclohexylalanine or 2-amino-3-cyclohexylpropionic acid; *p*-CF_3Bza-OH, 4-trifluoromethylbenzoic acid; *p*-TflPhAc-OH, 3-(4-trifluoromethylbenzyl)acetic acid; *p*-TflCinn-OH, 3-(4-trifluoromethylbenzyl)acrylic acid; Cl-TrtCl-resin, 2-chlorotrityl chloride resin; Dha, 2-aminoacrylic acid; *Z*-Dhb, α,*β*-didehydro-α-aminobutyric acid; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EDC·HCl, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; Fmoc, 9 fluorenylmethoxycarbonyl; 6,6-dFHep-OH, 6,6-difluoroheptanoic acid; 3,5-dFPhAc-OH, (3,5-difluorophenyl)acetic acid; 4-GuBut-OH, 4-guanidinobutyric acid; GI_{50}, growth inhibition at 50%; hCh, homocyclohexylalanine or 2-amino-4-cyclohexylbutyric acid; Hep-OH, heptanoic acid; HOAc, acetic acid; HOAt, 1-hydroxy-7-azabenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo[4,5-*b*]pyridine); HOBt, 1 hydroxybenzotriazole; Icos-OH, icosanoic acid; KF, kahalalide F; LC_{50}, lethal concentration at 50%; Lit-OH, litocholic acid; MeHex-OH, methyhexanoic acid; (c/t)-MecHex-OH, (*cis*/*trans*)-4-methylcyclohexanecarboxylic acid; *p*-MeBza-OH, 4-methylbenzoic acid; MeOH, methanol; Mst-OH, myristic acid or tetradecanoic acid; NaI, 2-amino-3-naphthalen-2-ylpropionic acid; Oct-OH, octanoic acid; 6-Ohep-OH, 6-oxoheptanoic acid; Oic, octahydroisoindole-1-carboxylic acid; Phf-OH, perfluoroheptanoic acid; Phg, aminophenylacetic acid; (5*R*)-Ph-Pro, 5-(*R*)-phenylpirrolidine-2-carboxilic acid; Pip, pipecolic acid; Pipe-OH, benzo[1,3]dioxole-5-carboxilic acid; SPS, solid-phase synthesis; SRB, sulphorhodamine B; TCA, trichloroacetic acid; Tfa, trifluoroacetyl; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; TGI, total growth inhibition; Thi, 2-amino-3-thiophen-2-ylpropionic acid; Tic, 1,2,3,4-tetraisoquinoline-3-carboxilic acid; Und-OH, undecanoic acid.

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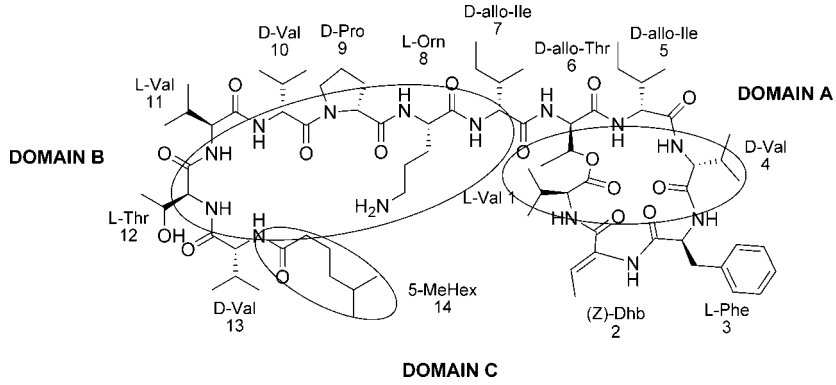
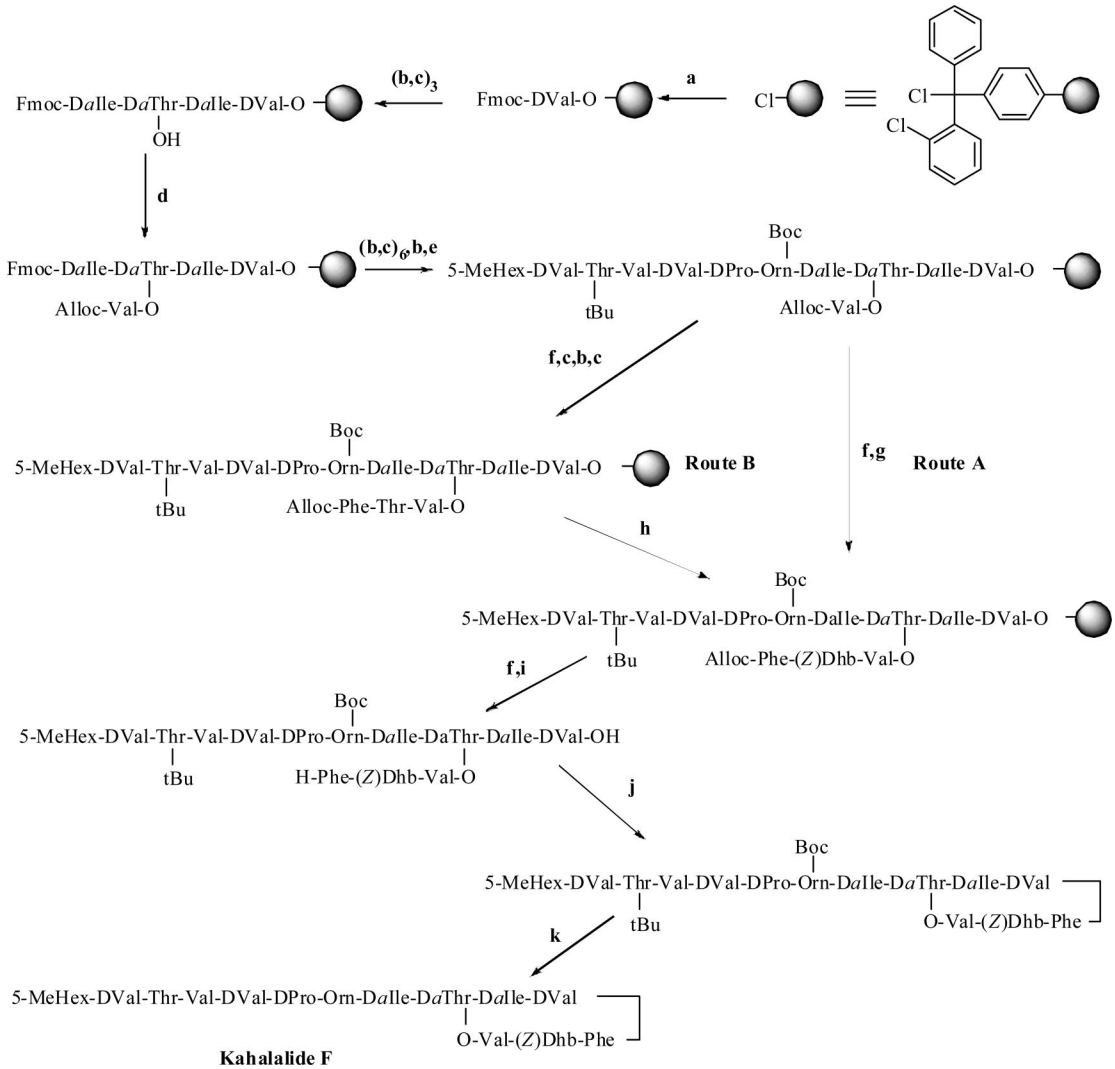


Figure 1. KF structure with its three domains highlighted.

Scheme 1. Synthetic Strategies for Solid-Phase Synthesis of KF Analogues[a]



[a] Conditions: (a) Fmoc-D-Val-OH, DIEA, DMF; (b) piperidine−DMF (2:8); (c) Fmoc-aa-OH, DIPCDI, HOBt, DMF; (d) Alloc-Val-OH, DIPCDI, DMAP; (e) 5-MeHex acid, DIPCDI, HOBt, DMF; (f) $Pd(PPh_3)_4$, $PhSiH_3$, DCM; (g) Alloc-Phe-(*Z*)-Dhb-OH, DIPCDI, HOAt, DMF; (h) EDC·HCl, CuCl, DMF−DCM (1:9); (i) TFA−DCM (1:99); (j) DIPCDI, HOBt, DIEA, DCM; (k) TFA−DCM (19:1).

phase elongation (SPPS) instead of azabenzotriazole-based reagents and (ii) the use of DIPCDI/HOBt/DIEA for the cyclization step instead of PyBOP/DIEA. Azabenzotriazole derivatives are expensive, and therefore, their substitution greatly reduces the cost of the synthesis. Moreover, these changes imply that the epimerization on the DVal(4) is prevented during cyclization. For the synthesis of most of the analogues, one or more amino acids, or the 5-MeHex acid, were either removed or substituted by another properly protected amino acid or acid.

