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Solution-Phase Parallel Synthesis of a Pharmacophore Library of HUN-7293 Analogues: A General Chemical Mutagenesis Approach To Defining Structure−Function Properties of Naturally Occurring Cyclic (Depsi)peptides

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Abstract: HUN-7293 (**1**), a naturally occurring cyclic heptadepsipeptide, is a potent inhibitor of cell adhesion molecule expression (VCAM-1, ICAM-1, E-selectin), the overexpression of which is characteristic of chronic inflammatory diseases. Representative of a general approach to defining structure−function relationships of such cyclic (depsi)peptides, the parallel synthesis and evaluation of a complete library of key HUN-7293 analogues are detailed enlisting solution-phase techniques and simple acid−base liquid−liquid extractions for isolation and purification of intermediates and final products. Significant to the design of the studies and unique to solution-phase techniques, the library was assembled superimposing a divergent synthetic strategy onto a convergent total synthesis. An alanine scan and *N*-methyl deletion of each residue of the cyclic heptadepsipeptide identified key sites responsible for or contributing to the biological properties. The simultaneous preparation of a complete set of individual residue analogues further simplifying the structure allowed an assessment of each structural feature of **1**, providing a detailed account of the structure−function relationships in a single study. Within this pharmacophore library prepared by systematic chemical mutagenesis of the natural product structure, simplified analogues possessing comparable potency and, in some instances, improved selectivity were identified. One potent member of this library proved to be an additional natural product in its own right, which we have come to refer to as HUN-7293B (**8**), being isolated from the microbial strain F/94-499709.

The naturally occurring cyclic heptadepsipeptide HUN-7293 (**1**, Figure 1) was first isolated in 1992 from a fungal broth in a screen for inhibitors of inducible cell adhesion molecule expression.[1] Its structure was subsequently established by ^{1}H NMR spectroscopic methods and confirmed by X-ray analysis.[2] Independently, the same cyclic depsipeptide was isolated by a Japanese group from a different fungal species based on a screen for anti-HIV compounds.[3] Endothelial cell associated molecules including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin play important roles in the immune response by recruiting and regulating leukocyte migration and cell-to-cell interactions at a site of inflammation. Modulation of these interactions through the inhibition of such cell adhesion molecule expression should prove useful for the treatment of chronic inflammatory and autoimmune diseases characterized by cell adhesion molecule overexpression or upregulation. As detailed in recent studies,[4] diverse signals act on endothelial cells to activate members of the nuclear factor of Kappa B (NF-κB) transcription factor family. NF-κB family members are cytoplasmic homo- or heterodimer proteins inactivated when complexed with members of a family of inhibitor proteins, IκB. Phosphorylation of IκB, releasing the active NF-κB, results in translocation to the nucleus and initiation of transcription of its targeted genes including the cell surface adhesion molecules. Aside from antisense[5] and antibody[6] approaches, only a limited number of other groups have detailed work on small molecule inhibitors[7] of the expression of cell surface adhesion molecules and, unlike HUN-7293 (**1**),[1] each target the NF-κB signal transduction pathway.

We recently disclosed the first total synthesis of HUN-7293 (**1**) by a convergent approach which served to define several

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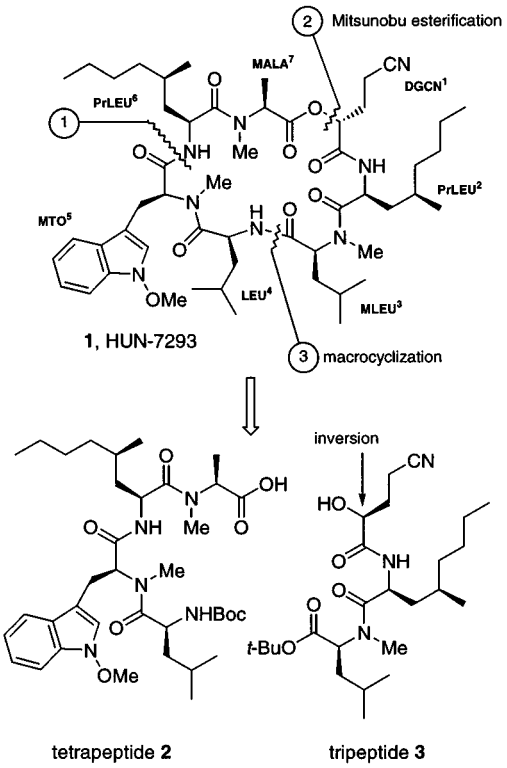

Figure 1.

subtle elements essential to its preparation including backbone ester formation by a Mitsunobu displacement and the identification of MLEU[3]-LEU[4] as an effective macrocyclization site.[8] In addition to identifying the tetrapeptide **2** and tripeptide **3** as key intermediates in the convergent synthesis, the use of a Mitsunobu esterification with inversion of the DGCN α-center avoided racemization of the sensitive NMe-Ala (residue 7) observed upon carboxylate activation required of a conventional esterification, and permitted the utilization of a readily available L- versus D-amino acid precursor to the D-α-hydroxy carboxylic acid residue. Herein, we detail the implementation of this approach in the parallel synthesis of a library of key analogues of **1** used to define in detail the structure−function relationships of the natural product.

A solution-phase approach to the simultaneous (parallel) preparation of the library was utilized enlisting simple acid−base liquid−liquid extractions for isolation and purification of the synthetic intermediates and final products. This approach, which we introduced with the multistep preparation of simpler amide-based small molecules libraries,[9−14] provided each intermediate and final product sufficiently pure for subsequent use even in the multistep synthesis of the natural product **1** and its structurally challenging analogues. The library described (**4**−**34**) consists of nearly 40 natural product analogues,[15−21] each prepared using a convergent,[12−14] yet divergent[22] total synthesis, and provides an alanine scan of the seven residues, an *N*-methyl deletion of the three *N*-methyl residues, and fundamental simplifications in each of the nonstandard amino acid side chains allowing the identification of key residues and structural features contributing to the biological properties. The simultaneous inclusion of key analogues of the nonstandard amino acid side chains permitted the identification of not only essential structural features, but also nonobvious and subtle structural features. It is this combination of the alanine scan and *N*-methyl deletion library with the systematic single site deletion modifications of structural features found in each of the individual side chains that we have come to refer to as a pharmacophore library.

Significant to the design of such studies, the library was assembled superimposing a divergent synthetic strategy[22] onto a convergent total synthesis. This combination of the beneficial attributes of a conventional convergent synthetic strategy with the divergent preparation of a series of structurally related analogues (e.g., use of one prep of **2** to assemble all residue 1−3 analogues) can only be conveniently conducted using solution-phase, not solid-phase, techniques and avoids the more repetitive independent linear synthesis of each analogue.

