Journal of Medicinal Chemistry

Volume 46, Number 26 December 18, 2003

Award Address

Design and Synthesis of Novel Bioactive Peptides and Peptidomimetics

Roger M. Freidinger†

Department of Medicinal Chemistry, Merck Research Laboratories, West Point, Pennsylvania 19486

Received September 25, 2003

Introduction

Ralph F. Hirschmann has been a leader in efforts to harness the therapeutic potential of peptides for 40 years. He recognized the importance of synthetic organic chemistry and peptide conformation in peptide research, and he and his collaborators have made numerous important contributions in this field. He has been an inspiration to my research in peptides and related areas, and I am honored to join the distinguished group of chemists who have received the Ralph F. Hirschmann Award in Peptide Chemistry. This award is especially significant for me as well because my own research in peptide chemistry began when I joined Merck in 1975 while Ralph Hirschmann was head of the Department of Medicinal Chemistry in West Point.

Biologically active peptides are recognized to have significant therapeutic potential but serious limitations, especially for oral dosing. The challenge continues to be to overcome these limitations in order to develop useful therapeutic agents. Our group at Merck has taken two general approaches to this problem. The first is a structure/conformation/activity strategy based on the structure of the native peptide. This approach requires the integration of chemical synthesis, biological assays, and conformational studies, including spectroscopic analyses and molecular modeling. A number of our programs have begun with a second approach involving screening of natural product and synthetic collections for novel structural leads. The majority of these latter projects targeted peptide receptors, but they involved mostly non-peptide chemistry and will not be covered in this article. An unrelated approach has utilized peptide substrates for enzymes in a prodrug tumor targeting strategy for parenterally administered cancer chemotherapy.

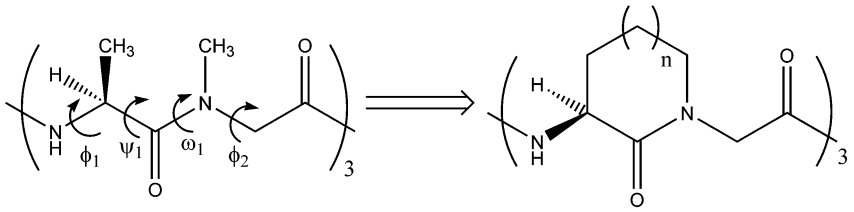
Covered in this article are two areas of research under the first general approach involving our design and synthesis of dipeptide lactams as conformational constraints for peptides and our development of cyclic hexapeptide somatostatin agonists. Under the third strategy, the design and synthesis of prostate-specific antigen substrate−cytotoxic drug conjugates as applied to doxorubicin and vinblastine will be described.

Dipeptide Lactams

The idea to utilize dipeptide lactams as conformational constraints in peptides originated in a study of C3 symmetric cyclic hexapeptides that were of interest for potentially improving feed efficiency in ruminant animals. These cyclic hexapeptides, typified by cyclo-(Ala-Sar)$_3$ (**1**), have cation binding properties, and they shift ruminant stomach bacterial fermentation toward the production of less volatile products.[1] As part of an extensive structure−activity study of these peptides, we wanted to explore the effect of peptide backbone conformational restriction on activity. We began to use the Merck Molecular Modeling System[2] to study conformations of these peptides, and we found it useful for generating hypotheses to be tested in the laboratory. In addition to the common strategies of incorporating D-amino acids and other unnatural amino acids, molecular modeling studies suggested that building a bridge from the α-position of alanine to the nitrogen of the succeeding sarcosine in the prototype structure would be an effective constraint. The resultant lactam would restrict the peptide bond (ω) to the trans conformation, and the local small ring conformation would restrict the

† Phone: 215-652-5665. Fax: 215-652-3971. E-mail: roger_freidinger@merck.com.

10.1021/jm030484k CCC: $25.00
Published on Web 11/21/2003



1

Figure 1. Initial application of dipeptide lactams.

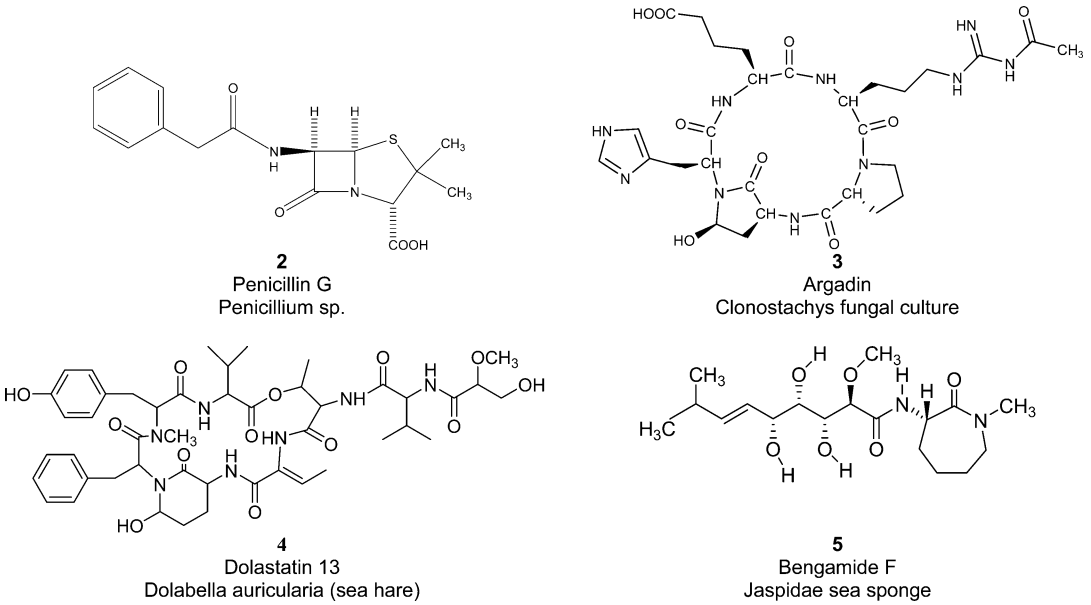


Figure 2. Natural products with dipeptide lactams.

ψ_1 backbone dihedral angle. The proximity of the lactam ring would also restrict conformations of dihedral angles ϕ_1 and ϕ_2. Furthermore, the size of the lactam ring could potentially bias the peptide backbone conformation in different ways (see Figure 1).

It is of interest that examples of such lactam rings in peptide backbones have appeared in natural products (Figure 2). At the time we were planning our studies, the only known examples were the β-lactam antibiotics such as penicillin G (**2**).[3,4] Recently, an example of a γ-lactam was found in the fungal product argadin (**3**),[5,6] and a δ-lactam was characterized in the marine natural product dolastatin 13 (**4**).[7] Although not a complete dipeptide lactam, the ϵ-lactam in the marine natural product bengamide F (**5**) is structurally related.[8] Very recently, a novel sulfenyl amide containing δ-lactam dipeptide was shown to play a key role in the redox regulation of protein tyrosine phosphatase 1B.[9]

In considering synthetic approaches to dipeptide lactams of various ring sizes, we established several objectives. The lactams should be readily synthesized in protected form. The synthetic methods should produce optically pure products, and these compounds should be readily incorporated into peptides using standard protocols. Commercially available, chirally pure, protected α-amino acids were attractive starting materials for achieving these objectives. Three general lactam ring-forming approaches were developed involving key steps of intramolecular acylation, intramolecular alkylation, and a condensation of an appropriate amino acid derivative with a carbonyl compound.[10]

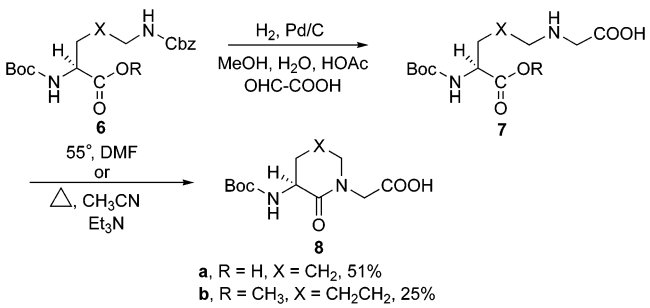


Figure 3. Synthesis of δ- and ϵ-lactams.

The first dipeptide lactam was prepared in late 1976 by way of the intramolecular acylation approach utilizing N^{α}-Boc,N^{δ}-Cbz-L-ornithine (**6a**) as the starting material (Figure 3). Catalytic hydrogenolysis was employed to remove the side chain protecting group. By including glyoxylic acid in the reaction mixture, the newly freed amine was reductively alkylated in the same pot. The reaction was stopped when the desired monoalkylation product was maximal. This carboxymethylated compound **7a** smoothly cyclized to the δ-lactam dipeptide **8a** with warming in dimethylformamide (DMF) in an overall yield of 51%. The chiral center remained intact during these operations. The corresponding lysine precursor **6b** was converted to the ϵ-dipeptide lactam **8b**. In this case, the cyclization was

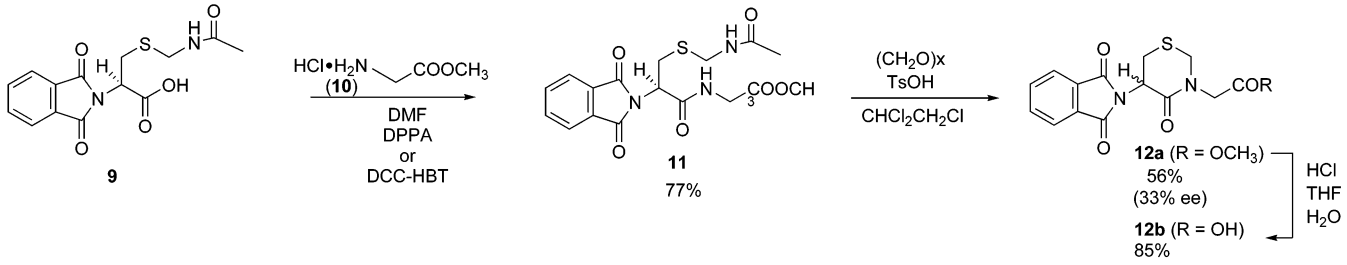


Figure 4. Synthesis of thiazine lactams.