In Scheme 1, two alternative routes to incorporate the nonproteinogenic amino acid *Z*-didehydroaminobutyric acid [(*Z*)-Dhb] are shown. Route A was chosen for most of the analogues. This procedure involves the incorporation of a presynthesized dipeptide, Alloc-Phe-(*Z*)-Dhb-OH, to the peptide

Table 1. Mean Activity of KF in Assay A (Values in Molar (M))

		GI_{50}	TGI	LC_{50}
prostate	DU-145	9.31×10^{-7}	1.72×10^{-6}	3.72×10^{-6}
	LN-caP	9.60×10^{-7}	1.96×10^{-6}	4.26×10^{-6}
ovary	IGROV	4.93×10^{-7}	1.18×10^{-6}	3.22×10^{-6}
	IGROV-ET	5.50×10^{-7}	1.30×10^{-6}	3.28×10^{-6}
breast	SK-BR3	3.63×10^{-7}	8.10×10^{-7}	1.98×10^{-6}
melanoma	SK-MEL-28	1.19×10^{-6}	2.26×10^{-6}	4.50×10^{-6}
lung (NSLC)	A549	1.16×10^{-6}	2.20×10^{-6}	4.52×10^{-6}
leukemia	K-562	1.93×10^{-6}	3.32×10^{-6}	5.28×10^{-6}
pancreas	PANC1	1.77×10^{-6}	3.33×10^{-6}	5.07×10^{-6}
colon	HT29	4.32×10^{-7}	9.15×10^{-7}	2.24×10^{-6}
	LOVO	3.34×10^{-7}	6.14×10^{-7}	1.51×10^{-6}
	LOVO-DOX	2.99×10^{-7}	4.85×10^{-7}	1.47×10^{-6}
cervix	HELA	9.65×10^{-7}	1.97×10^{-6}	4.73×10^{-6}
	HELA-APL	9.75×10^{-7}	2.02×10^{-6}	4.29×10^{-6}

sequence as a regular amino acid. Route B, which involves a β-alcohol solid-phase dehydration, takes longer and gives a lower yield[12] and was therefore used only when the modification was on the amino acids (*Z*)-Dhb or Phe.

Finally, another set of analogues was obtained by reaction of KF or an intermediate with diverse reactants through the amino group of Orn (acylation with common coupling reagents) or the hydroxyl group of Thr (acylation with DIPCDI in the presence of DMAP).[13]

Antitumoral Screening and Data Representation. The cytotoxicity of each peptide was evaluated against a panel of up to 14 human tumor cell lines. The cytotoxic assay (assay A in methods section) gives an output of three parameters: GI_{50} (concentration at which the growth is inhibited by 50%), TGI (concentration at which the growth is totally inhibited), and LC_{50} (lethal concentration that will kill 50% of the cells).[14] Of these parameters, only GI_{50} is shown in Tables 2–5 to facilitate comparison. The mean activity for KF is shown in Table 1.

Moreover, the activity of several analogues was taken from another assay (assay B in methods section), which was performed at the very beginning of this study. The output data of this assay is the IC_{50} (half-maximal inhibitory concentration). Compounds that did not show relevant activity were not tested again in assay A. For the sake of simplicity, the activity of these compounds has been included in the table with the other analogues and can be distinguished by an asterisk.

Activity data are shown in Tables 2–5 using a color palette to describe GI_{50} concentration in a logarithmic scale.

SAR. From the 132 KF analogues synthesized and tested, 32 had modifications at domain A (analogues **1**−**32**, Table 2), 59 at domain B (**33**−**91**, Table 3), and 34 at domain C (analogues **92**−**125**, Table 4). In addition, seven had modifications in more than one domain (analogues **126**−**132**, Table 5). The chemical structure of each substitution is given in Figures 2–4.

Several compounds in Tables 2 (domain A) and 3 (domain B) had two modifications on their structure: one related to the explored domain and the other corresponding to the N-terminal moiety. The 5-MeHex in these compounds was substituted by 4(*S*)-MeHex in position 14. This switching did not affect the in vitro activity, as demonstrated in parallel experiments that showed that these two acids led to similar in vitro activity. In contrast, the compound with 4(*S*)-MeHex (**100**, Table 4) exhibited higher in vivo efficacy in breast and prostate xenografts.[19]

Domain A Analogues. This domain contains the macro ring formed by the six C-terminal amino acids closed by an ester bond between the carboxylic acid of Val (1) and the β-hydroxyl function of D-*allo*-Thr (**6**). Given the absence of activity of the acyclic natural analogue of KF, kahalalide G, it is known that the ring is essential for biological activity.[4]

The presence of D-amino acids, the non-natural amino acid (*Z*)-Dhb, and the lack of a C-terminal amino acid as a result of the lactamization confer a high degree of enzymatic stability to this domain.[15] These features also increase the rigidity of the domain, which may be of relevance for activity.[15]

The most explored residue in this domain is Phe (**3**), with 20 analogues, the most active being those that introduce halogen groups on the aromatic ring, with the greatest of the activity in compounds **22** [Phe(2-Cl)], **23** [Phe(3-Cl)], **24** [Phe(4-Cl)], **25** [Phe(3,4-F2)], and **26** (NaI). Interestingly, **11** [Phe(3,4-Cl2)] with two chlorines on the aromatic ring was not as active as the monochlorinated compounds **22**, **23**, or **24**, and the position of the chlorine did not appear to be relevant. In contrast, **25** [Phe(3,4-F2)] with two fluorines was more active than the analogue with a single fluorine (**15**) and the analogue with five fluorines (**12**).

The analogues at Phe (**3**) suggest that a hydrophobic residue is required in that position. Almost all halogen derivatives of Phe were more active than KF. Compounds in which Phe was replaced by Trp (**9**), hCh (**10**), and methylated Tyr (**16**) were more active, while hydrophilic compounds such as Thi (**17**) and Tyr (**19**) showed lower activity. Analogues that restricted the rotation of the aromatic ring through N-alkylation either did not improve activity (*N*-MePhe, **21**) or blocked it (Oic **20**, Tic **18**). Finally, the Phg derivate (**28**), which can be considered a Phe lacking the β-methylene atom, was slightly less active.

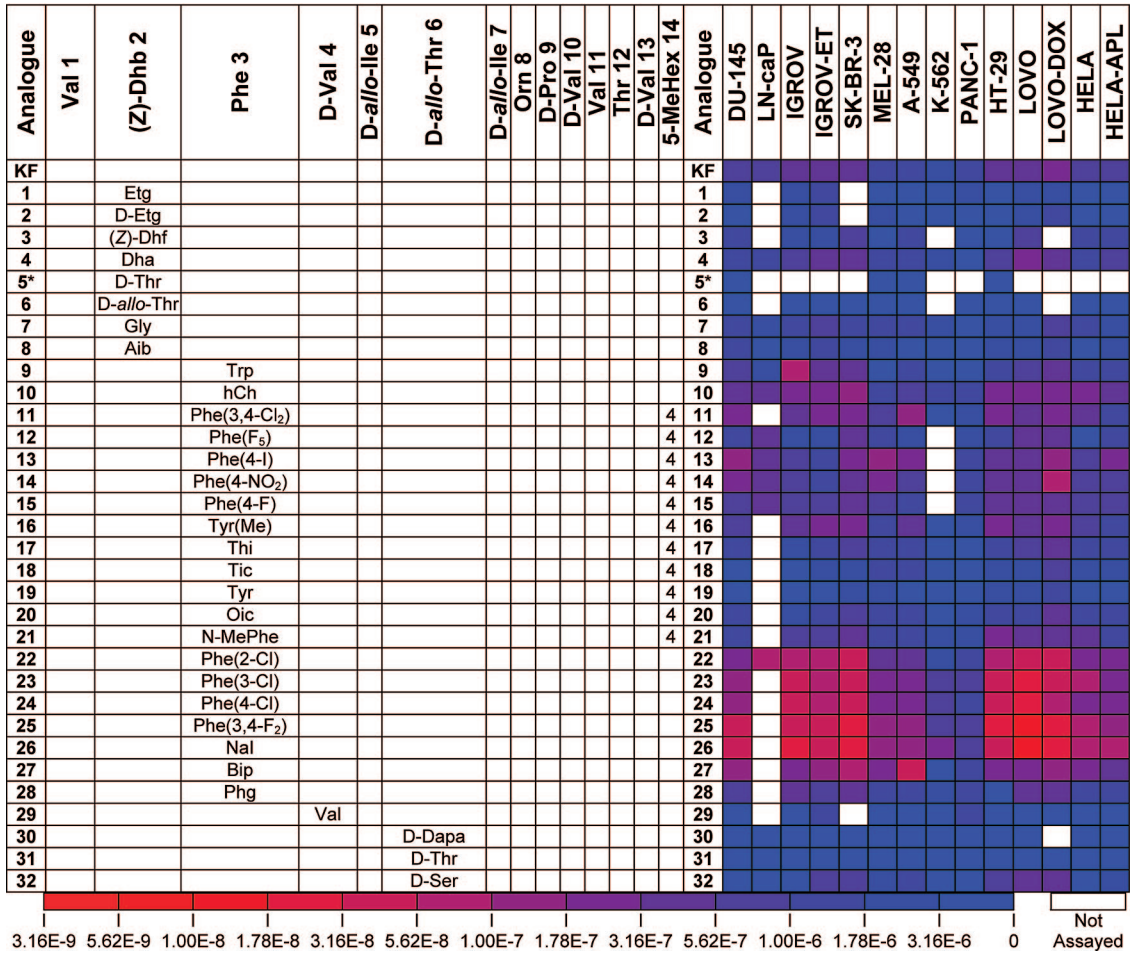
Given the ring nature of domain A, the L/D configuration of the α-carbon of the backbone is crucial. Compound **29** explored the possibility of Val(4) instead of D-Val.[25] This analogue lost all activity, thereby confirming the importance of the conformation of the cycle.