Solution-Phase Synthesis of HUN-7293 and Aza-HUN-7293. In preliminary studies designed to optimize the solution-phase synthesis protocols for implementation in the library

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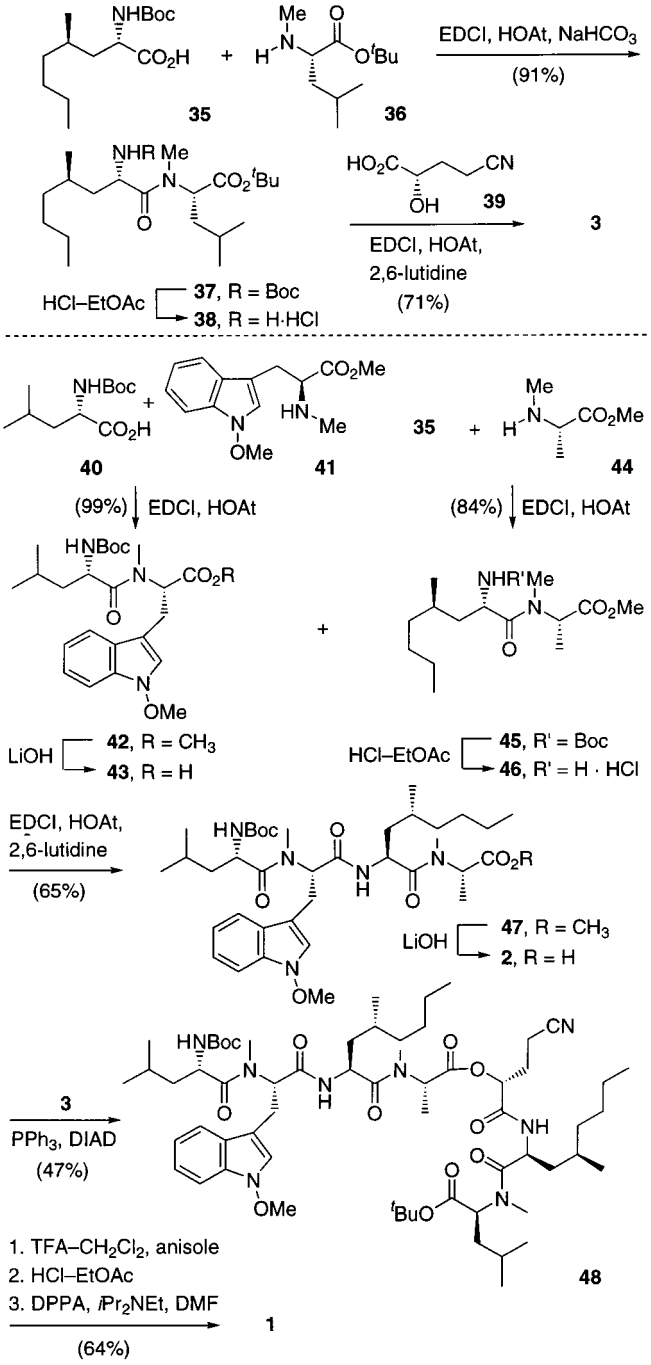
Scheme 1



synthesis, the preparations of HUN-7293 (**1**) and its backbone aza analogue **4** were conducted enlisting acid−base liquid−liquid extractions for the purification of the intermediates. The results obtained with the synthesis of **1** are detailed in Scheme 1 and details of the synthesis of **4** are provided in the Supporting Information.[23,24] The initial preparation of **4** along with **1** permitted an early assessment of the importance of the backbone depsipeptide ester resulting in its maintenance in the library.

The tetrapeptide **2** and tripeptide **3** were prepared starting with the five amino acids and α-hydroxycarboxylic acid previously disclosed.[8] To obtain pure products from the extraction isolations with minimal formation of undesired diastereomers, the peptide coupling conditions were optimized with the amines used in excess to ensure the complete consumption of the intermediate activated esters derived from the carboxylic acids. The three dipeptides **37**, **42**, and **45** were obtained cleanly in good yields after simple acid−base liquid−liquid extractions of couplings promoted by EDCI-HOAt (Scheme 1). The excess starting amine, any residual unreacted carboxylic acid, reagents (EDCI-HOAt), and the reagent byproducts (from EDCI) were removed in the extractions. Selective deprotection of the *N*-Boc group in the presence of the *t*-Bu ester in **37** with HCl-EtOAc[25] provided the amine HCl salt **38**, which was used directly in the next coupling reaction. Tripeptide **3** was obtained in good yield and purity from **38** and the α-hydroxycarboxylic acid **39** using EDCI-HOAt (2,6-lutidine) and the acid−base liquid−liquid extraction for purification and isolation. Similarly, tetrapeptide **2** was prepared employing EDCI-HOAt (2,6-lutidine) for the coupling of dipeptides **43** and **46** following *N*-Boc deprotection of **45** (HCl-EtOAc) and methyl ester hydrolysis of **42** (LiOH). Under these conditions, the diastereomeric ratio favoring the desired tetrapeptide **47** was increased to 32:1 compared to 13:1 using HATU-$NaHCO_3$.[8] Notably, this coupling entails activation of a racemization prone *N*-acyl *N*-methyl amino acid and was carefully monitored in the synthesis of analogues containing modifications in residues 4−6. Mitsunobu esterification of **2**, derived from LiOH hydrolysis of **47**, with the tripeptide **3** provided the key linear heptadepsipeptide **48** (47%). Whereas the product of the amide coupling reaction utilized to assemble the linear heptapeptide precursor to aza-HUN-7293 (**4**) was isolated and purified through the acid−base liquid−liquid extraction protocol,[25] the Mitsunobu esterification product **48** and those leading to **5**−**32** were further purified by chromatography. In principle this may not have been necessary, but it did simplify the isolation and improved the purity of the final products. Linear heptadepsipeptide **48** was deprotected upon treatment with TFA, and converted to the amine HCl salt. Final macrolactamization to provide **1** (64%) was achieved by the treatment with DPPA-*i*Pr_2NEt in 1 mM DMF. These cyclization conditions were first developed for the preparation of aza-HUN-7293 (**4**),[23] which was established to close much less effectively than **1** itself, and subsequently established to be optimal for synthesis of the HUN-7293 analogues. In contrast, treatment of the deprotected linear heptadepsipeptide derived from **48** with HOAt-EDCI in the absence of base afforded $C_2{}^3$-*epi*-**1** exclusively (32−44%) without the detection of **1**. Analogous observations were made with the cyclization of the linear heptapeptide leading to **4** (aza-HUN-7293) although this substrate failed to close as effectively as **1**. Cyclization conducted with DPPA (0 °C, 5 mM DMF) in the presence of *i*Pr_2NEt (16:1, 27%) or $NaHCO_3$ (21:1, 10%) provided **4** with little competitive $C_2{}^3$ epimerization, whereas closure promoted by BOP (25 °C, 5 mM CH_3CN) in the presence of DMAP (5:1, 22%), *i*Pr_2NEt (3:1, 25%), or $NaHCO_3$ (1:2.5, 5%) suffered substantial $C_2{}^3$ epimerization, and those promoted by EDCI-HOAt (0−25 °C, 5 mM DMF) with or without $NaHCO_3$ suffered near complete $C_2{}^3$ epimerization (1:18−32, 40−85%).