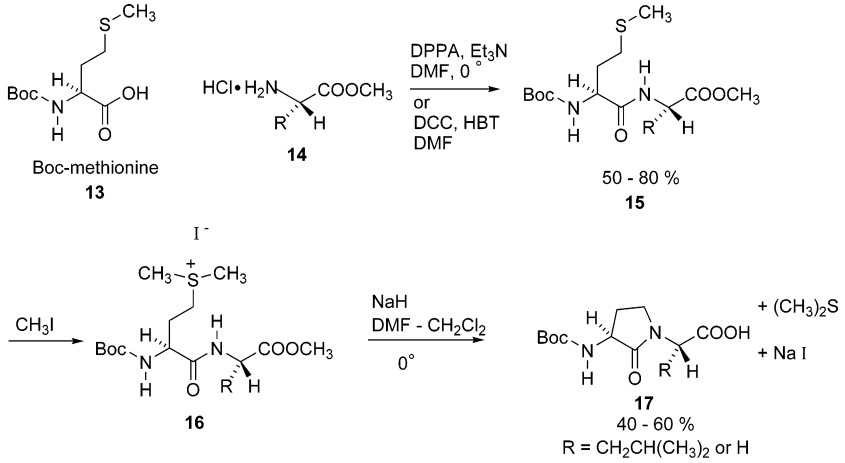


Figure 5. Synthesis of γ-lactams.

more difficult, and heating of the methyl ester in acetonitrile in the presence of triethylamine was required.

An alternative dipeptide δ-lactam contains a thiazinone ring and is synthesized by condensing a cysteine derivative with paraformaldehyde (Figure 4). The starting material, N^{α}-phthaloyl-*S*-acetamidomethyl (Acm) cysteine (**9**), was coupled with glycine methyl ester (**10**). The resultant dipeptide (**11**) was condensed with paraformaldehyde in the presence of *p*-toluenesulfonic acid with heating to form the thiazinone **12a**. The Acm protecting group was removed under the reaction conditions to free the thiol for reaction to produce the heterocyclic lactam. The methyl ester was readily hydrolyzed to the carboxylic acid **12b** with HCl in aqueous tetrahydrofuran. A potential advantage of this synthetic route is that substitution of glycine with other amino acids would permit the introduction of a variety of side chains. A disadvantage is that significant racemization occurs at the lactam asymmetic center in the course of the synthesis. As a result, we have limited our use of this particular dipeptide lactam in subsequent applications.

Intramolecular alkylation was employed in the synthesis of dipeptide γ-lactams (Figure 5). Boc-L-methionine (**13**) was coupled with an amino acid methyl ester of choice (**14**) utilizing either diphenylphosphoryl azide (DPPA) or dicyclohexylcarbodiimide (DCC) as the coupling agent. With the resultant dipeptide **15** in hand, the key step was conversion of the side chain of methionine into a leaving group. Methylation of the methylthio group with methyl iodide produced the methyl sulfonium salt (**16**). Treatment with sodium hydride resulted in cyclization with expulsion of dimethyl sulfide to form the lactam **17**. This synthesis allows a variety of side chains to be incorporated in the second amino acid (illustrated for leucine and glycine) of the lactam dipeptide. The asymmetric centers from the starting amino acids are preserved through the synthesis.

A method was also developed for incorporating an α-substituent on the γ-lactam as exemplified for the Trp-Lys analogue (Figure 6).[11] N^{ϵ}-Cbz-lysine methyl ester (**18**) was warmed with the electrophilic cyclopropane **19** in dimethylformamide to form the lactam ring in a single step. The reaction is thought to proceed via initial opening of the cyclopropane by the α-amine of lysine followed by cyclization of this amine onto one of the carbonyl groups with loss of acetone, thus freeing a carboxyl group. The lactam **20** can then be alkylated with gramine methiodide (**21**) to incorporate the side chain of tryptophan. Two diastereomers (**22a** and **22b**) were produced that are separable by chromatography. Each diastereomer was subjected to the DPPA-mediated Curtius rearrangement to yield the fully protected L-Trp-L-Lys (**23a**) and D-Trp-L-Lys lactam dipeptides (**23b**).

Returning to the C3 symmetric cyclic hexapeptides that sparked the original interest in dipeptide lactams, we found that the lactam-constrained analogues were readily prepared in a single step from the dipeptide lactam amino acid **24** by cyclotrimerization (Figure 7). The reaction was mediated by DPPA in the presence of triethylamine in DMF at −20 °C, and the major product in each case was the cyclic hexapeptide, obtained in 26% yield in the example of the δ-lactam (**25b**).[1] With the lactam enforcing a trans amide linkage in the dipeptide, the cyclic monomer diketopiperazine is energetically unfavorable. The cyclic tetrapeptide was also not ob-

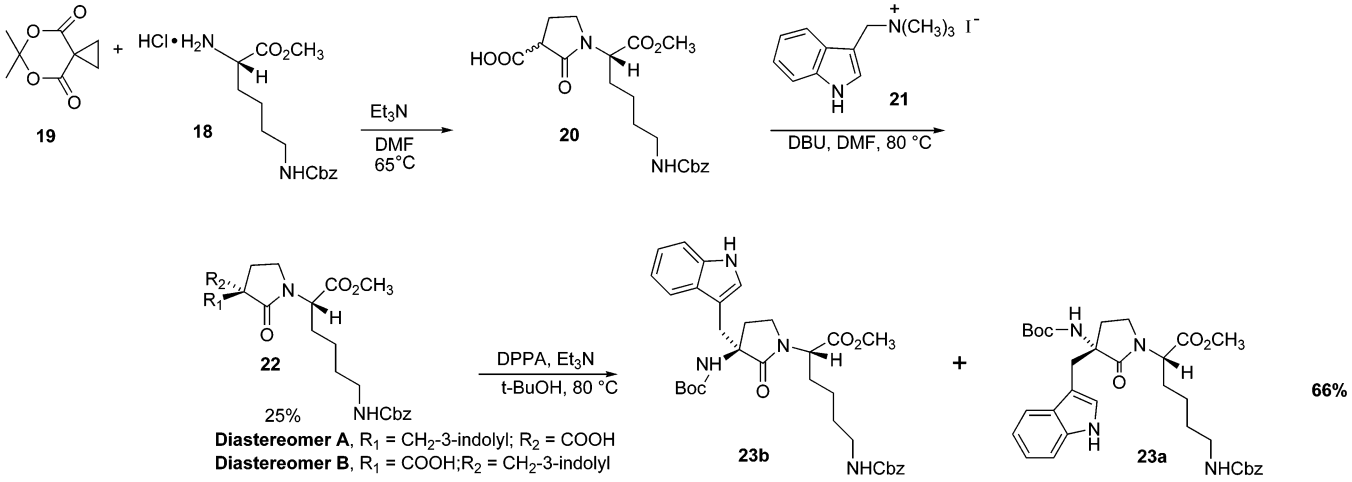


Figure 6. Synthesis of γ-lactams with an α-substituent.

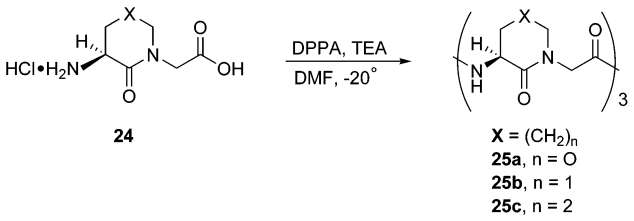


Figure 7. Cyclic hexapeptides via lactam dipeptide cyclotrimerization.

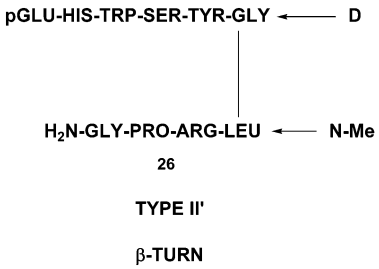


Figure 8. Luteinizing hormone−releasing hormone (LH−RH).

served, although small quantities of the cyclic octapeptide could be isolated.

Interestingly, only the δ-lactam-containing cyclic hexapeptide is active in the ruminant fementation assay with activity comparable to that of the parent cyclo-(Ala-Sar)$_3$. The corresponding γ- and ϵ-lactam analogues **25a** and **25c** are not active. This case represents the first example of the use of small lactam rings to bias peptide backbone conformation and demonstrates that lactams can be sensitive conformational probes in biologically active peptides.

We next turned our attention to the application of a dipeptide lactam constraint to luteinizing hormone−releasing hormone (LH−RH, **26**), an important mammalian fertility hormone. At that time, several lines of evidence supported a Tyr-Gly-Leu-Arg type II′ β-turn in the receptor bound conformation. For example, replacement of Gly with D-Ala results in an increase in potency, while incorporation of L-Ala at the same position decreases potency. In addition, the analogue with N^{α}-methylleucine in place of Leu retains activity (Figure 8). Conformational energy calculations by Momany suggest that this β-turn is a favored low-energy conformation for LH−RH.[12] Examination of a model of

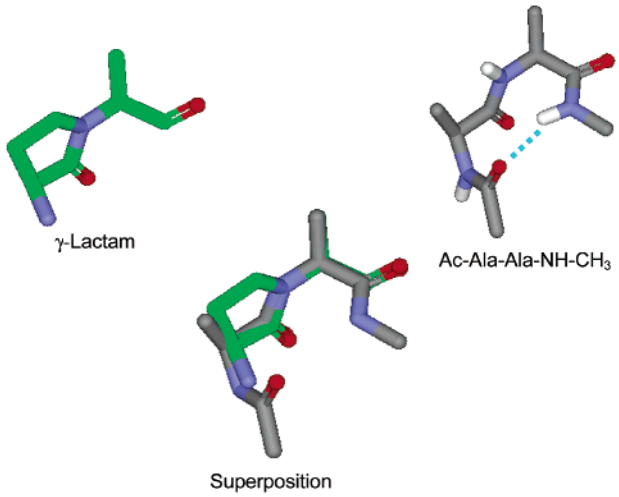


Figure 9. Bridging a type II′ β-turn with a γ-lactam.

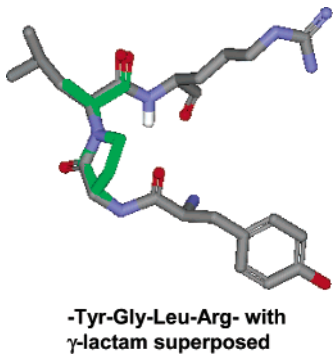


Figure 10. γ-Lactam constraint of a LH−RH type II′ β-turn tetrapeptide.

acetyl-alanyl-alanine methylamide in a type II′ β-turn (Figure 9) suggests that a γ-lactam should bridge effectively from the first Ala methyl to the second Ala nitrogen. For the proposed LH−RH β-turn, the lactam would bridge from the pro-S hydrogen C^{α}-position of Gly to the nitrogen of Leu. The prediction that the S (L)-lactam cyclic constraint should stabilize the turn contrasts with the known stabilization by R (D)-Ala (acyclic constraint) and presents a good test for the utility of the lactam (Figure 10).