A presumably key amino acid in this domain is (*Z*)-Dhb. Analogues at this residue included other didehydroamino acids such as (*Z*)-Dhf (**3**) and Dha (**4**). These two compounds were as active as KF, thereby indicating that its role revolves around the rigidness and special configuration that didehydroamino acids confer to the main chain,[15] with no major relevance of their side chains. This point could be reinforced by the observation that analogues **1** and **2**, which contain L and D ethylglycine (Etg), the saturated versions of (*Z*)-Dhb, were less active than KF. Analogues **5** and **6**, which incorporate D-Thr and D-*allo*-Thr, respectively, in that position showed almost no activity. Compounds with aquiral amino acids such as Gly (**7**) and Aib (**8**) did not show activity either.

Finally, the D-*allo*-Thr that closes the ring was the subject of a set of analogues with D-Dapa (**30**), D-Thr (**31**), and D-Ser (**32**) (**30** is the aza version of **32**). Analogue **32** (D-Ser) maintained the activity of KF, which could indicate that the β-methyl group of the D-*allo*-Thr is not relevant for the stabilization of the structure. However, when this methyl group was misplaced in analogue **31**, activity was lost. When D-Ser (**32**) was substituted by D-Dapa (**30**), activity was lost again. These observations indicate that the pattern of hydrogen bond donors/acceptors or the freedom around the heterodetic bond may be relevant to the conformation.

So far, the term "activity" has been referred to the overall activity of the cell lines compared to KF. On closer inspection, we can rank activity depending on the tissue from which the tumor derives. Cell lines were as follows, in order of sensitivity from high to low: colon (HT-29, LOVO and LOVO-DOX), ovary (IGROV and IGROV-ET), breast (SK-BR-3), prostate (DU-145 and LN-caP) and melanoma, lung, bone marrow

Table 2. Domain A KF Analogues[a]



[a] The chemical structure of each modification can be found in Figure 2. "4" in 5-MeHex column indicates the presence of 4(*S*)-MeHex. Colored squares represent the GI_{50} (or IC_{50} on marked (∗) compounds) of each compound on the cells lines tested. Colored values at the bottom are expressed in mol/L.

(leukemia), and pancreas (MEL-28, A-545, K-562, and PANC-1). Depending on the analogue, this selectivity was altered; for example, for **9** (Trp3), selectivity for ovary cell lines was greater than for colon. For **11** [Phe(3,4-Cl2)] the selectivity for lung cell line was increased. For **27** (Bip3) the activity in lung was higher than in any other cell line.

Domain B Analogues. This domain is composed of seven amino acids. It is rich in aliphatic residues including a D-*allo*-Ile, three Val (two D and one L), and one D-Pro. The domain is completed with one Thr and one Orn, which is the only charged amino acid in the peptide at physiological pH. It is also noticeable that four of the residues have configuration D.

All analogues in which the chirality of any residue was switched lost their activity [Table 3, analogues **54** (Pro), **66** (D-Val11), and **76** (D-Thr)] except for **86** (Val13), which is the closest residue to the N-terminal aliphatic acid. The importance of the configuration of the α carbons of the amino acids suggests that this domain is not merely a link without structure between the other two domains but rather adopts or induces some sort of folding and/or interactions with another molecule.

One set of compounds we synthesized explored the elimination of residues starting from D-*allo*-Ile 7 (analogues **46**−**52**). None of these analogues showed activity. In all these analogues the positive charge of Orn was missing, which may explain the loss of activity. To confirm this assumption, we synthesized compound **53** in which the Orn was kept but the last three residues were removed. This synthesis resulted in no activity. To confirm that the loss of activity was not induced by the loss of the total chain length, compound **130** (Table 5), which included the C_{14} myristic acid at the N-terminus, was also explored simulating the approximate length of the total chain. This also resulted in no activity. These observations corroborate the need for chiral information on that part of the peptide.

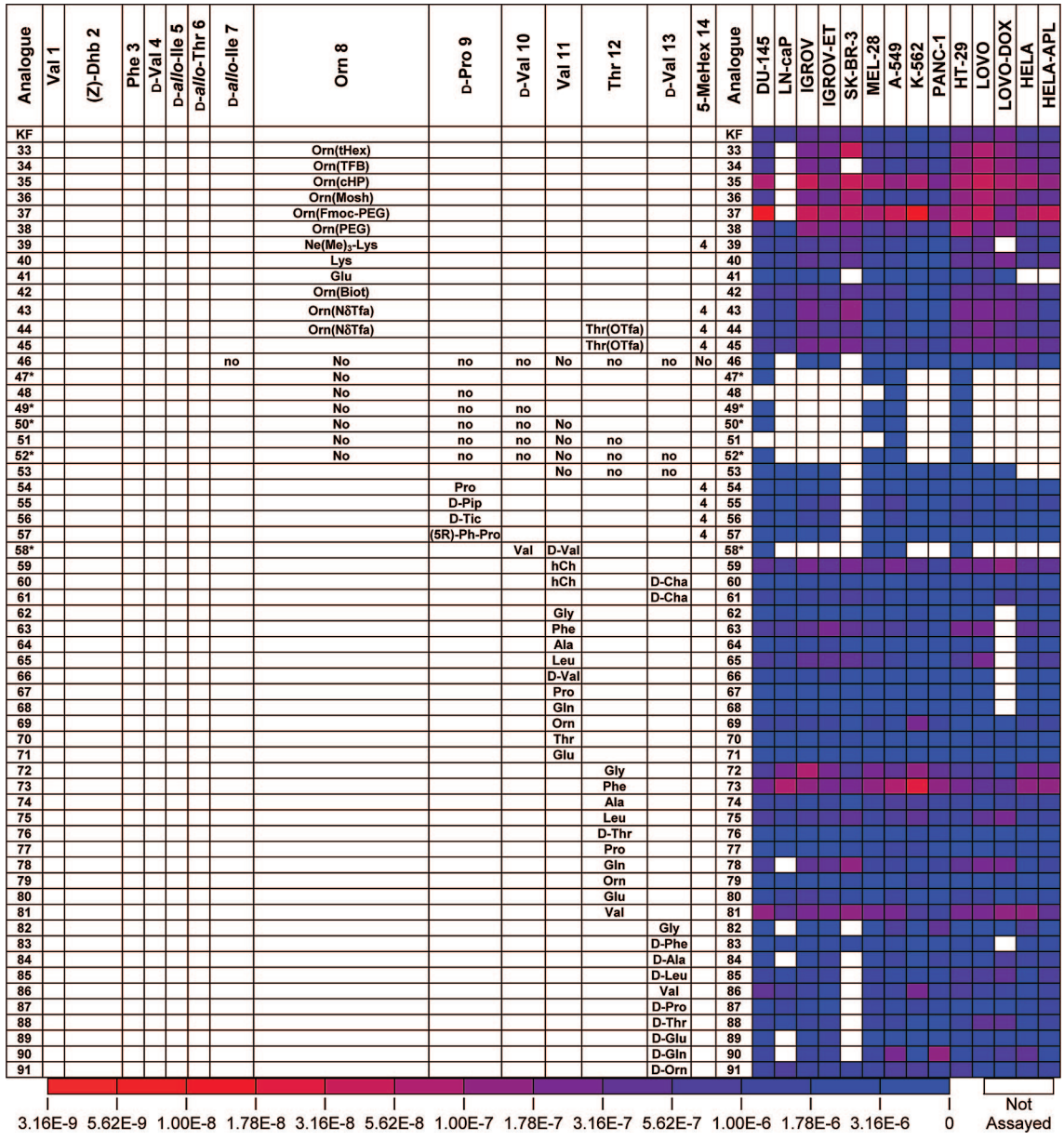
Moreover, when a residue was substituted by a distinct amino acid, the activity was conserved or even increased when nonpolar amino acids with the same chirality were used (analogues **59**, **63**, **65**, **73**, **75**, **81**, **83**, **85**, and **87**). The exception to this observation was, again, at position 13, where D-Val could not be replaced by the more hindered amino acid D-Cha (analogues **60** and **61**).