Aza-HUN-7293 (**4**) and its $C_2{}^3$ epimer were compared with **1** and its $C_2{}^3$ epimer in the ability to inhibit inducible cell

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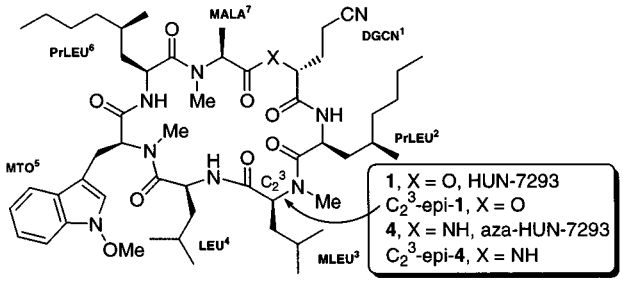


Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)

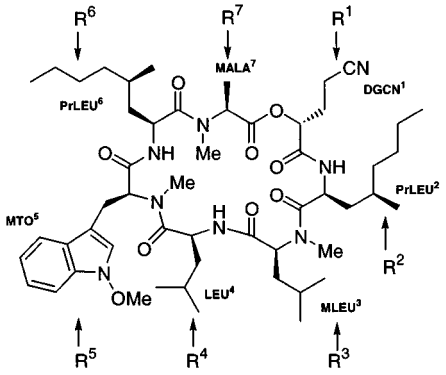
compound	VCAM-1		ICAM-1	selectivity: ICAM-1/VCAM-1
1	1 (1)[a]	(1)[b]	24 (12)[a]	24
C_2^3-epi-**1**	2 ± 1	(0.5)[b]	120 ± 40	70
4	58	(0.02)[b]	950	16
C_2^3-epi-**4**	550	(0.002)[b]	>3000	nd

[a]Synthetic **1**. [b]Relative potency.

Figure 2.

adhesion molecule expression (VCAM-1, ICAM-1, E-selectin) in ELISA assays previously disclosed (Figure 2).[1,2] In each case, **4** proved to be 20- to 60-fold less potent than **1** at inhibiting VCAM-1, ICAM-1, or E-selectin expression.[23] The natural product exhibits a 24-fold selectivity for VCAM-1 versus ICAM-1 expression and an even greater 44-fold selectivity versus E-selectin. This same selectivity, albeit reduced, was observed with **4**[23] and the subsequent screening of our library was conducted comparing only the inhibition of inducible VCAM-1 and ICAM-1 expression. In addition, the C_2^3 epimer of aza-HUN-7293 exhibited a further 10-fold loss in potency relative to **4** suggesting that the C_2^3 stereochemistry is important to the properties of **1**. In contrast to this behavior of **4** versus C_2^3-*epi*-**4**, C_2^3-*epi*-**1** was only 2-fold less potent than **1** against VCAM-1 and only 5-fold less potent against ICAM-1.[26] Although this result was not expected, it is consistent with subsequent observations made with a series of such C_2^3 epimers that we incorporated into the following studies. Consequently, the C_2^3 stereochemistry was critically assessed following library macrocyclization in light of the potential epimerization. Having established that the depsipeptide ester is important to the biological properties of **1** and facilitated macrocyclization,[23] the library was assembled incorporating the backbone ester. In principle, this did not alter the reliance on extraction purification. However, we did find that chromatographic purification following the Mitsunobu esterification facilitated the isolation of the pure final products by removing minor contaminant byproducts and diastereomers accumulating throughout the syntheses. In addition, and in order to ensure confidence in the testing results, the final analogues **4**−**34** were purified by HPLC chromatography prior to assay ensuring removal of any C_2^3-epimer and other potential diastereomer contaminants.

Alanine Scan. Alanine-scanning mutagenesis is an informative method for defining epitopes in proteins.[27] By replacing



Alanine Scan. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)[a]

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1, HUN-7293[b]	1	24	24	1
8, R^1 = Me	2.3 ± 0.5	178 ± 18	77	0.4
10, R^2 = Me	100 ± 34	6300 ± 370	63	0.01
13, R^3 = Me	56 ± 11	4100 ± 2500	73	0.02
14, R^4 = Me	16 ± 1.4	700 ± 20	43	0.06
15, R^5 = Me	1800 ± 10	2800 ± 210	1.6	0.0006
27, R^6 = Me	270 ± 40	1400 ± 300	5.2	0.004

[a]Unless indicated, the substituents R^1–R^7 are those found in **1**. [b]R^7 = Me, HUN-7293.

Figure 3.

each amino acid side chain with a methyl group while retaining the stereochemistry of the peptide backbone, the significance of each individual side chain can be identified.[28] Although this approach has its limitations, it has been used to provide insight into structure−function relationships of a protein, especially when the functional mechanism is unknown or its 3D structure is unavailable.[29]Similarly, in the absence of a defined target, the importance of each residue of the cyclic heptadepsipeptide structure of HUN-7293 could not be anticipated. Along with additional modifications and simplifications in each of the individual side chains, an alanine scan of the structure was conducted. The synthesis of each analogue constituting the alanine scan is presented in the following treatments of each residue. However, the results of their examination are summarized in Figure 3 since they provide an initial, albeit surprisingly incomplete, overview of the relative importance of each residue. With the exception of residue 7, which already contains a methyl group in the natural product (R^7 = Me), the replacement of each side chain with a methyl group led to a reduction in potency. However, the magnitude of this loss in activity varied over a 1000-fold range with the R^1 replacement being only ca. 2-fold lower, the R^2−R^4 replacements leading to 10- to 100-fold reductions, and the R^5 and R^6 replacements leading to even larger >100- to 1000-fold reductions with R^5 being by far the most significant residue. Just as interesting and despite the potency reductions, the methyl substitutions for the R^1−R^4 side chains enhanced the VCAM-1 versus ICAM-1 selectivity, indicating that the changes in this half of the

(26) The C_2^3 epimer of **1** has also been prepared by degradation of **1** through selective formation of the residue 3 thioamide, thioimidate formation, hydrolysis, and subsequent macrolactamization with partial C_2^3 epimerization. Its activity was found to be identical with that of our synthetic sample of C_2^3-*epi*-**1**, IC_{50} = 1.5 nM (VCAM-1), 84 nM (ICAM-1). We thank Berndt Oberhauser (Novartis) for sharing these observations in advance of publication.