The requisite γ-lactam-constrained analogue of LH−RH was synthesized starting from the Boc-γ-lactam acid **27** containing the leucine side chain in the second amino

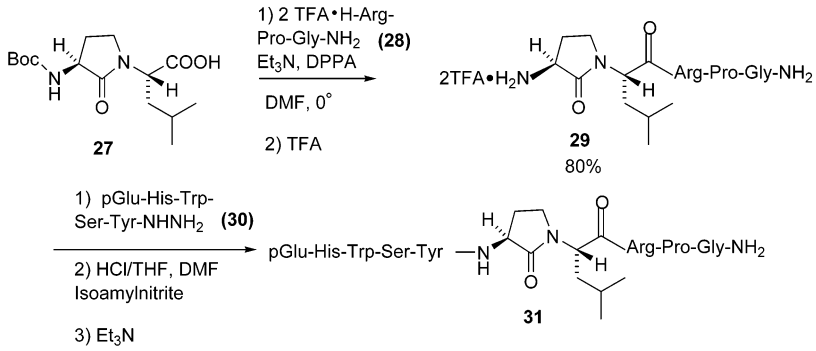


Figure 11. Synthesis of a lactam-constrained LH−RH analogue.

acid (Figure 11). Condensation with the carboxy terminal fragment of LH−RH (H-Arg-Pro-Gly-NH_2, **28**) in the presence of DPPA followed by Boc removal with trifluoroacetic acid (TFA) gave the pentapeptide fragment **29**. Condensation with the amino terminal pentapeptide fragment (pGlu-His-Trp-Ser-Tyr-$NHNH_2$, **30**) via the azide method provided the desired decapeptide analogue **31**.[13]

This compound proved to be a LH−RH agonist with 8.9 times the potency of that of the native hormone for release of LH in a pituitary cell culture system. Given intravenously to the rat, the analogue's potency was 2.4 times that of LH−RH. The high potency in the in vitro assay where the action of proteolytic enzymes should be minimized suggests that the increase is due to a more favorable interaction with the receptor. Overall, these results support the proposed type II′ β-turn in the biologically active conformation and again illustrate the utility of lactam conformational constraints. Kopple studied the solution conformation of lactam-constrained analogue **31** by NMR and concluded that the preferred conformation is also consistent with the proposed β-turn.[14]

These early results and others from our laboratory[15,16] indicate that dipeptide lactams are useful for potency enhancement, greater receptor selectivity, provision of information about a peptide's biologically active conformation, and increased peptidase stability. In addition, Thorsett pioneered the application of dipeptide lactams in the context of angiotensin converting enzyme inhibitors,[17] and Wyvratt synthesized the first bicyclic lactam dipeptides for the same project.[18] These compounds are synthetically accessible and can be readily incorporated into peptide analogues. Since the initial work in this area, other researchers have considerably broadened the scope of the chemistry and applications of dipeptide lactams.[19−22]

Cyclic Hexapeptide Somatostatin Agonists

Somatostatin (**32**) is a 14-amino acid peptide containing a 38-membered macrocyclic ring first isolated at the Salk Institute in 1973.[23] Among its biological activities are the inhibition of release of insulin, glucagon, and growth hormone. On the basis of these activities, the therapeutic target of interest in our laboratories was type I diabetes. Somatostatin is also found in the central nervous system and is of interest as a neuropeptide as well. By the time I became involved with the somatostatin project, another part of Daniel Veber's group had been working on the problem for some time, and

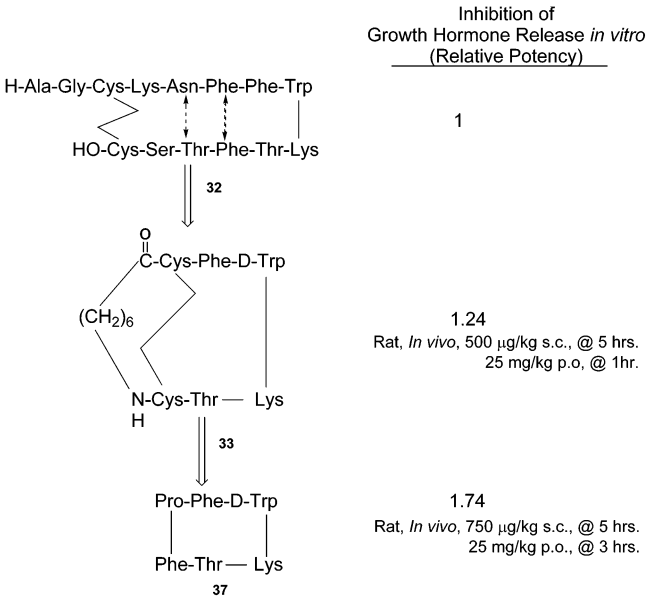


Figure 12. Constrained, simplified analogues of somatostatin.

substantial progress had been made toward constrained, simplified analogues (Figure 12).

Structure/conformation/activity studies had suggested that a β-turn composed of Phe^7-Trp^8-Lys^9-Thr^{10} is important for the biologically active conformation, and correlations with conformation of analogues in solution had proven useful. Much of the remainder of the hormone apparently functions as scaffold and can be replaced with simpler structural units. One of the most simplified and advanced structures was the bicyclic heptapeptide **33**, which retains only four of the original amino acids of somatostatin with D-Trp substituting for L-Trp and yet has 1.24 times the potency of somatostatin in an in vitro growth hormone release assay in a pituitary cell culture system.[24,25] The two cysteines and the aminoheptanoic acid in this compound are thought to function as scaffolding to stabilize the β-turn conformation, which would be type II′ in this case. Compound **33** is also more stable in vivo and shows a much longer duration of action than somatostatin after administration to rats. The challenge at this point was to design even simpler analogues with further improved biological properties.

We began by constructing a computer model of compound **33** with the Merck Molecular Modeling System utilizing NMR data for the preferred solution conformation. The model was then broken apart at two bonds as indicated by arrows in Figure 13, the scaffold

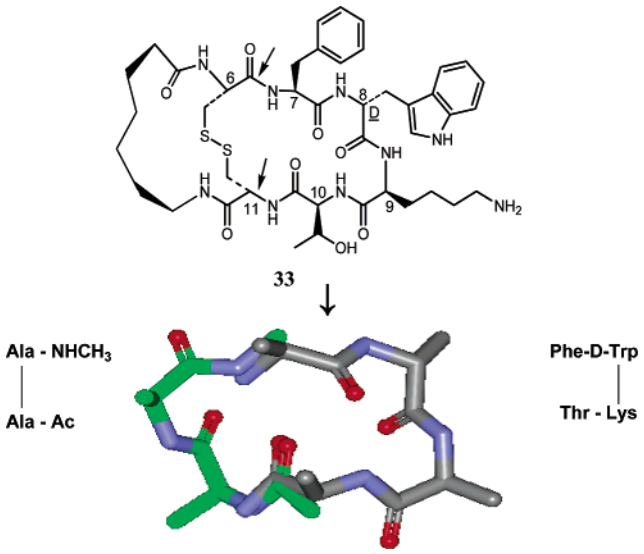


Figure 13. Design of cyclic hexapeptide somatostatin analogues.

Table 1. Cyclo-(A-B-Phe-D-Trp-Lys-Thr)

compound A	compound B	no.	δ (ppm) Lys, γ-CH_2	inhibition of growth hormone release (relative potency)
Cys-Aha-Cys		33	0.32, 0.48	1.24
Ala	Pro	34	0.39, 0.55	0.06
Pro	Pro	35	0.33, 0.53	0.008
D-Ala	D-Pro	36	0.2−0.7	0.006
Phe	Pro	37	0.4−0.55	1.74
D-Phe	D-Pro	38	0.56, 0.71	<0.002

portion was discarded, and the Phe-D-Trp-Lys-Thr β-turn with side chains simplified to methyls was retained for superposition studies with simpler scaffold structures. Among such structures explored were dipeptides (e.g., Ac-Ala-Ala-NHMe) in various turn conformations that would produce a cyclic hexapeptide containing two β-turns upon combination with the somatostatin tetrapeptide. Good matches were obtained with some of these dipeptides as illustrated in Figure 13, thus encouraging us to design a series of cyclic hexapeptide analogues for synthesis and biological evaluation.

Examples of five of these cyclic hexapeptides compared with compound **33** are shown in Table 1. The cyclic peptides were synthesized from linear precursors with D-Trp at the amino terminus by azide cyclization of acyl hydrazides. The linear hexapeptides were prepared by solid-phase peptide synthesis followed by removal from the resin with hydrazine. All of these compounds exhibit upfield-shifted Lys γ-methylene protons, demonstrating that they can achieve proximity of the Lys side chain and the indole of D-Trp indicative of the β-turn conformation in solution. In the in vitro growth hormone release assay, the Ala-Pro compound **34** exhibits about 6% of the activity of somatostatin and gives a complete biological response. The Pro-Pro and D-Ala-D-Pro compounds **35** and **36**, respectively, show less than 1% of the activity of somatostatin. We speculated that these cyclic hexapeptides were less active than **33** because of the absence of a hydrophobic binding element potentially represented by the aminoheptanoic acid of **33**. This hypothesis was supported when Ala was replaced by Phe in **34** to produce compound **37**. The latter compound is more potent than the starting

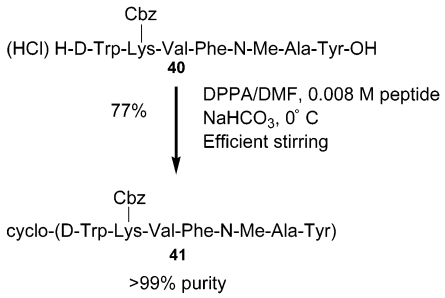


Figure 14. Improved peptide cyclization procedure.

Table 2. Optimization of Cyclic Hexapeptides

	Relative Potency for Inhibition of Release of: Insulin	Glucagon	Growth Hormone (*in vitro*)
Pro-Phe-D-Trp Phe -Thr - Lys **37**	5.2	8.0	1.7
N-Me-Ala-Tyr-D-Trp Phe -Val-Lys MK-678 **39**	70.0	117.0	52.3

bicyclic peptide **33** with 1.74 times the potency of somatostatin. The same strategy did not work for compound **36** (see **38**), however, indicating the importance of the conformation of the new turn region for proper orientation of the hydrophobic receptor binding element.[26]

The discovery of the potent cyclic hexapeptide **37** was a key advance for the program. This compound is more readily synthesized and exhibits a duration of action comparable to that of compound **33** in the rat. As a result, we turned our attention to optimization of this new lead class by variation of each of the six amino acids with a combination of the best substitutions. From a number of highly potent compounds, the optimal structure is **39** (MK-678), which contains *N*-Me-Ala for Pro, Tyr for Phe, and Val for Thr of **37** (Table 2**)**. This compound has 52 times the potency of somatostatin in the in vitro growth hormone release assay and is 70- to 117-fold more potent than somatostatin for inhibition of release of insulin and glucagon in the rat.[27] MK-678 was selected for human clinical trials.