It is also of note that the hydroxyl group of the Thr was not required for antitumoral activity. Furthermore, the activity was increased with aliphatic substitutions (analogues **63** and **65**).

The substitution of D-Pro by L-Pro (**54**) and (*R*)-5Ph-L-Pro (**57**) led to no activity, pointing again to the existence of some sort of folding or recognition. Moreover, neither did the substitution by six-member ring D-Pro analogues D-Pip (**55**) and D-Tic (**56**) produce activity, which introduces another twist to the peptide conformational sensitivity in that region, since increasing from five- to six-member ring had less impact on the orientation of the groups than changing the chirality of the amino acid.

Compound **58** has historical relevance in the determination of KF structure. The first report on KF stereochemistry assigned the configurations D and L to Val (10) and Val (11), respec-

Table 3. Domain B KF Analogues[a]



[a] The chemical structure of each modification is shown in Figure 3. "4" in 5-MeHex column indicates the presence of 4(*S*)-MeHex. "no" indicates the absence of that residue in the compound. Colored squares represent the GI_{50} (or IC_{50} on marked (∗) compounds) of each compound on the cell lines tested. Colored values at the bottom are expressed in mol/L.

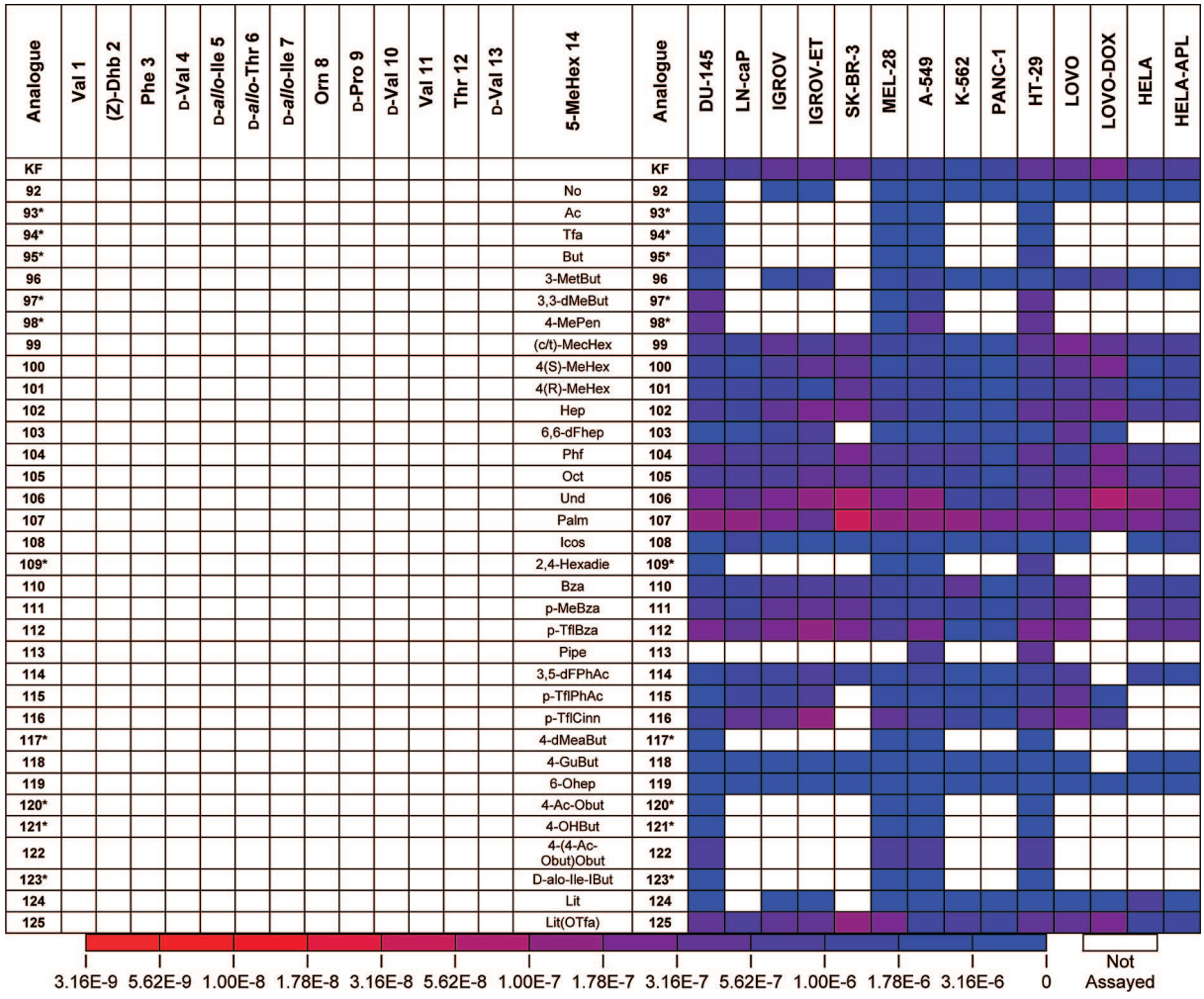
tively.[16] A review of this work originated a second assignment where the stereochemistry in these two residues was switched.[17] The two compounds were synthesized and compared to natural KF by NMR and by activity assay, solving the correct assignation without any doubt.[11] NMR spectra of these two compounds showed a single set of signals for KF, while analogue **58** presented two sets corresponding to cis/trans Pro isomerization. This was the first example of the sensitivity of the structure of domain B to the stereochemistry of its residues and how these affected its activity.

Furthermore, several analogues were prepared by direct reaction on the amino group of Orn in KF with diverse reagents, taking advantage of the regioselectivity of this group over the rest of the molecule as previously done on KF by other groups (analogues **33**−**39** and **42**−**44**).[18] We completed this set with the introduction of distinct amino acids instead of Orn (analogues **40** and **41**).

Here, we found the most active compounds, analogues **35** and **37**. In general, compounds with aliphatic groups coupled to the amino group of Orn were the most active (**34**−**37**). In analogue **37** the activity fell when the Fmoc group was cleaved, thereby generating a positive charge (analogue **38**). This is surprising, since this is the only charged amino acid in the whole sequence, and it seems to be not only unnecessary but undesirable. Moreover, these compounds open the door to conjugates with aliphatic drugs at that point that cannot only act in synergy with KF but can also increase its inner activity in the same way as analogues **35** and **37** do.

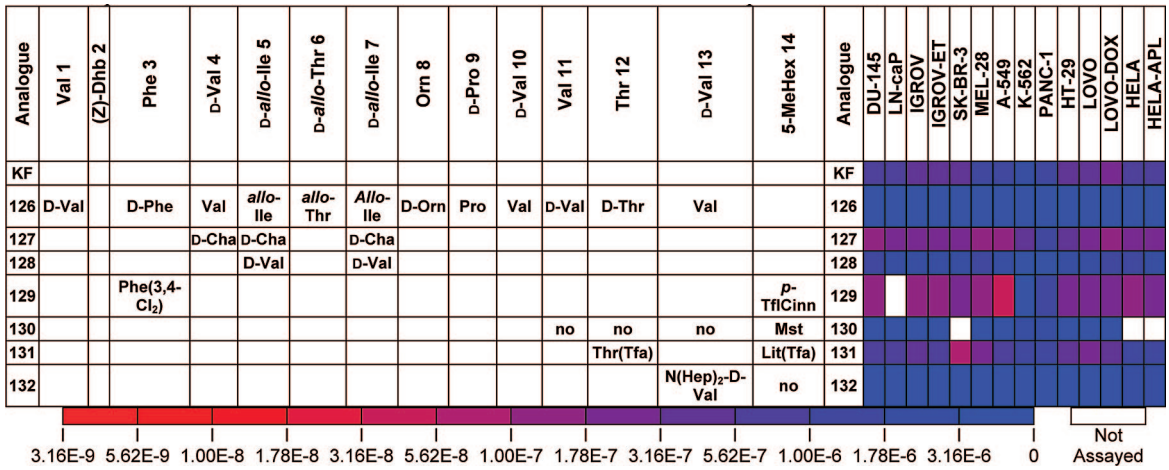
Another important datum is that Lys (**40**) is as active as Orn. This may seem strange because Lys is a coded amino acid and therefore more likely to be used in a natural product. Recently, it has been reported that this compound is also present (called as kahalalide S) in small amounts in *Elysia grandifolia* and the algae it feeds on, *Bryopsis plumose*.[3]

Table 4. Domain C KF Analogues[a]



[a] The chemical structure of each modification is shown in Figure 4. "no" indicates the absence of that residue in the compound. Colored squares represent the GI_{50} of each compound on the cell lines tested. Colored values at the bottom are expressed in mol/L.