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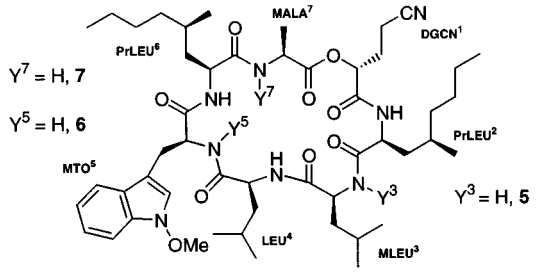
(29) For examples of such an *N*-methyl deletion scan, see: Boger, D. L.; Yohannes, D.; Myers, J. B. *J. Org. Chem.* **1992**, *57*, 1319−1321. Boger, D. L.; Yohannes, D.; Zhou, J.; Patane, M. A. *J. Am. Chem. Soc.* **1993**, *115*, 3420−3430. Boger, D. L.; Patane, M. A.; Zhou, J. *J. Am. Chem. Soc.* **1994**, *116*, 8544−8556. Boger, D. L.; Patane, M. A.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7357−7363. Boger, D. L.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7364−7378.

molecule diminished inhibition of ICAM-1 expression more significantly, whereas this selectivity was lost along with the activity with methyl substitutions at R^5 and R^6 with the change at R^5 again being especially detrimental. From these results alone, one could anticipate that substantial changes in the R^1 side chain could be tolerated, including efforts to enhance selectivity or physiochemical, distribution, stability, and toxicity profiles, whereas changes to R^2-R^4 might prove more challenging, and those to R^5 and R^6 are likely to be especially detrimental. Consequently, the results of the more subtle side chain modifications which are detailed in the following sections proved especially valuable at addressing inaccuracies in interpreting such an incomplete data set.

***N*-Methyl Deletion.** Peptides containing *N*-methyl groups often display special properties, including increased hydrophobicity, increased stability and bioavailability, along with promoting the adoption of cis amide bonds or disrupting intramolecular H-bonds which are key to establishing the conformation of the molecules.[28,29] HUN-7293 adopts a single, well-defined conformation in solution (>90% in $CDCl_3$/DMSO and MeOH/H_2O, $\geq$60−70% in $CDCl_3$) which is very similar to that found in the X-ray structure.[2] Prominent structural features of this major conformation are two cis amide bonds between residues 2 and 3 and residues 4 and 5 where two of the *N*-methyl amides are found. Two strong transannular H-bonds are also observed (N(2)−H/O═C(6), N(6)−H/O═C(3)). This *N*-methylation state and intramolecular H-bonding along with the depsipeptide ester leaves only one amide NH available to serve as a H-bond donor for intermolecular H-bonding (N(4)−H). One key set of analogues is **5**−**7** which constitute the *N*-methyl deletion at each of the three *N*-methyl amides of **1**. Upon reflection on the conduct of the work, we would now recommend extending such studies to include an *N*-methyl scan of each secondary amide residue of the native cyclic depsipeptide which could reveal the additional importance of the structural or H-bonding characteristics of the secondary amides.[30,31]However, at the time our work was conducted, the potential benefit of such a *N*-methyl scan escaped us.

The synthesis of these three key analogues requires the incorporation of the parent NH versus *N*-methyl derivative of residues 3, 5, and 7. Those of residues 3 and 7 are simply L-Leu and L-Ala and that of residue 5 constituted an intermediate in our synthesis of the MTO[5] residue of HUN-7293.[8] The details of the synthesis of **5**−**7** are provided in the Supporting Information.

The evaluation of **5**−**7** was revealing (Figure 4). The *N*-methyl amide linking residues 4 and 5 was found to be essential as its removal with **6** resulted in the complete loss of activity (>10 000-fold). This is consistent with the amide adopting a cis amide stereochemistry serving as a critical structural element establishing the conformation of the molecule. Consistent with this expectation, the strong shielding of one of the Leu[4] β_1 protons (δ −0.37), indicative of the stacking interaction with the MTO[5] indole seen in the NMR and X-ray structures, is no longer observed in the ^{1}H NMR of **6**. Although it adopts a well-defined conformation as judged by its sharp single resonance ^{1}H NMR, this conformation differs from that of **1**. This largest loss of activity with a modification to residue 5, like that observed in the alanine scan, indicates a critical role for this residue.



***N*-Methyl Deletion**. Inhibition (IC_{50}, nM ±SD) of VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)[a]

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1, HUN-7293	1	24	24	1
5, Y^3 = H	11 ± 1.3	600 ± 270	54	0.09
6, Y^5 = H	>10,000	>10,000	nd	<0.0001
7, Y^7 = H	6.8 ± 0.3	1200 ± 530	180	0.15

[a]Unless indicated, the substituents Y^3, Y^5, and Y^7 = Me as found in **1**.

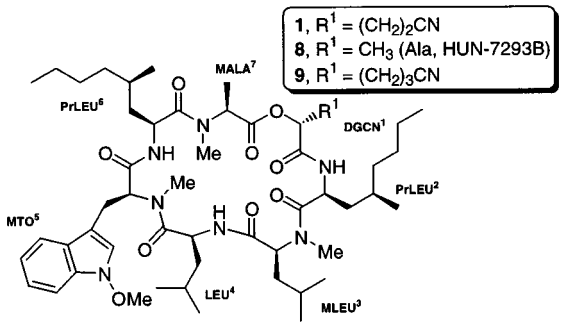
Figure 4.

The removal of the additional two *N*-methyl amides also reduced activity, albeit not nearly as substantially, but the changes also productively enhanced the VCAM-1 selectivity. The most interesting of these was the modest effect of removing the residue 7 *N*-methyl group with **7**. This only reduced the VCAM-1 potency 7-fold (IC_{50} = 6.8 nM versus 1 nM), providing an analogue with a sub 10 nM IC_{50}, with a much improved VCAM-1 versus ICAM-1 selectivity (180-fold versus 24-fold for **1**). Thus, removal of the residue 7 *N*-methyl group impacted the inhibition of ICAM-1 expression (50-fold reduction), but had a much more modest effect on inhibition of VCAM-1 expression (7-fold). Consistent with this behavior, **7** adopts at least two major and nearly equivalently populated conformations as judged by ^{1}H NMR. The ^{1}H NMR of one of these conformations exhibits the strongly shielded Leu[4] β_1 proton (δ −0.50) identical with that found in **1** whereas that of the second is less significantly, but still substantially, shielded (δ 0.00).