Our group performed the initial large-scale synthesis of **39**, and in the course of this effort, we developed an improved peptide cyclization protocol that has general utility (Figure 14). We had previously found DPPA in DMF to be an effective coupling agent in the macrocyclization step of cyclic peptide synthesis.[28] With the published reaction conditions, an organic base such as triethylamine is typically added in increments to maintain slightly basic conditions. The correct balance is important to allow the reaction to progress and to avoid racemization. For the synthesis of **39**, the protected linear hexapeptide **40** from a solution synthesis is cyclized to the penultimate intermediate **41**. To optimize this reaction, we explored a range of conditions includ-

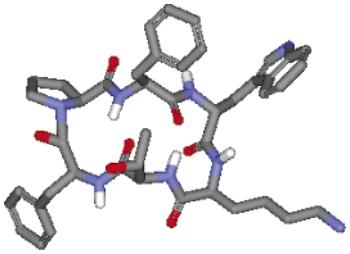


Figure 15. Model of bioactive conformation: cyclo-(Pro-Phe-D-Trp-Lys-Thr-Phe).

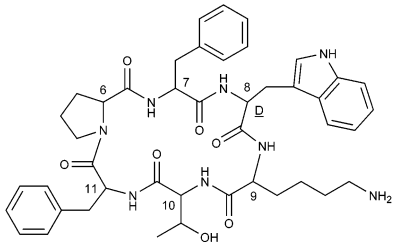
ing a variety of bases. One of the most effective bases was $NaHCO_3$. The reaction in this case is heterogeneous, and efficient stirring is essential. After a simple filtration through silica gel, the cyclized product is obtained in 77% yield and >99% purity. This new method has several advantages over the previous procedure. Polymer formation and side reactions at higher solution pH with soluble base are suppressed. The excess insoluble base eliminates frequent reaction monitoring, and minimal racemization is observed. The procedure is scalable, and we have performed it from milligram to 100 g scale.[29]

Returning to the lead cyclic hexapeptide **37**, we focused on developing a working model of its solution/bioactive conformation and used this model to design analogues with further improved biological properties. Compound **37** exhibits a preferred conformation in solution, and NMR data and conformational energy calculations were used to construct a model. The model, shown in Figure 15, contains the expected Phe-D-Trp-Lys-Thr type II′ β-turn with the Trp and Lys side chains in proximity. Surprisingly, the second β-turn comprising Thr-Phe-Pro-Phe is type VI containing a cis Phe-Pro amide linkage. The remaining side chains are positioned on the basis of observed chemical shifts and nuclear Overhauser effects.

One test of this model, which could also lead to more proteolytically stable analogues, would be to N-methylate amide linkages. Four of the amides in **37** were targeted. On the basis of the working model, appropriate *N*-methylamino acid replacements for Pro[6], Lys[9], and Phe[11] should be compatible with minimal conformational change and result in active compounds (**42**, **43**, and **45**, respectively). N-methylation at the Thr[10] position, however, would disrupt the type II′ β-turn with a predicted loss of activity. These analogues were synthesized by our usual procedures, and the biological results shown in Table 3 were in accord with predictions.[16,30] The *N*-Me Ala[6] analogue **42** is essentially a ring-opened Pro derivative. A key feature of this compound is that the Phe-*N*-Me-Ala amide can still attain the cis conformation required for the type VI β-turn in the model. At the 10-position, it had previously been shown that α-amino butyric acid (Abu) could substitute for Thr with full retention of activity. For reasons of synthetic accessibility, the *N*-Me-Abu[10] analogue **44** was prepared, and it is essentially inactive. These results strengthened support for the working model of **37**.

While considering sources of the *N*-Me amino acids required for the synthesis of these analogues, in particular N^{α}-Me-Lys, we realized a need for a more general preparation of *N*-alkylamino acids protected on nitrogen with an acid stable group. A new route that we developed is shown in Figure 16.[31] Refluxing a 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid **46** with paraformaldehyde or a higher aldehyde and *p*-toluenesulfonic acid in toluene produces an oxazolidinone **47**. The oxazolidinone is then reduced with excess triethylsilane in 1:1 chloroform/trifluoroacetic acid to yield the protected *N*-alkylamino acid **48**. The strongly acidic conditions presumably generate an *N*-acylimminium intermediate from opening of the oxazolidinone. Donation of a hydride from triethylsilane to this species completes the conversion. The reaction conditions permit access to many *N*-Me amino acids.[32] While the first step is most efficient with paraformaldehyde, other N-alkylated amino acids have been obtained from higher aldehydes. The protected *N*-alkylamino acids produced in this synthesis are ready for incorporation into peptides by standard methodology.

Table 3. *N*-Methylamino Acid Substitutions



amino acid	no.	Relative Potency for Inhibition of Release of: insulin	glucagon	growth hormone (in vitro)
N-Me-Ala[6]	42	6.0 (3.8, 10.9)	16.7 (4.3, 200)	3.5 (2.6,4.5)
N-Me-Lys[9]	43	0.5 (0.1, 1.8)	active	1.6 (0.7, 3.4)
N-Me-Abu[10]	44			0.004 (0.002, 0.008)
N-Me-Phe[11]	45	1.1 (0.6, 1.7)	2.0 (1.1, 4.0)	0.53 (0.34, 0.82)
	37	5.2 (2.4, 11)	8.0 (1.4, 60.2)	1.74 (1.31, 2.32)

Returning to the working conformational model of **37**, we wanted to further test the importance of the type VI β-turn with the cis amide linkage. Cyclic side chain and backbone constraints were employed. In the former case, Phe[11]-Pro[6] was replaced with Cys-Cys. The new amide linkage in the resultant analogue **49** is not N-alkylated, but the eight-membered disulfide-containing ring is still expected to prefer a cis amide between the cysteines based on conformational energy calculations and literature precedent.[33,34] Compound **49** is highly active as shown in Table 4 in accord with the prediction about its conformation. On first analysis, however, the high activity of this compound may appear surprising because an important binding element in the form of the Phe[11] side chain has been removed. The model indicates, however, that the Cys-Cys disulfide linkage occupies a similar region of space as that proposed for the Phe[11] side chain. The disulfide is likely to be playing the role of the key hydrophobic receptor binding element.

For backbone constraint of the Phe[11]-Pro[6] region of **37**, we incorporated γ- and δ-dipeptide lactams of L- and D-configuration.[16] This design retains the N-alkylated central amide linkage, but the lactam forces the amide to be trans. All four of these compounds (**50**−**53**) were essentially inactive (Table 4). The analogues are lacking the Phe[11] side chain, and their activity is more properly compared to that of the Ala[11]-Pro[6] analogue **34**. The most active analogue **50**, which contains the L-γ-lactam, is over 10-fold less potent than **34**. These results provide

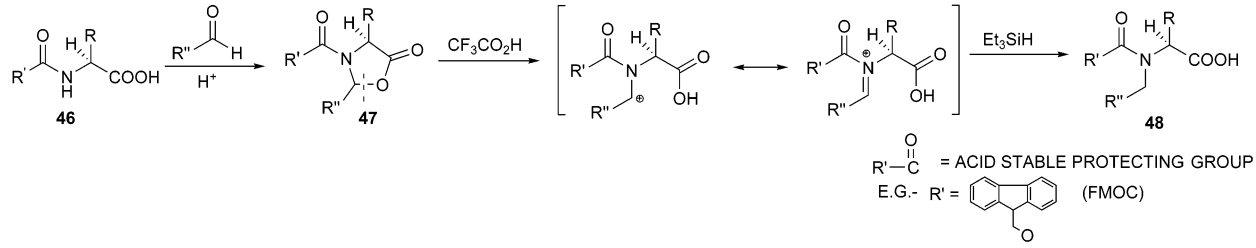
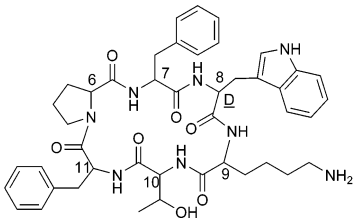
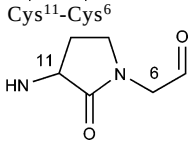
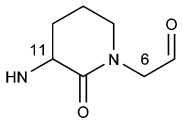


Figure 16. Route to protected *N*-alkylamino acids.

Table 4. Bridging Substitutions



substitution	no.	Relative Potency for Inhibition of Release of: insulin	glucagon	growth hormone (in vitro)
Cys^{11}-Cys^6	49	10.6 (5.5, 19.9)	10 (3, 26)	2.47 (1.66, 3.81)
	50	L, <0.07	<0.07	0.004 (0.001, 0.013)
	51	D, <0.2	<0.2	0.001 (0.0012, 0.004)
	52	L, <0.07	<0.07	<0.005
	53	D, <0.07	<0.07	<0.005
ALA^{11}	34	0.05 (0.03, 0.1)	0.08 (0.04, 0.21)	0.06 (0.03, 0.12)
	37	5.2 (2.4, 11)	8.0 (1.4, 60.2)	1.74 (1.31, 2.32)

additional support for the working model and the importance of the type VI β-turn in the bioactive conformation of **37**.

The structure/conformation/activity correlations that we had developed to this point suggested that the side chains of **37** were the key structural elements for binding to the somatostatin receptors with the peptide backbone functioning primarily as a scaffold. To test this hypothesis, we designed retrocycloisomeric analogues in which the direction of the backbone is reversed and the chiral centers of the amino acids are inverted.[30,35] The goal was to retain the overall side chain topography with a new scaffold. Such compounds with a majority of D-amino acids would potentially have the advantage of increased protease stability as well. The reference cyclic hexapeptide for this study was the *N*-Me-Ala^6 analogue **54**, which has 3.5 times the potency of somatostatin in the in vitro growth hormone secretion assay. The pyrrolidine ring of Pro^6 in **37** would have been a complicating factor for this approach when the direction of the peptide backbone is reversed. The direct retrocycloisomer of **54** is compound **55**. This compound was synthesized by our standard protocols and found to be 1000-fold less potent than **54** for inhibition of growth hormone release (Figure 17). A potential explanation for this poor activity is that the N-methylated amino acid and, as a result, the cis amide linkage are now out of position. The preferred backbone conformation of **55** may not allow the correct positioning of side chains for good receptor binding. In an attempt to remedy this problem, the analogue **56** in which the methyl group is moved from D-Ala to D-Phe (see arrow on **55**) was synthesized. The cis amide bond can now be in the correct location in the peptide backbone, and we were pleased to find that **56** recovered 100-fold of the lost potency and has 25% of the potency of somatostatin. A final modification in this design focused on the key β-turn containing the Trp and Lys side chains. Analogue **56** contains D-Lys and L-Trp, and the β-turn is likely to be type II′, the same as in the reference compound **54**. The enantiomeric β-turn is type II, and it should result from a structure containing L-Lys and D-Trp. It was of interest to compare the activity of the resultant analogue **57** with that of **56**. Compound **57** was synthesized, and it was found to be 3 times more potent than **56**. The cyclic hexapeptide **57**, which contains five D-amino acids and has a reversed peptide backbone, is comparable in potency to somatostatin and has 25% of the potency of **54** in the in vitro growth hormone release assay. Compound **57** is more stable to trypsin in vitro, but it was not longer acting in vivo compared to **37**. We concluded that other processes must be controlling the duration of action.