Table 5. KF Analogues in More Than One Domain[a]



[a] The chemical structure of each modification is shown in Figures 2–4. "no" indicates the absence of that residue in the compound. Colored squares represent the GI_{50} of each compound on the cell lines tested. Color values at the bottom are expressed in mol/L.

The broad range of active compounds modified on Orn suggests that this residue is not involved in any recognition interface.

When we examined the changes in cell line selectivity, we observed that the analogues with hydrophobic molecules capping the δ-amino group of Orn not only were the most active but also showed the most changes in selectivity. Thus, analogue **37** [Orn(Fmoc-PEG)] was as active or even more active on prostate, lung, bone marrow, and pancreas cell lines than on colon, breast, and ovary cell lines. This effect was also observed

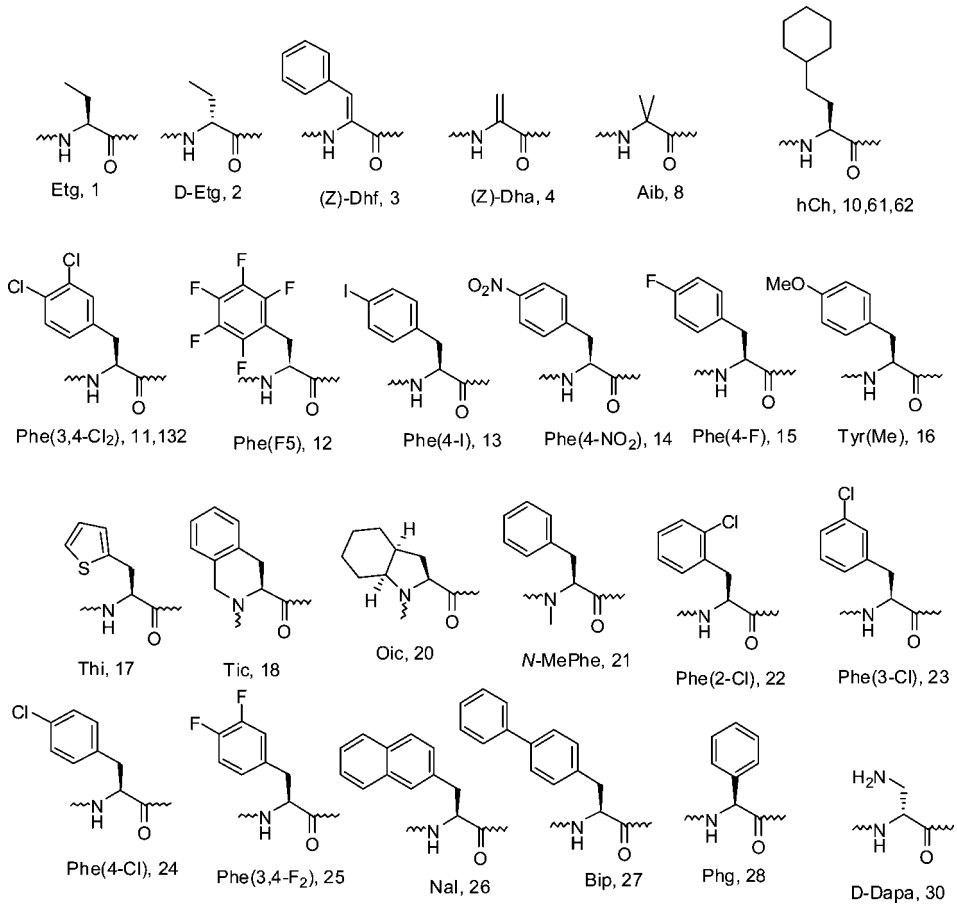


Figure 2. Building blocks used for the substitution of residues at domain A.

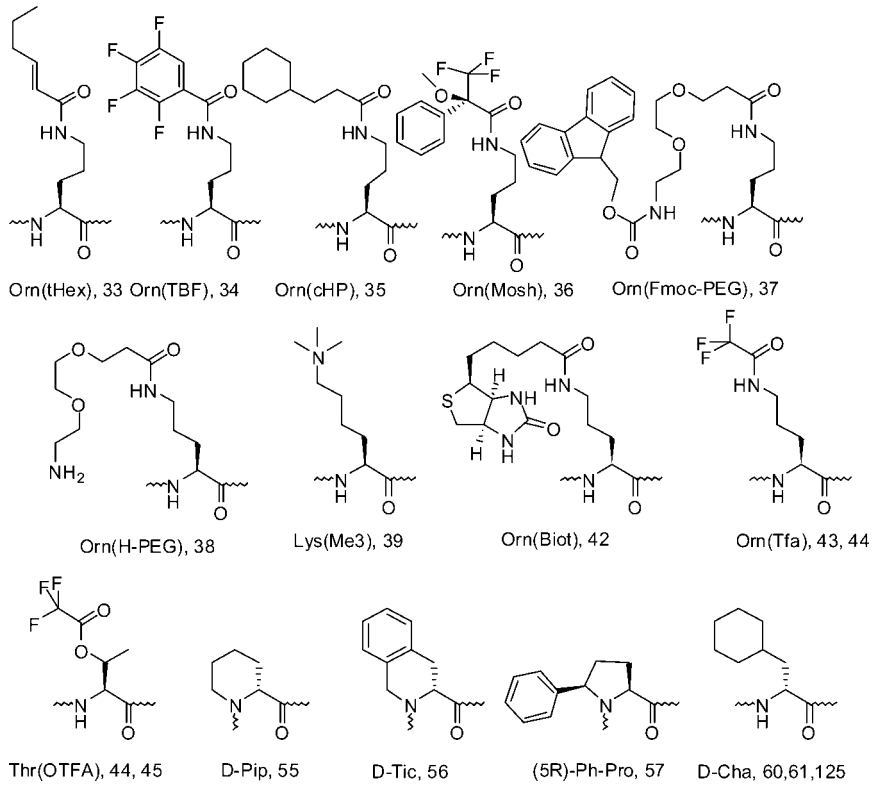


Figure 3. Building blocks used for the substitution of residues at domain B.

in analogue **35** [Orn(cHP)]. Analogues **33**, **36**, and **43** increased their activity in breast and colon cell lines with no major effect on the rest of cell lines. A remarkable case was compound **69** (Orn11 instead of Val). This compound not only broke the rule