Removal of the residue 3 *N*-methyl group providing **5** more significantly reduced activity (11-fold) and had a significant but less pronounced impact on the selectivity improving it beyond that observed with **1** (54- vs 24-fold for **1**). Interestingly, **5** adopts a single, well-defined solution conformation as judged by ^{1}H NMR that does not appear on the surface to differ substantially from that of **1**. For example, the diagnostic Leu[4] β_1 proton exhibits the same strongly shielded chemical shift (δ −0.28 for **5** vs −0.37 for **1**). However, the ^{1}H NMR spectra differs considerably in the δ 4.0−5.5 region (α-protons), indicating a more subtle conformational change, likely the result of adoption of a trans amide between residues 2 and 3. Thus, while the role of the residue 3 *N*-methyl amide is not yet defined, it is clearly important.

Residue 1. Residue 1, because of its side chain functionality, is the one residue that has been extensively explored through derivatization of the natural product itself.[32] Consequently, this residue was not extensively examined in our work and a number of obvious modifications that would have ordinarily been

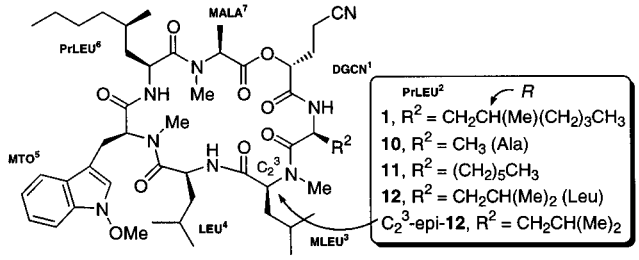
(30) Rajeswaran, W. G.; Hocart, S. J.; Murphy, W. A.; Taylor, J. E.; Coy, D. H. *J. Med. Chem.* **2001**, *44*, 1305−1311 and 1416−1421.
(31) For examples of such an *N*-methyl scan, see: Boger, D. L.; Teramoto, S.; Cai, H. *Bioorg. Med. Chem.* **1996**, *4*, 179−194. Boger, D. L.; Teramoto, S.; Cai, H. *Bioorg. Med. Chem.* **1997**, *5*, 1573−1590.



Residue 1. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1, HUN-7293	1	24	24	1
8, R^1 = Me	2.3 ± 0.5	178 ± 18	77	0.4
9, $R^1 = (CH_2)_3CN$	5 ± 0.1	210 ± 50	42	0.2

Figure 5.



Residue 2. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1, HUN-7293	1	24	24	1
10, R^2 = Me	100 ± 34	6300 ± 370	63	0.01
11, $R^2 = (CH_2)_5CH_3$	5.9 ± 1.3	210 ± 50	36	0.17
12, $R^2 = CH_2CH(Me)_2$	6.2 ± 2.3	230 ± 10	37	0.16
C_2^3-epi-**12**	19 ± 1	1900 ± 540	100	0.05

Figure 6.

incorporated were not pursued. The two analogues prepared, **8** and **9**, constitute the removal of the $-CH_2CN$ of the side chain ($R^1 = CH_3$) and the side chain extension by one carbon ($R^1 = CH_2CH_2CH_2CN$). These analogues would not be easily prepared from **1** itself yet are revealing in the importance of the presence of the side chain or its functionality. Their synthesis required the incorporation of commercially available (*S*)-2-hydroxypropionic acid and (*S*)-5-cyano-2-hydroxypentanoic acid (prepared from L-homoglutamine), into the modified tripeptide **3**, Mitsunobu esterification with tetrapeptide **2**, deprotection, and macrocyclization.

The examination of **8** and **9** was especially revealing (Figure 5). Simple alanine substitution with **8** for the more elaborate side chain had a modest ca. 2-fold effect lowering the inhibition of VCAM-1 providing a simplified analogue that still exhibited potent (IC_{50} = 2.3 nM) inhibition of VCAM-1 expression. A greater 7-fold loss in potency against ICAM-1 was observed, and the VCAM-1/ICAM-1 selectivity increased accordingly. In contrast, **9** was 5- and 8-fold less potent than **1** in reducing VCAM-1 and ICAM-1 expression, respectively, indicating that simple extension of the side chain by one carbon was modestly detrimental. Of these two analogues, **8** exhibits a potency that approaches that of **1**, and exhibits a better VCAM-1 selectivity. Consequently, it was remarkable that ad this work was being conducted, the potent residue 1 alanine analogue **8** was discovered to be a natural product in its own right, being isolated from the microbial strain F/94-499709,[32] and we have come to refer to it as HUN-7293B. Our sample of synthetic **8** was identical in all compared respects with naturally derived **8** (^{1}H NMR).[32]

Residue 2. The results of the alanine scan for residue 2, which indicated a 100-fold reduction in potency with **10**, along with the unique structure of the side chain incorporating a chiral center suggested this residue might be highly specific and resistant to productive changes. Consequently, the results of the concurrent examinations of **11** and **12** proved especially revealing (Figure 6).

The preparation of **10**−**12** required L-Ala, (*S*)-2-aminooctanoic acid,[33] and L-Leu, respectively, for incorporation into analogues of **37**, and the subsequent modified tripeptide **3**. Mitsunobu esterification with tetrapeptide **2**, subsequent deprotection, and macrocyclization provided **10**−**12**. C_2^3-*epi*-**12** was also synthesized and used to assess the importance of the C_2^3 center relative to the biological activity of **12**. The modified linear heptadepsipeptide **48** was deprotected and treated with EDCI-HOAt in the absence of added base to give C_2^3-*epi*-**12** in 44% isolated yield (C_2^3-*epi*-**12**:**12** = 5.5:1). Under comparable conditions, **48** itself closed to provide exclusively C_2^3-*epi*-**1** without the detection of **1**. Because of its availability, an examination of the macrocyclization reaction was also conducted with the linear heptadepsipeptide leading to **12** and the results are representative of other analogues studied. Three cyclization conditions were tested: EDCI-HOAt ($NaHCO_3$), which gave **1** in 72%,[8] BOP-DMAP, which gave **1** in 80% yield,[8] and DPPA-*i*Pr_2NEt, which also gave **1** without detection of C_2^3-*epi*-**1** (vide supra). The DPPA-*i*Pr_2NEt conditions proved optimal for this specific substrate and gave **12** in 59% without detection of C_2^3-*epi*-**12**, while EDCI-HOAt ($NaHCO_3$) afforded **12** in 47% with **12**:C_2^3-*epi*-**12** = 1:2, and BOP-DMAP provided **12** in 56% with **12**:C_2^3-*epi*-**12** = 6:1.