The correspondence of side chain topography must be quite good between **54** and **57** because the difference in potency corresponds to less than 1 kcal in binding energy. The solution conformation of **57** was determined by NMR, and a model was built. Comparison of the

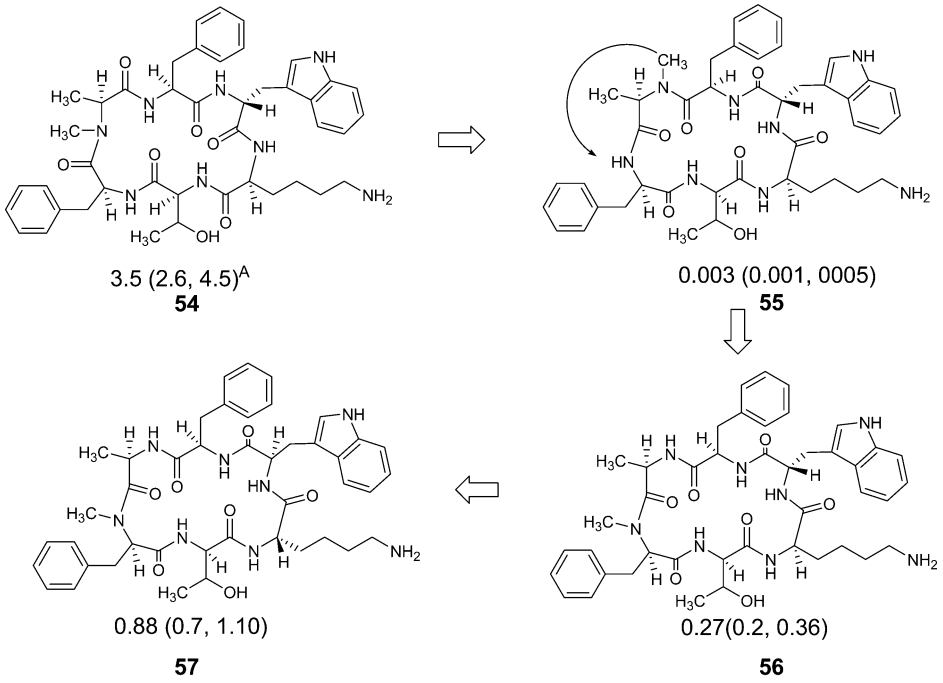


Figure 17. Retrocycloisomeric analogues.

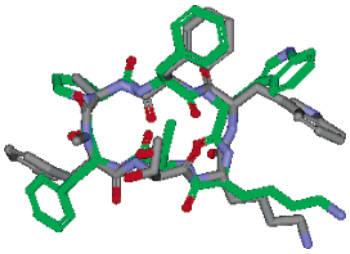


Figure 18. Comparison of solution/bioactive conformational models cyclo-(Pro-Phe-D-Trp-Lys-Thr-Phe) and cyclo-(*N*-Me-D-Phe-D-Thr-Lys-D-Trp-D-Phe-D-Ala).

models of **37** and **57** shows their side chains to be in the same regions of space, but the backbone amide hydrogen bonding elements are oriented very differently (Figure 18). These results lend further support to the hypothesis that the peptide backbone contributes, at most, a small amount to receptor binding. Its primary role in these cyclic hexapeptides is largely as a scaffold to position the side chains for binding to the receptor. After our work, two groups utilized the bioactive conformational information for these cyclic hexapeptides as a starting point for discovery of active somatostatin agonist analogues with non-peptide scaffolds, thus confirming that a peptide scaffold is unnecessary for this hormone's biological activity.[36,37] All of this research indicates that when sufficient structural and conformational information is available for a biologically active peptide, these data can be used to design and discover peptidomimetics and non-peptide ligands.

Prostate Specific Antigen (PSA)−Cytotoxic Drug Conjugates

A pioneering example of prodrug research was reported by Ralph Hirschmann and colleagues in 1964.[38] The title of this paper is "An Approach to an Improved Antiinflammatory Steroid. The Synthesis of 11β,17-Dihydroxy-3,20-dione-1,4-pregnadien-21-yl 2-Acetamido-2-deoxy-β-D-glucopyranoside", and the following statement was made: "It seemed possible to reduce all of these side effects if an inactive steroid could be prepared which is preferentially converted into an active drug at the site of its therapeutic actions". The concept relied on cleavage of an inactive sugar derivative of the steroid by β-*N*-acetylglucosaminidase at the inflamed site to liberate the active steroid. Proof of concept was demonstrated in the rat. This research has inspired many subsequent approaches to the design of prodrugs.

We have attached peptide substrates for prostate specific antigen (PSA) to cytotoxic agents in an attempt to target these drugs to prostate tumors. PSA is a serine protease with the function of liquefying semen by its ability to cleave several seminal fluid proteins including semenogelin I and II. This enzyme is only produced in prostate epithelium and in metastatic prostate cancer cells. PSA is active only in seminal fluid and in the microenvironment surrounding prostate cancer cells. PSA is not active once in circulation because of complexing with circulating α-1-antichymotrypsin and α-2-macroglobulin. In circulation, PSA remains immunologically active, and PSA levels in serum correlate with the extent of prostatic cancer in prostate cancer patients.

The overall objective of our research was to improve the therapeutic index of existing chemotherapeutics for prostate cancer by designing a peptide−cytotoxic drug conjugate that is selectively cleaved by PSA, thus activating the cytotoxic agent in the prostate cancer cell microenvironment to effect selective killing. Attainment of this objective requires a rapid and specific rate of cleavage by PSA and highly selective potency against PSA-secreting cells. The peptide substrate also must be resistant to nonspecific proteolysis, and the conjugate itself must have substantially reduced cytotoxicity compared to the parent drug. The latter result would be achieved if attachment of the peptide prevents the

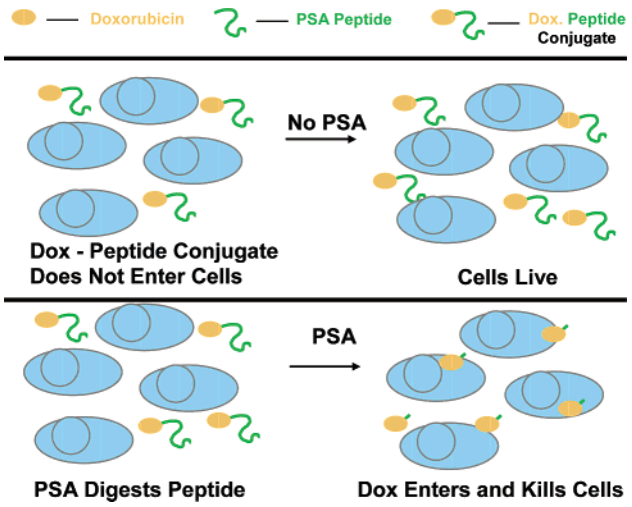


Figure 19. Proposed mode of action of doxorubicin−peptide conjugates.

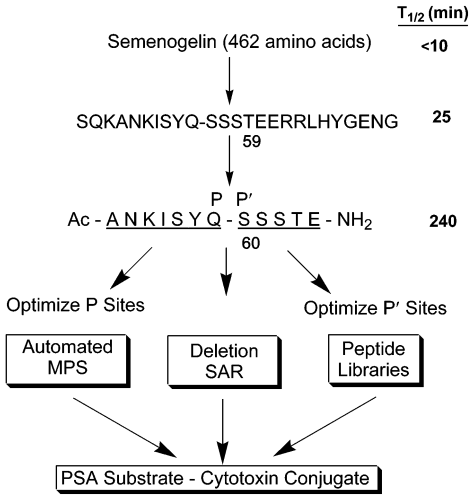


Figure 20. Cytotoxic substrate development scheme.

conjugated drug from entering cells. If these requirements were met, we expected to observe efficacy against PSA-secreting tumors. The proposed mode of action is illustrated for a doxorubicin−peptide conjugate in cartoon form in Figure 19.

Our initial efforts focused on doxorubicin−peptide conjugates. While doxorubicin (**58**) has several potential sites for attachment of peptides, the amino group on the sugar portion of the structure proved best for our purposes. PSA substrate development began with the 462 amino acid sequence of semenogelin I[39] and proceeded as outlined in Figure 20. Five unique sites at which PSA cleaves in the sequence have been identified. Primary cleavage occurs at the carboxy terminus of Gln, which appears to be unique among known mammalian proteases, although it has been observed for viral proteases.[40] The selective cleavage at Gln represents an advantage toward the development of PSA-specific prodrugs where nonspecific proteolysis would be minimized. To begin, a 25 amino acid sequence (**59**) representing P10−P15′ of the most efficient PSA cleavage site in semenogelin I was synthesized. This sequence is still a substrate for PSA. Incubation of a molar ratio of 1:100 PSA/peptide gave a time for 50% hydrolysis ($t_{1/2}$) of 25 min compared to a $t_{1/2}$ for semenogelin of <10 min. A carboxy and amino terminal blocked 12-mer (**60**) from this sequence is a poorer substrate, $t_{1/2} = 240$ min. The acetyl and amide groups at the termini will limit cleavage by amino and carboxy proteases. This compound was chosen as the base sequence for extensive modification aimed at identifying simpler and more efficient substrates.

Libraries of **60** at P1′, P2′, P3′, and P4′ were synthesized by the simultaneous incorporation of an equimolar mixture of 19 natural amino acids at each position.[41] The four individual libraries were subjected to PSA digestion for 60 min. Following quenching, the digests were evaluated by automated Edman sequence analysis. Only those peptides that are cleaved by PSA will give products with a free amino terminus; therefore, from these results, it was possible to determine the relative susceptibility to PSA hydrolysis of sequences with the various amino acid substitutions.

A similar approach was not readily accessible for evaluating P1−P4 substitution because PSA hydrolysis would not generate a unique set of hydrolytic products. To determine which amino acids, within the context of substrate **60**, contributed most to improved PSA cleavage, single point substitution analogues of **60** were synthesized using a multiple peptide synthesizer. The same set of 19 amino acids used for P1′−P4′ was introduced individually in P1−P4. The resultant 76 peptides after isolation from the solid-phase resin were treated with PSA to determine their relative rates of cleavage.