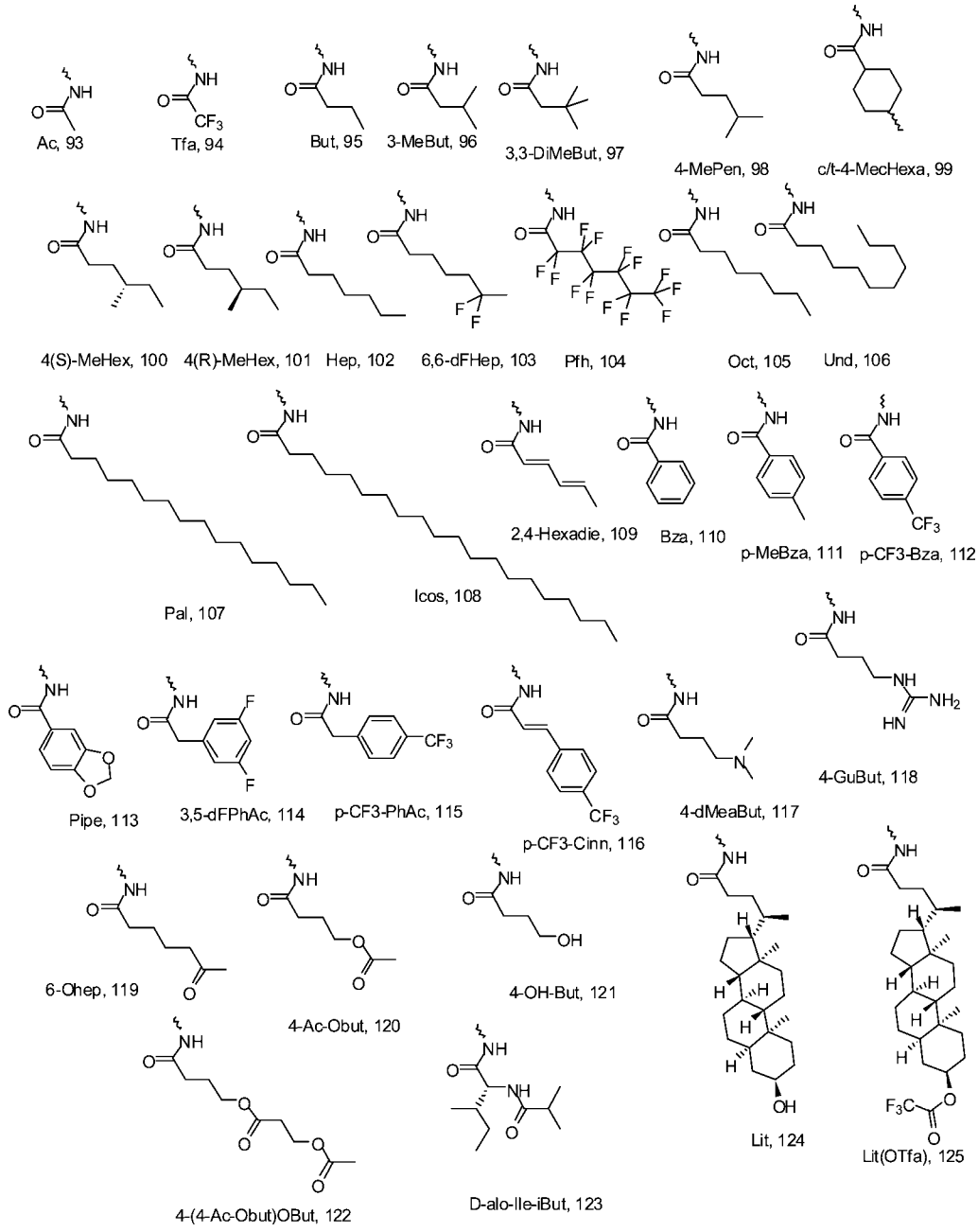


Figure 4. Building blocks used for the substitution of residues at domain C.

that aliphatic changes are preferred over polar substitutions but also affected mainly leukemia and showed very low activity against the rest of the panel cell lines.

Domain C Analogues. The analogues generated at this domain required an aliphatic group in this position. Any inclusion of polar groups able to generate hydrogen bonds decreased the activity (analogues **117**−**123**). Compounds with no terminal acid (**92**) or a short aliphatic group (analogues **93**−**98**) had lower activity than KF. Finally, the activity was maintained or increased when the N-terminal acid was substituted by a more aliphatic group (**99**−**116**). An exception to this rule was analogue **108** (icosanoic acid, C_{20}), where the low activity can be explained by the poor solubility of the compound. In compound **125** [Lit(OTfa)] the ester group generated hydrogen bonds but the relative size of the molecule must be considered.

A practical example of this is the finding of two new natural kahalalides called kahalalide R (KR) and kahalalide S (KS) found in the organism *Elysia grandifolia*.[2] In KR the 5-methylhexanoic acid in position 14 is substituted by 7-methyloctanoic acid, while in KS it is substituted by 5-hydroxy-7-methyloctanoic acid. KR is as active as KF, while KS is 20-fold less active than KF. It is noted that references 2 and 3 both use letters R and S to describe four new kahalalide compounds.

Compounds **100** and **101** showed an interesting effect. These two compounds incorporated the two enantiomers of 4-metylhexanoic acid, which is the compound closest to 5-methylhexanoic acid with a chiral center. The activity of the compound that incorporated the *R* enantiomer was lower than that which incorporated the *S* enantiomer, the activity of the latter being similar to the parent peptide. Further in vivo studies on these compounds showed that compound **100** has enhanced efficacy against breast and prostate xenografts.[19] On the basis of this observation, we chose to incorporate 4(*S*)-MeHex instead of 5-MeHex into other analogues.

Several analogues clearly showed higher in vitro activity than the parent peptide. Here, we highlight compounds **102**, **104**−**107**, **111**, **112**, **116**, and **125**, the most active analogues being **106** (undecanoic acid, Und), **107** (palmitic acid), **112** (*p*-TflBza), **116** (*p*-TflCinn), and **125** [Lit(OTfa)]. Compounds **111** and **112** were very similar, the only difference being that **112** had a trifluoromethyl group instead of a methyl group attached to the aromatic ring. These two groups exerted an opposite inductive effect on the aromatic ring, with enhanced electronic density on compound **111** and lower electronic density on compound **112**, the latter being more active.

The wide array of molecules that maintained or increased the activity in domain C and the common characteristic of their high aliphatic behavior suggest that this domain does not interact selectively with any other molecule and that its main function is as an aliphatic buoy.

As seen in other domains, a number of compounds showed a change in their selectivity against the cell lines assayed. Analogues **102**, **106**, **107**, **112**, **116**, and **125** had predominant activity against ovary cell lines. Compound **107** also significantly increased activity against prostate cell lines.

Multidomain Analogues. Table 5 shows the analogues modified in more than one domain. The first entry (**126**) corresponds to the KF enantiomer, which showed no activity.

Analogues **127** and **128** showed once again that more hydrophobic residues (D-Cha) than those of Val or Ile increased the activity, and when the number of methylenes was reduced (e.g., substituting D-*allo*-Ile by Val), the activity diminished. This behavior can also be explained by conformational changes induced by the volume of the side chains, since Val is less voluminous than D-*allo*-Ile, thereby allowing more conformations.

Analogue **129**, which is the combination of analogues **11** and **116**, showed increased activity compared to KF but not significantly higher than that of compound **11**.

Finally, in compound **132**, the amino group of D-Val13 was alkylated with two heptane chains. This substitution led to no activity, which can be explained by the generation of a very stable positive charge at this nitrogen.

In this panel we observed changes in the cell line selectivity of compounds **127**, **129**, and **131**, where again the compounds were most active on ovary cell lines. Also worth mentioning is the increased activity against prostate cell lines in compound **127**. In compound **129** the activity against lung cell line (A549) was very high because of the presence of Phe(3,4-Cl_2) at the third position, as with compound **11.**

Conclusions

Kahalalide F has a more defined structure than expected. This is not surprising in cyclic domain A, but astonishingly it also occurs in domain B, which a priori is much more flexible. KF is highly sensitive to stereotopical changes, namely, those changes that affect chirality at the α-carbon of the residue. KF is not sensitive to side chain substitutions in almost every residue, the electronic density of the substituting chain being more relevant than its volume. For almost every side chain, it was possible to find a distinct side chain that could preserve or even improve the activity.

Our results on enantiomer **126** suggest that KF interacts with at least one chiral compound to develop cytotoxicity. In this regard, we found a more hindered replacement in each side chain able to keep or improve the activity; the interactive region should be pointed at the backbone of the molecule, the cycle being the more appealing target.

The activity can be increased by enhancing the hydrophobicity at any position on the molecule, solubility in water being a limiting factor. Domain C is the most sensitive to this rule, since it does not allow the inclusion of electronic-donor atoms such as oxygen or nitrogen. Domain C accepts only highly hydrophobic groups with a low heteroatom load. This observation suggests that this domain interacts with a highly hydrophobic and unspecific environment such as a membrane, possibly with the lysosomal membrane since KF affects this organule.[7]

Analogues with a hydrophobic group attached to the ε-amino group of Orn open the door to conjugated drugs (i.e., **33**, **34**, **35**, and **37**). These compounds are more active than KF and compounds with polar groups. This finding implies that KF activity can be enhanced while simultaneously using KF as a carrier of a hydrophobic drug with solubility or transport limitations, thereby obtaining a synergic effect.

As an extension of the above discussion, we consider this set of analogues to be useful also as control peptides in research into the biological mechanism of KF. By examination of the distinct behavior displayed by some analogues compared to KF, it is possible to identify the role played by each part of the peptide in the overall activity. An example of this approach would be the enantiomer analogue **126**. Studying its capacity to internalize the cell would provide information as to whether KF is actively internalized by a carrier or unspecifically internalized.

Also relevant, this set of compounds opens up the possibility of modulating KF selectivity when used in distinct cell lines. It would therefore be feasible to develop analogues with different tissue specificity. This would allow the development of KF-related compounds targeted to a particular kind of tumor, thus obtaining a wider therapeutic window.

Finally, now that the impact of backbone stereochemistry on KF activity is known, analogues that affect this stereochemistry can be used to perform structural studies of KF by means of molecular modeling and NMR. These analogues are **1**, **2**, **3**, **7**, **8**, **30**−**32** in domain A and **54**−**58**, **62**, **66**, **76**, and **86** in domain B.

Experimental Section

Cl-TrtCl-resin, protected Fmoc-amino acid derivatives, HOBt, and HOAt were from ABI (Framingham, MA), Bachem (Bubendorf, Switzerland), and NovaBiochem (Läufelfingen, Switzerland). 4-MeHex derivatives were from Narchem. HATU was from ABI (Framingham, MA). DIPEA, DIPCDI, EDC·HCl, piperidine, and TFA were from Aldrich (Milwaukee, WI). DMF and CH_2Cl_2 were from SDS (Peypin, France). Acetonitrile (HPLC grade) was from Scharlau (Barcelona, Spain). All commercial reagents and solvents were used as received with the exception of CH_2Cl_2, which was passed through an alumina column to remove acidic contaminants. Alloc-amino acids were prepared essentially as described,[20] and alloc-*Z*-Dhb-Phe-OH compounds were prepared as described[11] and also in Supporting Information.