In contrast to the alanine analogue (100-fold loss), the removal of the side chain methyl group and the chiral center with **11** resulted in only a 6-fold loss of potency and an analogue that maintained or possessed a slightly enhanced VCAM-1 selectivity (Figure 6). Similarly, the removal of the propyl extension of the side chain with the incorporation of L-Leu with **12** (removal of three methylenes) resulted in an analogous 6-fold reduction in potency and a similarly maintained or slightly enhanced VCAM-1 selectivity. Thus, an aliphatic hydrophobic side chain is required, but the characteristics of this side chain do not appear to be as specific as its unique structure might imply. In contrast to conclusions that might be drawn from the alanine scan, considerable simplification of this residue may be possible including others not yet explored in our studies. Importantly,

(32) Dreyfuss, M.; Fehr, T.; Foster, C. A.; Geyl, D.; Oberhauser, B. PCT Internation Patent Application WO 9719104, 1997; *Chem. Abstr*. **1997**, *127*, 81792. Dreyfuss, M. M.; Foster, C. A.; Naegeli, H.-U.; Oberhauser, B. PCT Internation Patent Application WO 9603430, 1996; *Chem. Abstr*. **1996**, *125*, 34177. We thank Berndt Oberhauser (Novartis) for supplying the ^{1}H NMR of authentic HUN-7293B (IC_{50} = 2.1/220 nM for VCAM-1/ICAM-1) for comparison.

(33) Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc*. **1989**, *111*, 6354−6364.

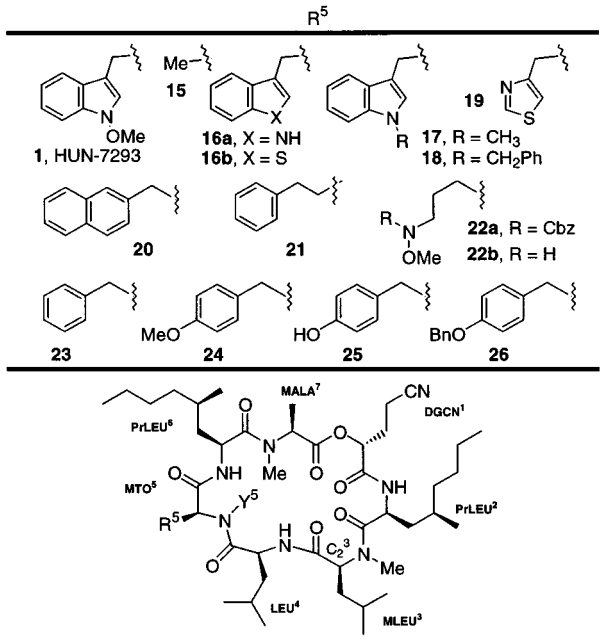
the simplification achieved with **11** and **12** was accomplished with the maintenance of single digit nM activity versus VCAM-1 expression with a slightly enhanced VCAM-1 selectivity. Analogous to the comparison of **1** and C_2^3-*epi*-**1**, C_2^3-*epi*-**12** was only 3-fold less potent at inhibiting VCAM-1 expression than **12** itself but 8-fold less effective at inhibiting ICAM-1 expression. Thus, C_2^3-*epi*-**12**, like C_2^3-*epi*-**1**, is slightly less potent, but more selective for inhibition of VCAM-1 expression.

Notably, the adopted conformation of **10** ($R^2 = CH_3$), as judged by near identical ^{1}H NMR's, is essentially indistinguishable from that of **1**, **11**, or **12**, indicating that the 100-fold reduction in activity with **10** is not derived from a structural perturbation, but rather that the side chain modifications in the series impact the extent to which **1** reaches or interacts with its biological target.

Residues 3 and 4. Because residues 3 and 4 are NMe-Leu and L-Leu, respectively, the changes examined were limited to alanine substitutions, **13** and **14** (Figure 3), the *N*-methyl deletion (**5**, Figure 4), and the examination of the C_2^3-epimer of **1** (C_2^3-*epi*-**1**, Figure 2). With the exception of the latter modification, each of these changes resulted in 10- to 60-fold losses in potency with that of the NMe-Ala substitution for NMe-Leu^3 being the most significant (60-fold). By contrast, the C_2^3-epimer of **1** was nearly as active as **1** against VCAM-1 expression.[26] Thus, while the presence of the hydrophobic side chain is important, its configuration is not. This suggests its impact does not lie with specific target interactions or conformational effects on the cyclic depsipeptide, but rather with properties that influence how well the compound reaches its biological target. In addition, each change enhanced the VCAM-1 selectivity, indicating that the modification had a more detrimental effect on ICAM-1 versus VCAM-1 expression.

The preparation of **13** and **14** simply required incorporation of NMe-Ala (residue 3, tripeptide **3**) and Ala (residue 4, tetrapeptide **2**), respectively, into the cyclic heptadepsipeptide synthesis. In the preparation of the modified tripeptide **3** required for **13**, the use of HCl-EtOAc for the selective deprotection of the *N*-Boc group in the modified dipeptide **37** turned out to be critical. The alternative use of neat formic acid followed by basic workup (aqueous $NaHCO_3$), which affords the free amine and was applicable to dipeptides containing the natural *i*-Bu R^3 side chain, resulted in formation of the cyclic diketopiperazine byproduct exclusively.

Residue 5. Residue 5 proved to be one of the most interesting and surprising sites in the molecule. Its very unusual side chain structure, incorporating both the backbone *N*-methyl amide and the *N*-methoxy functionalization of the indole side chain, suggested a very special role for this subunit. Moreover, the results of the alanine scan with **15** (1800-fold loss in activity) and the *N*-methyl deletion with **6** (>10 000-fold loss in activity) confirmed a critical role for this residue and implied that even subtle changes might have profound effects. As discussed earlier, the *N*-methylation promotes the formation of a backbone cis amide, which in turn is critical for adoption of the well-defined solution conformation of **1**. Consequently, the role of this structural feature of residue 5 is clear. However, the role of the side chain was not clear and the results of the examination of additional analogues containing simplifying modifications proved especially revealing. Fortunately, this residue was thoroughly examined with a series of key analogues **15**−**26** designed to address the contribution of each structural feature of this unusual side chain (Figure 7). With the exception of **6**, this required the preparation of the *N*-methyl amino acid precursor, which was typically assembled by *N*-methylation of the corresponding *N*-Boc carboxylic acid (NaH, CH_3I, THF) following the procedure of Coggins and Benoiton.[34] For the amino acid precursor required for **22a**, Ag_2O-CH_3I was employed and provided the *N*-methyl amino acid methyl ester directly. In each case, the extent of racemization was assessed by chiral phase HPLC and comparison with a racemic standard. The precursors to **15** (L-Ala), **16b**, **19**, **20**, **21**, **23** (L-Phe), and **24**−**26** (L-Tyr) were commercially available as *N*-Boc amino acids or unprotected amino acids. The precursors to **17** and **18** were prepared by indole *N*-methylation or *N*-benzylation enlisting commercially available tryptophan derivatives. The *N*-Boc precursor to **22** was prepared by intermolecular Mitsunobu displacement