With the preferred single substitutions in **60** identified for P4′−P4, multiple preferred substitutions and residue deletions were investigated. The best resultant substrates were then attached to the amino group of doxorubicin. Selected key conjugate analogues are shown in Table 5. At the PSA cleavage site (P1−P1′) in these analogues, the native amino acids Gln-Ser from semenogelin are preferred. To decrease chymotryptic susceptibility, cyclohexyl glycine in place of tyrosine at P2 is an effective substitution. Leucine is preferred at P2′, and the native serine is preferred at P3. The nonnative alanine is an excellent substitution at P4. Utilizing these amino acids plus the native lysine at P5 produced the heptapeptide conjugate **65** with a PSA $t_{1/2}$ of 10 min, comparable to that of semenogelin. Unfortunately, compound **65** and other conjugates containing basic groups were unacceptable for in vivo administration because of their stimulation of histamine release in dogs. The lysine at P5 could be successfully replaced by cyclic amino acids such as hydroxyproline to eliminate this undesirable activity. The resultant conjugate **66** has a good $t_{1/2}$ of 20 min, but its aqueous solubility is not sufficient for intravenous formulation. Replacement of the amino terminal acetyl group with an acyl group containing a carboxyl provided the optimal balance of PSA cleavage time and physical properties. The preferred compound is **68**, which has an amino terminal glutaryl and is 50% cleaved by PSA in 30 min.

The peptide−doxorubicin conjugates were prepared by a combination of solid-phase and solution synthesis methods as illustrated for **68** in Figure 21.[41] The peptides were synthesized by solid-phase synthesis using a Boc strategy on 4-(oxymethyl)phenylacetamidomethyl (PAM) resin. The cleavage of the peptides from the resin and removal of the side chain protecting

Table 5. Biological Data for Key Doxorubicin−Peptide Conjugates

	doxorubicin−conjugate structure	$t_{1/2}$ (min)	in vitro EC_{50} (μM) LNCaP	DuPRO
58	doxorubicin (Dox)		0.7	1.8
61	H-Ser-Leu-Dox			
62	H-Leu-Dox		6.7	14.0
63	Ac-Ala-Asn-Lys-Ala-Ser-Tyr-Gln-Ser-Ser-Leu-Dox	100	6.3	>100
64	Ac-Lys-Ala-Ser-Tyr-Gln-Ser-Leu-Dox	30	4.8	100
65	Ac-Lys-Ala-Ser-Chg-Gln-Ser-Leu-Dox	10	4.5	>100
66	Ac-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox	20	4.5	>100
67	succinyl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox	55	5.7	100
68	glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox	30	5.0	>100

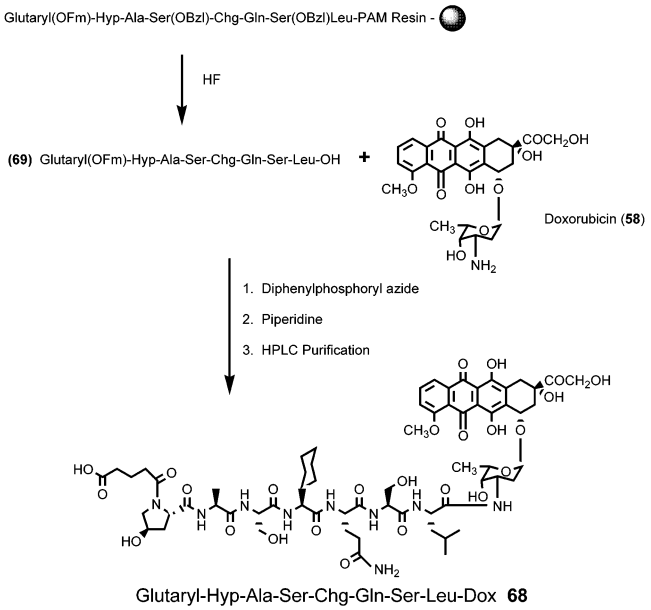


Figure 21. Synthesis of peptide−doxorubicin conjugate **68**.

groups were carried out in anhydrous HF to yield intermediate **69**. Fmoc was utilized for side chain protection of amino groups and OFm (fluorenylmethyl ester) for acidic amino terminal groups such as glutaryl. Compound **69** was used without purification for coupling with doxorubicin **58**. The coupled product was treated with 20% piperidine in DMF at 0 °C to remove the OFm protecting group. Final conjugate **68** was purified by reverse-phase high-performance liquid chromatography, and the identity and purity of the final product were established by amino acid analysis, HPLC, and FAB mass spectrometry.

Table 5 also contains data for the selected conjugates in prostate cancer cell cultures.[39] The LNCaP line secretes PSA, and the DuPRO line does not. Cells were incubated with doxorubicin or various peptide−doxorubicin conjugates, and the EC_{50} for cell kill was determined. As expected, doxorubicin itself killed the cells from the two lines indiscriminately with an EC_{50} of about 1 μM. We were quite pleased to find that all of the peptide conjugates in Table 5, however, were selectively toxic to the PSA-secreting LNCaP cells. For example, the optimal compound **68** has an EC_{50} of 5.0 μM for killing LNCaP cells but has >100 μM for killing DuPRO cells. These results suggest that the conjugates are performing as designed, but some additional explanation of mechanism is necessary. We expect that cleavage by PSA is occurring at the Gln-Ser bond to produce H-Ser-Leu-doxorubicin. The microenvironment of prostate cancer cells is rich in aminopeptidases, and

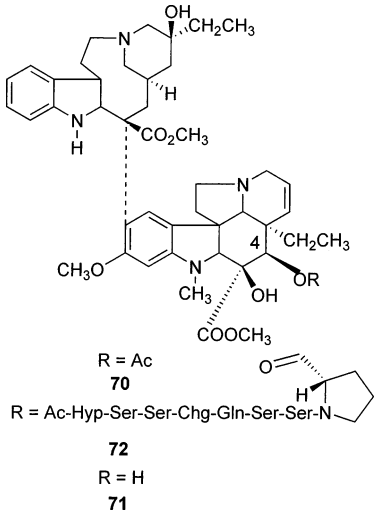


Figure 22. Peptide−vinblastine conjugate.

rapid removal of these two amino acids to yield free doxorubicin is anticipated. An intermediate in this process is H-Leu-doxorubicin, which is nonselectively cytotoxic but less potent than doxorubicin.

In nude mouse xenograft studies using LNCaP prostate cancer cells, results were also in accord with conjugate **68** functioning as a tumor-targeted prodrug. The compound reduced PSA levels by 95% and tumor weight by 87% at a dose below its maximum tolerated dose (MTD).[39] Both doxorubicin and Leu-Dox were not effective in reducing circulating PSA and tumor burden at their MTD values. In phase I clinical trials, **68** was generally well tolerated in patients with hormone refractory prostate cancer, and physiological activity was demonstrated on the basis of PSA level reductions.[42]

We were also able to apply this tumor targeting approach to the antitumor agent vinblastine **70** (Figure 22).[43] In evaluating the vinblastine structure for this strategy, the first consideration was identifying a suitable site for attachment of the peptide substrate for PSA. After some experimentation, the 4-position hydroxyl was chosen. This group is esterified with an acetyl group in vinblastine, and we considered that peptide esters could also be attached at this site. Furthermore, des-Ac vinblastine **71** is a major metabolite of the parent compound with comparable cytotoxicity. It was also established at an early point in this study that attachment of a peptide sequence at this site rendered the resultant molecule relatively nontoxic. A key feature of vinblastine is that it is more chemically robust over a wider pH range than doxorubicin, thus allowing the application of a wider range of reaction conditions in the preparation of peptide conjugates. This

Table 6. Biological Data for Vinblastine Conjugate

vinblastine−conjugate structure	$t_{1/2}$ (min)	in vitro EC$_{50}$ μM) LNCaP	Colo320	T47D	in vivo % PSA reduction	% tumor wt reduc	dose (μmol/kg)	MTD (μmol/kg)
vinblastine (**70**)		0.5						
des-acetyl vinblastine (dAc-VIN)[a] (**71**)		0.1	0.2	0.15	14	16	0.26	0.26
4-*O*-(Pro)dAc-VIN[a]		1.0	3.0	1.6	33	60	4.6	4.6
4-*O*-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-Pro)-dAc-VIN[a] (**72**)	12	1.6	14	>50	99	85	15.3	21.4

[a] VIN = vinblastine.

became important because the 4-position is sterically hindered, which mandates fairly vigorous conditions for both cleavage of the acetyl and subsequent esterification. On the basis of literature precedent, we found that the acetyl could be selectively removed with 25% hydrazine in ethanol at room temperature.

With a good preparation of des-Ac vinblastine in hand, we next investigated incorporation of peptide sequences. Even with simple amino acid derivatives, vigorous coupling conditions were required for good yields in esterification reactions. For example, the acid chloride of Fmoc-proline in pyridine was required for efficient reaction. These results also established that direct coupling of a peptide sequence to the 4-OH would not be a suitable strategy. The choice of an optimal peptide substrate for PSA relied heavily on our database developed in the doxorubicin studies.[43] In the optimized doxorubicin conjugates, an acidic group at the amino terminus was required for sufficient aqueous solubility for intravenous formulation. Because of the basic nature of vinblastine and its greater aqueous solubility, especially at pH less than 7, the amino terminal group could be a simple acetyl in this case. The substrate sequence Ac-Hyp-Ser-Ser-Chg-Gln-Ser-OH was initially chosen for coupling to H-Pro-des-Ac-vinblastine and other amino acid vinblastine derivatives; however, cleavage rates by PSA were unsuitably slow in all cases. An additional serine was incorporated at the carboxyl terminus. Experience in the doxorubicin research indicated that this sequence would be a good substrate. The target compound was prepared from the heptapeptide precursor (synthesized by solid-phase peptide synthesis) and 4-*O*-prolyl vinblastine with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)/3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (ODHBt)/*N*-methylmorpholine in DMF at 0 °C. We were pleased to find that compound **72** with the proline linker to vinblastine gave excellent cleavage rates with a $t_{1/2}$ for PSA cleavage of 12 min.