HRMS was performed in LTQ-FT Ultra from ThermoScientific in nanospray mode by infusion and H_2O/CH_3CN (1:1) + 0.1% formic acid as a solvent. MALDI-TOF and ES-MS analyses of peptide samples were performed in a PerSeptive Biosystems Voyager DE RP, using DHB matrix, on a Waters Micromass ZQ spectrometer and on an Agilent ion trap 1100 series LC/MSDTrap. Peptide−resin samples were hydrolyzed in 12 N aqueous HCl−propionic acid (1:1) at 155 °C for 1−3 h, and peptide-free samples were hydrolyzed in 6 N aqueous HCl at 155 °C for 1 h. Subsequent amino acid analyses were performed on a Beckman System 6300 autoanalyzer. ^{1}H NMR spectroscopy [^{1}H, NOESY, TOCSY at 278 K] was performed on a Varian Unity Plus (500 MHz). Chemical shifts (δ) are expressed in parts per million downfield from TMS. Coupling constants are expressed in hertz. Analytical HPLC was

carried out on a Waters instrument comprising two solvent delivery pumps (Waters 1525), automatic injector (Waters 717 autosampler), dual wavelength detector (Waters 2487), and system controller (Breeze, version 3.20) and on an Agilent 1100 instrument comprising two solvent delivery pumps (G1311A), automatic injector (G1329A), and DAD (G1315B). UV detection was at 215 or 220 nm, and linear gradients consisted of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA).

Here, we describe the synthesis of compound **100** as a general example. Detailed synthesis and characterization of the rest of the compounds can be found in Supporting Information.

(4*S*)-MeHex-D-Val-ThrVal-D-Val-D-Pro-Orn-D-*allo*-Ile-ciclo[D-*allo*-Thr-D-*allo*-Ile-D-Val-Phe-(*Z*)-Dhb-Val], [(4*S*)-MeHex^{14}]KF, Compound 100. Step 1 of 7. H-D-Val-O-TrtCl-Resin. Cl-TrtCl-resin (1 g, 1.64 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 × 0.5 min), and a solution of Fmoc-D-Val-OH (238 mg, 0.7 mmol) and DIPEA (0.41 mL, 2.41 mmol) in CH_2Cl_2 (2.5 mL) was added. The mixture was then stirred for 15 min. Extra DIPEA was then added (0.81 mL, 4.75 mmol), and the mixture was stirred for 45 min. The reaction was completed by addition of MeOH (800 μL, 19.78 mmol), after stirring for 10 min. The Fmoc-D-Val-O-TrtCl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), piperidine as indicated in the general procedures section, and DMF (5 × 0.5 min). The loading was 0.50 mmol/g calculated by Fmoc determination.

Step 2 of 7. Fmoc-D-*allo*-Ile-D-*allo*-Thr(Val-Alloc)-D-*allo*-Ile-D-Val-O-TrtCl-Resin. Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Thr-OH (free hydroxy group) (683 mg, 2 mmol, 4 equiv), and Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv) were added sequentially to the H-D-Val-O-TrtCl-resin described above using DIPCDI (310 μL, 2 mmol, 4 equiv) and HOBt (307 mg, 2 mmol, 4 equiv) in DMF (2.5 mL). In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of the Fmoc group and washings were carried out as described in the Supporting Information. Alloc-Val-OH (502 mg, 2.5 mmol, 5 equiv) was coupled using DIPCDI (387 mg, 2.5 mmol, 5 equiv) in the presence of DMAP (30.6 mg, 0.25 mmol, 0.5 equiv) and DIPEA (88 μL, 0.5 mmol, 1 equiv) for 45 min. This coupling was repeated under the same conditions twice. An aliquot of the peptidyl-resin was treated with TFA, and the HPLC results (t_R = 7.8 min, conditions S, column D) of the crude product obtained after evaporation showed a purity of >98%. ESMS, calcd for $C_{45}H_{63}N_5O_{11}$, 849.45. Found: *m*/*z* 850.1 $[M + H]^+$.

Step 3 of 7. Fmoc-D-Val-D-Pro-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(Val-Alloc)-D-*allo*-Ile-D-Val-O-TrtCl-Resin. The Fmoc group was removed from the peptidyl-resin obtained in step 2 using piperidine/DMF 20:80 for 1 × 1 min, 2 × 5 min, 1 × 10 min, and Fmoc-Orn(Boc)-OH (912 mg, 2 mmol, 4 equiv), Fmoc-D-Pro-OH (843 mg, 2.5 mmol, 5 equiv), and Fmoc-D-Val-OH (255 mg, 2.5 mmol, 5 equiv) were sequentially incorporated using DIPCDI (310 μL, for 2.0 mmol and 4 equiv; 388 μL, for 2.5 mmol and 5 equiv) and HOBt (307 mg, for 2.0 mmol and 4 equiv; 395 mg, for 2.5 mmol and 5 equiv) for 90 min. The ninhydrin test after incorporation of Orn and D-Pro was negative. The chloranil test after incorporation of D-Val was slightly positive, and this residue was recoupled with Fmoc-D-Val-OH (678 mg, 2.0 mmol, 4 equiv), DIPCDI (310 μL, 2.0 mmol, 4 equiv) and HOBt (307 mg, 2.0 mmol, 4 equiv) for 90 min. An aliquot of the peptidyl-resin was treated with TFA, and the HPLC results (t_R = 10.1 min, conditions S, column D) of the crude product obtained after evaporation showed a purity of >98%. MALDI-TOF-MS, calcd for $C_{65}H_{97}N_9O_{16}$, 1,259.71. Found: *m*/*z* 1282.16 $[M + Na]^+$.

Step 4 of 7. (4*S*)-MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(Val-Alloc)-D-*allo*-Ile-D-Val-O-TrtCl-resin. The Fmoc group was removed, and Fmoc-Val-OH (678 mg, 2 mmol, 4 equiv), Fmoc-Thr(*t*Bu)-OH (992 mg, 2.5 mmol, 5 equiv), Fmoc-D-Val-OH (678 mg, 2 mmol, 4 equiv), and (4*S*)-MeHex-OH (195 mg, 1.5 mmol, 3 equiv) were added sequentially to the peptidyl-resin (step 3) using DIPCDI (233 μL, for 1.5 mmol and 3 equiv; 310 μL, for 2 mmol and 4 equiv; 388 μL, for 2.5 mmol and 5 equiv) and HOBt (230 mg, for 1.5 mmol and 3 equiv; 307 mg, for 2 mmol and 4 equiv; 395 mg, 2.5 mmol and 5 equiv) for 90 min. In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of the Fmoc group and washings were carried out as described in the general procedures section.

Step 5 of 7. (4*S*)-MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(Val-*Z*-Dhb-Phe-Alloc)-D-*allo*-Ile-D-Val-O-TrtCl-resin. The Alloc group was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv) under Ar atmosphere, and Alloc-Phe-*Z*-Dhb-OH (666 mg, 2 mmol, 4 equiv) and HOAt (273 mg, 2 mmol, 4 equiv) were dissolved in DMF (1.25 mL) and added to the peptidyl-resin. DIPCDI (310 μL, 2 mmol, 4 equiv) was then added, and the mixture was stirred for 5 h, after which the ninhydrin test was negative. After washings with DMF and CH_2Cl_2, an aliquot of the peptidyl-resin was treated with TFA−H_2O (1:99) for 1 min and the product was characterized by MALDI-TOF-MS, calcd for $C_{88}H_{146}N_{14}O_{21}$, 1735.08. Found: *m*/*z* 1758.67 $[M + Na]^+$, 1,774.62 $[M + K]^+$.

Step 6 of 7. (4*S*)-MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(Val-*Z*-Dhb-Phe-H)-D-*allo*-Ile-D-Val-OH. After washings with DMF and CH_2Cl_2, the Alloc group was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv) under Ar atmosphere. The protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL), and the H_2O was partially removed under reduced pressure, appearing as a light-yellow precipitation in the remaining water. Acetonitrile was then added to dissolve the solid that appeared during H_2O removal until the solution was clear. Then lyophilization gave 639 mg (387 μmol, 77% yield) of the title compound with a purity of >95% as shown by HPLC (condition R, column C, t_R = 10.5 min).