Residue 5. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)[a]

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1, HUN-7293	1	24	24	1
6, Y^5 = H	>10,000	>10,000	nd	<0.0001
15, R^5 = Me	1800 ± 10	2800 ± 210	1.6	0.0006
16a	44 ± 11	510 ± 140	12	0.02 (0.06)[b]
16b	1.7 ± 1.6	19 ± 1.2	11	0.6
17	21 ± 1.6	186 ± 3	9	0.05 (0.3)[b]
18	19 ± 1.2	16 ± 13	0.8	0.05 (0.6)[b]
19	38 ± 5	2100 ± 170	55	0.03
20	11 ± 2.6	71 ± 35	6	0.09
21	29 ± 6	94 ± 13	3	0.03
22a	380 ± 120	890 ± 340	2.3	0.003
22b	1400 ± 230	>7000	>5	0.0007
23	2.3 ± 0.6	43 ± 6	19	0.4
24	63 ± 4	720 ± 90	11	0.02
C_2^3-epi-**24**	190 ± 20	9700 ± 3300	51	0.005
25	810 ± 130	8200 ± 120	10	0.001
26	280 ± 7	480 ± 65	1.7	0.004
C_2^3-epi-**26**	1100 ± 240	2600 ± 440	2.4	0.0009

[a]Unless indicated, Y^5 = Me, the substituent found in **1**. [b]See text and ref 38.

Figure 7.

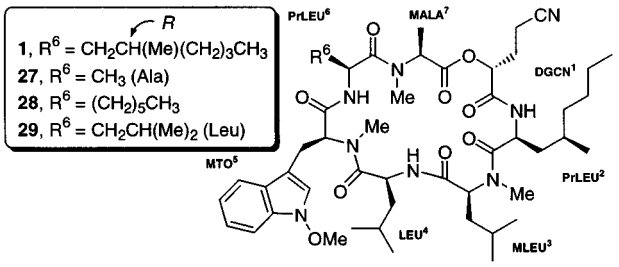
(34) Coggins, J. R.; Benoiton, N. L. *Can. J. Chem.* **1971**, *49*, 1968−1971.

of the primary alcohol derived from Boc-L-Glu(O*t*Bu) ($ClCO_2$-Et, Et_3N; $NaBH_4$, 87%)[35] with CbzNHOMe[36] (DEAD, PPh_3, 57%) as a key step. Notably, the coupling of the modified dipeptide **43** with **46** to afford the modified tetrapeptide **47** was conducted with EDCI-HOAt (2,6-lutidine, −30 °C, 3−5 h) minimizing the competitive epimerization of the intermediate active ester of the *N*-acyl-*N*-methyl carboxylic acid. Finally, **16a** (92%), **22b** (99%), and **25** (89%) were obtained by hydrogenation (H_2, Pd-C, CH_3OH or EtOAc, 25 °C, 1−4 h) of **1**, **22a**, and **26**, respectively.

The important key analogue comparison of **16a** with **1** revealed that removal of the indole *N*-methoxy group reduced activity 40-fold. Although this might initially suggest an important interaction with the biological target, it was also found to substantially impact the stability of the compound. Thus, whereas **1** is stable to conventional handling and storage, **16a** rapidly discolors under analogous conditions. This observation along with those discussed below suggest the role of the *N*-methoxy group may not involve a productive interaction with the biological target itself, but rather that it may be as simple as improving compound stability or masking a free indole NH. Unexpectedly and consistent with such an interpretation, a number of simplified substitutions for residue 5 were found to be potent and selective inhibitors of VCAM-1 expression. Most notably, the NMe-L-Phe substitution **23** (IC_{50} = 2.3 nM) proved to be only 2-fold less potent than **1**. Also surprisingly potent were the related analogues **20** and **21**, and the comparisons in this series indicate that both the presence and position of the hydrophobic aromatic side chain substituent are important (**23** > **20** > **21**). In contrast to **1** versus **16a**, the incorporation of polar substituents with **24**−**26** including a methoxy group substantially reduced the activity of **23**. It is the unanticipated potent activity of **23** that highlights how easy it might be to naively assign a special importance to an unusual residue that can even be apparently validated by degradation studies (**16a**) or an alanine substitution (**15**). Thus, the unbiased examination of a reasonable range of alternative substitutions in a first pass study accessible using this systematic chemical mutagenesis of the cyclic depsipeptide can reveal important unanticipated insights into structure−function properties. Not only is residue 5 key to the activity of **1**, but a subsequent focus of SAR studies on this residue is likely to be highly productive.

Like the observations made with **1** and **12**, the $C_2{}^3$ epimers of **24** and **26** are only 3- to 4-fold less potent against VCAM-1 expression. In the case of **24**, its $C_2{}^3$ epimer was >10-fold less effective against ICAM-1, resulting in a greater VCAM-1 versus ICAM-1 selectivity analogous to observations made with **1** and **12**.

After the evaluation of the first round of compounds, three additional residue 5 analogues (**16b**, **17**, and **18**) were prepared for examination. The intent was to define in greater detail the role of the structural features of the MTO residue indole. Like the parent indole **16a**, **17** and **18** proved to be modest inhibitors of VCAM-1 expression. Moreover, they also proved less robust on storage[37] than **1**, but easier to handle than **16a**. Thus, initial



Residue 6. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1, HUN-7293	1	24	24	1
27, R^6 = Me	270 ± 40	1400 ± 300	5.2	0.004
28, $R^6 = (CH_2)_5CH_3$	1.6 ± 0.3	28 ± 3	18	0.6
29, $R^6 = CH_2CH(Me)_2$	7.1 ± 1.8	100 ± 6	14	0.14

Figure 8.