Evaluation of other in vitro properties demonstrated the PSA-secreting tumor-targeted profile of **72**.[44] In cell culture systems, **72** selectively killed the PSA-secreting LNCaP cells compared to the non-PSA-secreting Colo320 and T47D cells (Table 6). In contrast, des-Ac vinblastine potently kills all of these cell types with no selectivity. As in the case of the optimal doxorubicin conjugates, this selectivity translated to improved in vivo efficacy. In nude mice impregnated with LNCaP tumor cells, des-Ac-vinblastine and 4-*O*-Pro-des-Ac-vinblastine show little effect on circulating PSA levels or tumor weight at their maximum tolerated doses. However, conjugate **72** at a dose below its MTD demonstrated 99% reduction of PSA levels and 85% reduction of tumor weight in these mice (Table 6).[44] This

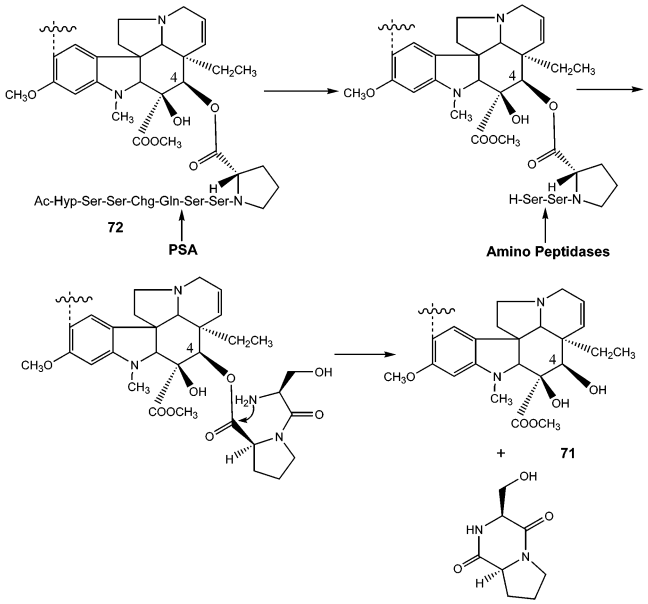


Figure 23. Proposed chemical release mechanism for vinblastine conjugate.

conjugate has promising properties for further evaluation of its antitumor efficacy.

The excellent biological properties of **72** and the important role of proline compared to nonsecondary amino acids as the linker to vinblastine suggested that a novel chemical cleavage mechanism could be operating for this conjugate. Further studies support the mechanism shown in Figure 23.[43] As anticipated, the first step in the in vivo release of des-Ac-vinblastine **72** involves cleavage by PSA at the Gln−Ser bond. Amino peptidase action would then remove the next residue (serine). Metabolism studies on substrates with primary amino acids as the linker to vinblastine indicate that subsequent protease and esterase cleavage is relatively slow. In the case of **72**, because of the tertiary amide linkage between Ser and Pro that lowers the energy of the cis amide conformation, the H-Ser-Pro-des-Ac-vinblastine can readily lose the dipeptide via a diketopiperazine cyclization to release des-Ac-vinblastine. This combination of enzymatic and chemical cleavage mechanisms is thought to be key to the efficient in vivo generation of **71** from **72**.

Conclusion

Three examples of our contributions to peptide chemistry have been exemplified in this article. We designed and synthesized the first dipeptide lactams as conformational constraints for biologically active peptides. Practical synthetic routes for five-, six-, and seven-membered lactams were developed to provide the target

materials in protected form ready for incorporation into peptide sequences. The utility of the lactams for providing bioactive conformational information was demonstrated in analogues of LH−RH, somatostatin, and rumen additive peptides. In a second example, we designed and synthesized the first cyclic hexapeptide somatostatin agonists with biological potency greater than that of the native hormone. Various conformationally constrained cyclic hexapeptide analogues were prepared to further define the biologically active conformation of somatostatin. With retrocycloisomeric analogues, it was shown that the peptide backbone was not critical for activity, thus paving the way for the development of non-peptide somatostatin analogues by other researchers. In the course of the somatostatin studies, we also developed a novel method for the synthesis of protected *N*-alkylamino acids and an improved peptide macrocyclization procedure. The third example of our research involved the design and synthesis of short peptide substrates for the serine protease prostate specific antigen (PSA). These peptides were conjugated to doxorubicin and vinblastine, and targeting of these cytotoxic agents to PSA-secreting tumors in nude mice was demonstrated.

The area of biologically active peptide research continues to be very active and holds considerable promise for the development of novel therapeutic agents. Ongoing research is utilizing some of our concepts and methodologies, and I hope that these tools will continue to be useful to workers in the field.

Acknowledgment. I thank the leaders of the Department of Medicinal Chemistry and the peptide research efforts at Merck (Ralph Hirschmann, Daniel Veber, Paul Anderson, and Joel Huff) for their support and guidance. The work described in this manuscript would not have been possible without the dedicated efforts of many Merck colleagues. They are coauthors of our publications that I have cited, and I thank them for their contributions. I thank Joel Huff and Victor Garsky for critical reading of the final draft, Chris Culberson for molecular modeling assistance, and Jean Kaysen for skilled preparation of the manuscript.

Biography

Roger M. Freidinger received a B.S. in Chemistry at the University of Illinois (1969) and a Ph.D. in Organic Chemistry under the direction of George Büchi at the Massachusetts Institute of Technology (1975). He served in the U.S. Army from 1970 to 1972. His professional career has been at the Merck Research Laboratories in West Point, Pennsylvania, where he is Executive Director, Medicinal Chemistry. Dr. Freidinger's research activities have focused on the chemistry and medicinal chemistry of peptides, peptidomimetics, small-molecule ligands for receptors, and enzyme inhibitors encompassing several therapeutic areas. He received the Vincent du Vigneaud Award of the American Peptide Society and is a fellow of the American Association for the Advancement of Science. He is currently serving as President of the American Peptide Society.

References

(1) Freidinger, R. M.; Veber, D. F.; Hirschmann, R.; Paege, L. M. Lactam Restriction of Peptide Conformation in Cyclic Hexapeptides Which Alter Rumen Fermentation. *Int. J. Pept. Protein Res.* **1980**, *16*, 464−470.

(2) Gund, P.; Andose, J. D.; Rhodes, J. B.; Smith, G. M. Three-Dimensional Molecular Modeling and Drug Design. *Science* **1980**, *208*, 1425−1431.

(3) Fleming, A. On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to Their Use in the Isolation of *B. Influenze. Br. J. Exp. Pathol.* **1929**, *10*, 226−236.

(4) Hodgkin, D. C. The X-Ray Analysis of the Structure of Penicillin. *Adv. Sci.* **1949**, *6*, 85−89.

(5) Arai, N.; Shiomi, K.; Yamaguchi, Y.; Masuma, R.; Iwai, Y.; Turberg, A.; Koelbl, H.; Omura, S. Argadin, a New Chitinase Inhibitor, Produced by *Clonostachys* sp. FO-7314. *Chem. Pharm. Bull.* **2000**, *48*, 1442−1446.

(6) Houston, D. R.; Shiomi, K.; Arai, N.; Omura, S.; Peter, M. G.; Turberg, A.; Synstad, B.; Eijsink, V. C. H.; van Aalten, D. M. F. High-Resolution Structures of a Chitinase Complexed with Natural Product Cyclopentapeptide Inhibitors: Mimicry of Carbohydrate Substrate. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 9127−9132.

(7) Pettit, G. R.; Kamano, Y.; Herald, C. L.; Dufresne, C.; Cerny, R. L.; Herald, D. L.; Schmidt, J. M.; Kizu, H. Antineoplastic Agent. 174. Isolation and Structure of the Cytostatic Depsipeptide Dolastatin 13 from the Sea Hare Dolabella Auricularia. *J. Am. Chem. Soc.* **1989**, *111*, 5015−5017.

(8) Adamczeski, M.; Quiñoà, E.; Crews, P. Novel Sponge-Derived Amino Acids. 5. Structures, Stereochemistry, and Synthesis of Several New Heterocycles. *J. Am. Chem. Soc.* **1989**, *111*, 647−654.

(9) (a) Salmeen, A.; Andersen, J. N.; Myers, M. P.; Meng, T.-C.; Hinks, J. A.; Tonks, N. K.; Barford, D. Redox Regulation of Protein Tyrosine Phosphatase IB Involves a Sulphenyl-amide Intermediate. *Nature* **2003**, *423*, 769−773. (b) van Montfort, R. L. M.; Congreve, M.; Tisi, D.; Carr, R.; Jhotl, H. Oxidation State of the Active-Site Cysteine in Protein Tyrosine Phosphatase IB. *Nature* **2003**, *423*, 773−777.

(10) Freidinger, R. M.; Perlow, D. S.; Veber, D. F. Protected Lactam-Bridged Dipeptides for Use as Conformational Constraints in Peptides. *J. Org. Chem.* **1982**, *47*, 104−109.

(11) Freidinger, R. M. Synthesis of γ-Lactam-Constrained Tryptophyllysine Derivatives. *J. Org. Chem.* **1985**, *50*, 3631−3633.

(12) Momany, F. A. Conformational Energy Analysis of the Molecule, Luteinizing Hormone−Releasing Hormone. 1. Native Decapeptide. *J. Am. Chem. Soc.* **1976**, *98*, 2990−2996.

(13) Freidinger, R. M.; Veber, D. F.; Perlow, D. S.; Brooks, J. R.; Saperstein, R. Bioactive Conformation of Luteinizing Hormone−Releasing Hormone: Evidence from a Conformationally Constrained Analog. *Science* **1980**, *210*, 656−658.

(14) Kopple, K. D. Peptide Backbone Folding in LHRH and Analogs. In *Peptides: Synthesis, Structure, Function*: *Proceedings of the Seventh American Peptide Symposium*; Rich, D. H., Gross, E., Eds.; Pierce Chemical Company: Rockford, IL, 1981; pp 295−298.

(15) Cascieri, M. A.; Chicchi, G. G.; Freidinger, R. M.; Colton, C. D.; Perlow, D. S.; Williams, B.; Curtis, N. R.; McKnight, A. T.; Maguire, J. J.; Veber, D. F.; Liang, T. Conformationally Constrained Tachykinin Analogs Which Are Selective Ligands for the Eledoisin Binding Site. *Mol. Pharmacol.* **1986**, *29*, 34−38.

(16) Freidinger, R. M.; Perlow, D. S.; Randall, W. C.; Saperstein, R.; Arison B. H.; Veber D. F. Conformational Modifications of Cyclic Hexapeptide Somatostatin Analogs. *Int. J. Pept. Protein Res.* **1984**, *23*, 142−150.

(17) (a) Thorsett, E. D.; Harris, E. E.; Aster, S. D.; Peterson, E. R.; Taub, D.; Patchett, A. A. Dipeptide Mimics. Conformationally Restricted Inhibitors of Angiotensin-Converting Enzyme. *Biochem. Biophys. Res. Commun.* **1983**, *111*, 166−171. (b) Thorsett, E. D.; Harris, E. E.; Aster, S. D.; Peterson, E. R.; Tristram, E. W.; Snyder, J. P.; Springer, J. P.; Patchett, A. A. Conformationally Restricted Inhibitors of Angiotensin Converting Enzyme. In *Peptides, Structure and Function: , Proceedings of the Eighth American Peptide Symposium*; Pierce Chemical Company: Rockford, IL, 1983; pp 555−558.

(18) Wyvratt, M. J.; Tischler, M. H.; Ikeler, T. J.; Springer, J. P.; Tristram, E. W.; Patchett, A. A. Bicyclic Inhibitors of Angiotensin-Converting Enzyme. In *Peptides, Structure and Function: Proceedings of the Eighth American Peptide Symposium*; Pierce Chemical Company: Rockford, IL, 1983; pp 551−554.