Step 7 of 7. (4*S*)-MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-D-*allo*-Ile-*cyclo*(D-*allo*-Thr-D-*allo*-Ile-D-Val-Phe-*Z*-Dhb-Val). The protected peptide from step 6 (639 mg, 387 μmol) was dissolved in CH_2Cl_2 (390 mL, 1 mM). Then we added compounds in the following order: HOBt (237 mg, 1.55 mmol) dissolved in the minimum volume of DMF, DIPEA (203 μL, 1.16 mmol, 2.3 equiv), and DIPCDI (240 μL, 1.55 mmol, 4 equiv). The mixture was stirred for 1 h, and the course of the cyclization step was monitored by HPLC. The solvent was removed by evaporation under reduced pressure. The protected cyclic peptide was dissolved in TFA−H_2O (19:1, 85 mL), and the mixture was stirred for 1 h. The solvent was removed by evaporation under reduced pressure, dioxane was added (30 mL), and the solvent was removed by evaporation under reduced pressure (the process was repeated three times). H_2O (40 mL) was then added, and the solution was lyophilized. The crude product was purified by HPLC (Kromasil C_8 5 μm, 205 mm × 50 mm), isocratic 44% acetonitrile (+0.05% TFA) in water (+0.05% TFA), 55 mL/min, detection at 220 nm, to give the title product (192 mg, 0.13 mmol, 26% yield, 92.3%). HRMS *m*/*z*: found $(M + H)^+$ 1477.937 91, calcd for $C_{75}H_{125}N_{14}O_{16}$ $(M + H)^+$ 1477.939 25. The ^1H NMR (2.5 mM, 500 MHz, H_2O-D_2O (9:1) data of the compound are shown in Table 6.

Bioassays for Antitumoral Screening. Since activity data had been collected over several months, the activity value of each compound was normalized using the activity of KF on the assay day and the mean KF activity.

Assay A. The objective of this assay is to interrupt the growth of an in vitro tumor cell culture by means of continued exposure of the cells to the sample to be tested. Cancer cell lines used are listed in Table 7.

Inhibition of Cell Growth by Colorimetric Assay. A colorimetric type of assay using the sulforhodamine B reaction was adapted for quantitative measurement of cell growth and viability.[21] This assay employed 96-well cell culture microplates of 9 mm diameter.[22,23] Most of the cell lines were obtained from American Type Culture Collection (ATCC) derived from a range of types of human cancer. Cells were maintained in RPMI 1640 10% FBS,

Table 6. [4(*S*)-MeHex[14]]KF (Compound **100**) ^{1}H NMR Chemical Shifts

residue	N−H	Hα	Hβ	other
(*Z*)-Dhb	9.59 (s)		6.63 (q, J =7.5 Hz)	1.19 (d, γ-CH_3)
D-*allo*-Ile 1	8.82 (d, J = 9.0 Hz)	4.42	1.87	1.25, 1.09, 0.82 (γ-CH_2, γ-CH_3, δ-CH_3)
L-Phe	8.75 (d, J =5.5 Hz)	4.63	3.08 (m)	7.31 (2H Ar, t), 7.25 (3H Ar, d)
D-*allo*-Thr	8.67(d, J = 9.0 Hz)	4.64	5.05 (m)	1.21 (γ-CH_3)
D-Val 3	8.13 (d, J = 7.5 Hz)	4.33	2.01	0.90 (2 γ -CH_3)
L-Orn	8.29 (d, J = 7.5 Hz)	4.31	1.66 (2H)	1.88 (γ-CH_2), 2.96 (bs, δ-CH_2), 7.56 (ε-NH_3^+)
D-*allo*-Ile 2	7.92 (d)	4.18	1.80	1.25, 1.09, 0.81 (γ-CH_2, γ-CH_3, δ-CH_3)
D-Val 5	8.01 (d)	4.08	2.07	0.87 (2 γ-CH_3)
L-Thr	8.19 (d, J = 7.5 Hz)	4.29	4.14 (m)	1.13 (γ-CH_3)
D-Val 2	7.89 (d, J = 7.5 Hz)	4.32	2.11	0.78 (γ-CH_3)
L-Val 4	8.04 (d)	4.10	2.07	0.90 (2 γ-CH_3)
L-Val 1	7.19 (d, J = 9.0 Hz)	4.02	1.52	0.75 (γ-CH_3), 0.65 (d, γ-CH_3)
D-Pro		4.36	2.23, 1.99 (m, β-CH_2), 1.85 (m, γ-CH_2), 3.83 (1H, m, δ-CH_2), 3.64 (1H, m, δ-CH_2)	
4(*S*)-MeHex		2.26 (2H)	1.57 (β-CH_2), 1.26, 1.10, 1.33, 0.79 (δ-CH_2, δ-CH_3, γ-CH, ε-CH_3)	

Table 7. Cellular Lines Used in Assay A

name	no. ATCC	species	tissue	description
DU-145	HTB-81	human	prostate	prostate carcinoma, not androgen receptors
LN-caP	CRL-1740	human	prostate	prostate adenocarcinoma, with androgen receptors
IGROV		human	ovary	ovary adenocarcinoma
IGROV-ET		human	ovary	ovary adenocarcinoma, characterized as ET-743 resistant cells
SK-BR-3	HTB-30	human	breast	breast adenocarcinoma, Her2/neu+, (pleural effusion)
MEL-28	HTB-72	human	melanoma	malignant melanoma
A-549	CCL-185	human	lung	lung carcinoma "NSCL"
K-562	CCL-243	human	bone marrow;	chronic myelogenous leukemia
PANC-1	CRL-1469	human	pancreas	pancreatic epitheloid carcinoma
HT-29	HTB-38	human	colon	colon adenocarcinoma
LoVo	CCL-229	human	colon	colon adenocarcinoma
LoVo-Dox		human	colon	colon adenocarcinoma (MDR)
HELA	CCL-2	human	cervix	cervix epitheloid carcinoma
HELA-APL	CCL-3	human	cervix	cervix epitheloid carcinoma, characterized as aplidine resistant cells

supplemented with 0.1 g/L penicillin and 0.1 g/L streptomycin sulfate and then incubated at 37 °C, 5% CO_2, and 98% humidity. For the experiments, cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium before plating. Cells were seeded in 96-well microtiter plates, at 5×10^3 cells per well in aliquots of 195 μL medium, and allowed to attach to the plate surface by growing in drug-free medium for 18 h. Afterward, samples were added in aliquots of 5 μL in a range between 10 and 8 μg/mL, and dissolved in DMSO/EtOH/PBS (0.5:0.5:99). After 48 h of exposure, the antitumor effect was measured by the sulforhodamine B (SRB) methodology: cells were fixed by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid and then incubated for 60 min at 4 °C. Plates were washed with deionized water and dried. An amount of 100 μL of SRB solution (0.4 wt %/vol in 1% acetic acid) was added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, and the bound stain was solubilized with Tris buffer. Optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 490 nm. The values for mean ± SD of data from triplicate wells were calculated. Some parameters for cellular responses were calculated: GI = growth inhibition, TGI = total growth inhibition (cytostatic effect), and LC = cell killing (cytotoxic effect).

Assay B. This assay used 24-well multidishes of 16 mm in diameter. The tumor cell lines employed were A-549 (ATCC CCL 185) (monolayer culture of a human lung carcinoma), HT-29 (ATCC HTB-38) (monolayer culture of a human colon carcinoma), MEL-28 (ATCC HTB-72) (monolayer culture of a human melanoma), and DU-145 (ATCC HBTB-81) (monolayer culture of a human prostate carcinoma).

Cells were maintained in the logarithmic phase of growth in Eagle's minimum essential medium, with Earle's balanced salts, with nonessential amino acids, with 2.0 mM L-glutamine, without sodium bicarbonate (EMEM/neaa), supplemented with 10% fetal calf serum (FSC), 10^{-2} M sodium bicarbonate, and 0.1 U/L penicillin G + 0.1 g/L streptomycin sulfate. For the experiments, cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium before plating.

A-549, HT-29, MEL-28, and DU-145 cells were seeded into 16-mm diameter wells at 1×10^4 cells per well in 1 mL aliquots of EMEM 5% FCS containing a range of concentrations of the sample to be tested. A separate set of cultures without drug was seeded as control of growth to ensure that cells remained in an exponential phase of growth. All determinations were carried out in duplicate. After 3 days of incubation at 37 °C, 5% CO_2 in a 98% humid atmosphere, cells were stained with 0.1% crystal violet. An approximate IC_{50} was determined by comparing the growth in wells containing the drug to the growth in control wells.

For quantification of the activity, after the incubation time cells were trypsinized and counted in a Coulter counter ZM. All counts (net cells per well) represented the average of duplicate wells, % G, percent of growth relative to cultures without drug. The results of these assays were used to generate dose−response curves from which more precise IC_{50} values were determined (sample concentration that produces 50% cell growth inhibition).

The results obtained may predict the usefulness of a certain drug as a potential cancer treatment. For this technique, compounds that showed IC_{50} values smaller than 1 μM were selected for further studies. IC_{50} data allowed us to predict that a drug not only may be cystostatic but may also provide potential benefit in terms of tumor reduction.

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Supporting Information Available: Synthesis procedures and characterization by HPLC and MS, as well as in vitro assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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