(19) Aubé, J. Synthetic Routes to Lactam Peptidomimetics. In *Advances in Amino Acid Mimetics and Peptidomimetics*; JAI Press Inc.: Greenwich, CT, 1997; Vol. 1, pp 193−232.

(20) Halab, L.; Gosselin, F.; Lubell, W. D. Design, Synthesis, and Conformational Analysis of Azacycloalkane Amino Acids as Conformationally Constrained Probes for Mimicry of Peptide Secondary Structures. *Biopolymers (Pept. Sci.)* **2000**, *55*, 101−122.

(21) Simpson, J. C.; Ho, C.; Shands, E. F. B.; Gershengorn, M. C.; Marshall, G. R.; Moeller, K. D. Conformationally Restricted TRH Analogues: Constraining the Pyroglutamate Region. *Bioorg. Med. Chem.* **2002**, *10*, 291−302.

(22) Hoffmann, T.; Waibel, R.; Gmeiner, P. A General Approach to Dehydro-Freidinger Lactams: Ex-Chiral Pool Synthesis and Spectroscopic Evaluation as Potential Reverse Turn Inducers. *J. Org. Chem.* **2003**, *68*, 62−69.

(23) Brazeau, P.; Vale, W.; Burgus, R.; Ling, N.; Butcher, M.; Rivier, J.; Guillemin, R. Hypothalamic Polypeptide That Inhibits the Secretion of Immunoreactive Pituitary Growth Hormone. *Science* **1973**, *179*, 77−79.

(24) Veber, D. F.; Holly, F. W.; Paleveda, W. J.; Nutt, R. F.; Bergstrand, S. J.; Torchiana, M.; Glitzer, M. S.; Saperstein, R.; Hirschmann, R. Conformationally Restricted Bicyclic Analogs of Somatostatin. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2636−2640.

(25) Veber, D. F.; Holly, F. W.; Nutt, R. F.; Bergstrand, S. J.; Brady, S. F.; Hirschmann, R.; Glitzer, M. S.; Saperstein, R. Highly Active Cyclic and Bicyclic Somatostatin Analogues of Reduced Ring Size. *Nature* **1979**, *280*, 512−514.

(26) Veber, D. F.; Freidinger, R. M.; Perlow, D. S.; Paleveda, W. J., Jr.; Holly, F. W.; Strachan, R. G.; Nutt, R. F.; Arison, B. H.; Homnick, C.; Randall, W. C.; Glitzer, M. S.; Saperstein, R.; Hirschmann, R. A Potent Cyclic Hexapeptide Analogue of Somatostatin. *Nature* **1981**, *292*, 55−58.

(27) Veber, D. F.; Saperstein, R.; Nutt, R. F.; Freidinger, R. M.; Brady, S. F.; Curley, P.; Perlow, D. S.; Paleveda, W. J.; Colton, C. D.; Zacchei, A. G.; Tocco, D. J.; Hoff, D. R.; Vandlen, R. L.; Gerich, J. E.; Hall, L.; Mandarino, L.; Cordes, E. H.; Anderson, P. S.; Hirschmann, R. A Super Active Cyclic Hexapeptide Analog of Somatostatin. *Life Sci.* **1984**, *34*, 1371−1378.

(28) Brady, S. F.; Varga, S. L.; Freidinger, R. M.; Schwenk, D. A.; Mendlowski, M.; Holly, F. W.; Veber, D. F. Practical Synthesis of Cyclic Peptides, with an Example of Dependence of Cyclization Yield upon Linear Sequence. *J. Org. Chem.* **1979**, *44*, 3101−3105.

(29) Brady, S. F.; Freidinger, R. M.; Paleveda, W. J.; Colton, C. D.; Homnick, C. F.; Whitter, W. L.; Curley, P.; Nutt, R. F.; Veber, D. F. Large-Scale Synthesis of a Cyclic Hexapeptide Analogue of Somatostatin. *J. Org. Chem.* **1987**, *52*, 764−769.

(30) Freidinger, R. M.; Veber, D. F. Design of Novel Cyclic Hexapeptide Somatostatin Analogs from a Model of the Bioactive Conformation. In *Conformationally Directed Drug Design*; American Chemical Society: Washington, DC, 1984; pp 169−187.

(31) Freidinger, R. M.; Hinkle, J. S.; Perlow, D. S.; Arison, B. H. Synthesis of 9-Fluorenylmethyloxycarbonyl-Protected *N*-Alkyl Amino Acids by Reduction of Oxazlidinones. *J. Org. Chem.* **1983**, *48*, 77−81.

(32) Aurelio, L.; Box, J. S.; Brownlee, R. T. C.; Hughes, A. B.; Sleebs, M. M. An Efficient Synthesis of *N*-Methyl Amino Acids by Way of Intermediate 5-Oxazolidinones. *J. Org. Chem.* **2003**, *68*, 2652−2667.

(33) Brady, S. F.; Paleveda, W. J., Jr.; Arison, B. H.; Saperstein, R.; Brady, E. J.; Raynor, K.; Reisine, T.; Veber, D. F.; Freidinger, R. M. Approaches to Peptidomimetics Which Serve as Surrogates for the cis Amide Bond: Novel Disulfide-Constrained Bicyclic Hexapeptide Analogs of Somatostatin. *Tetrahedron* **1993**, *49*, 3449−3466.

(34) Hata, Y.; Matsuura, Y.; Tanaka, N.; Ashida, T.; Kakudo, M. *tert*-Butyloxycarbonyl-L-cysteinyl-L-cysteine Disulfide Methyl Ester. *Acta Crystallogr.* **1977**, *B33*, 3561−3564.

(35) Goodman, M.; Chorev, M. On the Concept of Linear Modified Retro-peptide Structures. *Acc. Chem. Res.* **1979**, *12*, 1−7.

(36) Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Leahy, E. M.; Salvino, J.; Arison, B.; Cichy, M. A.; Spoors, P. G.; Shakespeare, W. C.; Sprengeler, P. A.; Hamley, P.; Smith, A. B., III; Reisine, T.; Raynor, K.; Maechler, L.; Donaldson, C.; Vale, W.; Freidinger, R. M.; Cascieri, M. R.; Strader, C. D. De Novo Design and Synthesis of Somatostatin Non-Peptide Peptidomimetics Utilzing β-D-Glucose as a Novel Scaffolding. *J. Am. Chem. Soc.* **1993**, *115*, 12550−12568.

(37) Yang, L.; Berk, S. C.; Rohrer, S. P.; Mosley, R. T.; Guo, L.; Underwood, D. J.; Arison, B. H.; Birzin, E. T.; Hayes, E. C.; Mitra, S. W.; Parmar, R. M.; Cheng, K.; Wu, T.-J.; Butler, B. S.; Foor, F.; Pasternak, A.; Pan, Y.; Silva, M.; Freidinger, R. M.; Smith, R. G.; Chapman, K. Synthesis and Biological Activities of Potent Peptidomimetics Selective for Somatostatin Receptor Subtype 2. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10836−10841.

(38) Hirschmann, R.; Strachan, R. G.; Buchschacher, P.; Sarett, L. H.; Steelman, S. L.; Silber, R. An Approach to an Improved Antiinflammatory Steroid. The Synthesis of 11β,17-Dihydroxy-3,20-dione-1,4-pregnadien-21-yl 2-Acetamido-2-deoxy-β-D-glucopyranoside. *J. Am. Chem. Soc.* **1964**, *86*, 3903−3904.

(39) DeFeo-Jones, D.; Garsky, V. M.; Wong, B. K.; Feng, D.-M.; Bolyar, T.; Kaskell, K.; Kiefer, D. M.; Leander, K.; McAvoy, E.; Lumma, P.; Wai, J.; Senderak, E. T.; Motzel, S. L.; Keenan, K.; Van Zwieten, M.; Lin, J. H.; Freidinger, R.; Huff, J.; Oliff, A.; Jones R. E. A Peptide−Doxorubicin "Prodrug" Activated by Prostate-Specific Antigen Selectively Kills Prostate Tumor Cells Positive for Prostate-Specific Antigen in Vivo. *Nat. Med.* **2000**, *11*, 1248−1252.

(40) Anand, K.; Ziebuhr, J.; Wadhwani, P.; Mesters, J. R.; Hilgenfeld, R. Coronavirus Main Proteinase (3CL^{pro}) Structure: Basis for Design of Anti-SARS Drugs. *Science* **2003**, *300*, 1763−1767.

(41) Garsky, V. M.; Lumma, P. K.; Feng, D.-M.; Wai, J.; Ramjit, H. G.; Sardana, M. K.; Oliff, A.; Jones, R. E.; DeFeo-Jones, D.; Freidinger, R. M. The Synthesis of a Prodrug of Doxorubicin Designed To Provide Reduced Systemic Toxicity and Greater Target Efficacy. *J. Med. Chem.* **2001**, *44*, 4216−4224.

(42) DiPaola, R. S.; Ebbinghaus, S.; Nemunaitis, J.; McCullough, J.; Ciardella, M.; Adams, N.; Williams, A.; Garsky, V.; Wong, B, K.; Defeo-Jones, D.; Jones, R.; Schwartz, M.; Winchell, G.; Arena, C.; Deutsch, P.; Yao, S. Phase I and Pharmacokinetic Study of L377202, a Novel Peptide Doxorubicin Conjugate, in Patients with Advanced Prostate Cancer. *Proc. Am. Soc. Clin. Oncol.* **2000**, *19*, 348.

(43) Brady, S. F.; Pawluczyk, J. M.; Lumma, P. K.; Feng, D.-M.; Wai, J. M.; Jones, R.; DeFeo-Jones, D.; Wong, B. K.; Miller-Stein, C.; Lin, J. H.; Oliff, A.; Freidinger, R. M.; Garsky, V. M. Design and Synthesis of a Pro-Drug of Vinblastine Targeted at Treatment of Prostate Cancer with Enhanced Efficacy and Reduced Systemic Toxicity. *J. Med. Chem.* **2002**, *45*, 4706−4715.

(44) DeFeo-Jones, D.; Brady, S. F.; Feng, D.-M.; Wong, B. K.; Bolyar, T.; Haskell, K.; Kiefer, D. M.; Leander, K.; McAvoy, E.; Lumma, P.; Pawluczyk, J. M.; Wai, J.; Motzel, S. L.; Keenan, K.; Van Zwieten, M.; Lin, J. H.; Garsky, V. M.; Freidinger, R.; Oliff, A.; Jones, R. E. A prostate specific antigen (PSA) activated vinblastine prodrug selectively kills PSA secreting cells in vivo. *Mol. Cancer Ther.* **2002**, *1*, 451−459.

JM030484K