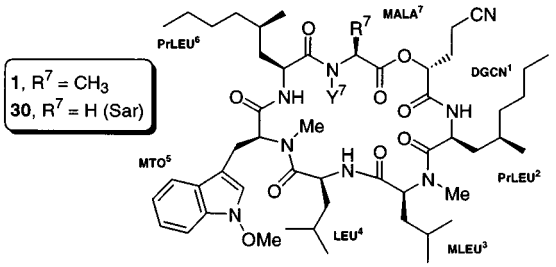
testing of shipped samples of **17** and **18** provided IC_{50}'s of 21 and 19 nM, respectively. However, independent testing of **16a**, **17**, and **18** employing in-house samples (Novartis) prepared by degradation of the natural product and semisynthesis[38] provided IC_{50}'s of 15, 3.6, and 1.7 nM,[38] respectively. One interpretation of these results is that the *N*-methoxy group of **1** is not integral to its activity, but that it does appear to convey a unique stability to the natural product contributing to its biological potency. Consistent with this interpretation, the analogue **16b** incorporating a benzothiophene in place of the *N*-methoxyindole proved stable and nearly equipotent with the natural product (IC_{50} = 2 nM).

Residue 6. Analogous to residue 2, the specialized structure of its side chain and the results of the alanine scan with **27** (270-fold loss in activity) suggested residue 6 was especially important to the properties of **1**, second only to residue 5. Consequently, the results of the examination of **27**−**29** (Figure 8) were especially interesting, revealing simplifications in the structure that do not impact the properties. Their preparation required the incorporation of L-Ala, (*S*)-2-aminooctanoic acid,[33] and L-Leu into the modified tetrapeptide **2**, Mitsunobu esterification with tripeptide **3**, deprotection, and macrocyclization.

In contrast to the results of the replacement of this side chain with a methyl group which resulted in a 270-fold reduction in potency and loss of VCAM-1 selectivity, the removal of the side chain methyl group and the chiral center resulted in an analogue **28** with essentially the same potency and selectivity as the natural product. Shortening the side chain with **29** removing the propyl extension of Leu reduced activity 7-fold. In this regard, the lack of sensitivity to removal of the side chain methyl group and chiral center with **28** contrasts the results with residue 2 where it was more significant, whereas the sensitivity to shortening the side chain with **29** was identical to the results observed with residue 2. Thus, despite the more pronounced reliance on the residue 6 side chain than residue 2 (compare alanine scan), a significant simplification could be made with **28** which had little impact on potency.

One feature that became apparent upon the final purification of the analogues was the dramatic impact that the residue 6, and residue 2, side chain had on the reverse phase HPLC

(35) Lee, B. H.; Gerfen, G. J.; Miller, M. J. *J. Org. Chem.* **1984**, *49*, 2418−2423.
(36) Kawase, M.; Kitamura, T.; Kikugawa, Y. *J. Org. Chem.* **1989**, *54*, 3394−3403.
(37) Compound **17** was found to be only 20% (Novartis) to 40% (Scripps) pure after several months of storage whereas **16a** discolors more rapidly.
(38) We thank Berndt Oberhauser (Novartis) for sharing these results and observations in advance of publication.



Residue 7. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)[a]

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1, $R^7 = Y^7 =$ Me	1	24	24	1
7, Y^7 = H	6.8 ± 0.3	1200 ± 530	180	0.15
30, R^7 = H	2.6 ± 0.1	150 ± 60	58	0.4

[a]Unless indicated otherwise, the substituents R^7, Y^7 = Me as found in **1**.

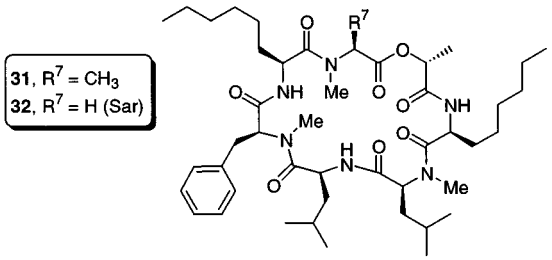
Figure 9.

retention times (82−100% MeOH−H_2O gradient) even though they did not appear to alter the conformational properties (^{1}H NMR). Retention times of **1** (21 min), **28** (19 min), **29** (14 min), and **27** (10 min) were observed mirroring the order in decreased potency. An analogous trend was observed with **1** versus the residue 2 analogues **11** (18 min), **12** (13 min), and **10** (9.6 min). Although the correlation between the two residues is not perfect, the dramatic impact of the two side chains on the hydrophobic character of the resulting analogue suggests their impact may go beyond their interaction with a biological target. They may additionally influence access to, transport to, or the ability to reach a biological target by virtue of substantially altering the hydrophobic character of the compound.

Residue 7. One of the most interesting and unexpected series of residue modifications centered on residue 7. The *N*-methyl deletion series revealed that removal of the residue 7 *N*-methyl group with **7** had a modest effect of lowering potency 7-fold, but substantially improved the VCAM-1 versus ICAM-1 selectivity (180-fold selective). The remaining simplification possible is removal of the side chain substituting Sar (NMe-Gly) for NMe-Ala and providing **30**. Consistent with the near identical ^{1}H NMR's of **1** and **30** indicating little conformational perturbation accompanying this change, **30** was found to be only 2- to 3-fold less potent than **1**, and it also exhibited an enhanced VCAM-1 selectivity (Figure 9).

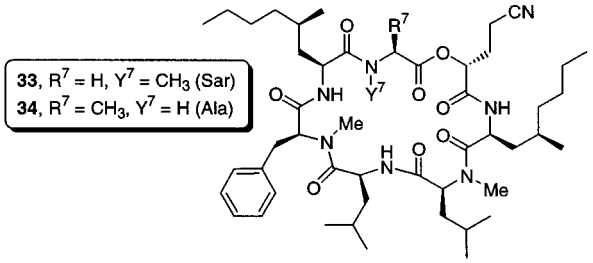
Cumulative Simplifications. Following the evaluation of the initial library, four additional analogues were prepared that incorporate the acceptable side chain simplifications or changes which enhanced the VCAM-1 selectivity. The first set (**31** and **32**) incorporate the residue 1 simplified methyl substitution (2-fold reduction), the residue 2 and 6 simplified (*S*)-2-aminooctanoic acid (6-fold and <2-fold reduction, respectively), and the remarkable residue 5 NMe-Phe substitution (2-fold reduction). One of these contained the authentic residue 7 NMe-Ala residue (**31**) while the second (**32**) incorporated the additional simplified Sar substitution (2-fold reduction).

The first of these analogues, **31**, which contained four side chain simplifications, proved to be especially interesting (Figure 10). It exhibited potent VCAM-1 expression inhibitory activity (IC_{50} = 104 nM) without impacting the intrinsic VCAM-1/ICAM-1 selectivity, albeit at a level that is approximately 100-fold less potent than **1**. The cumulative effect of the individual



Cumulative Simplifications. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1	1	24	24	1
31, R^7 = Me	104 ± 27	2600 ± 130	25	0.01
32, R^7 = H	1400 ± 200	>10,000	>7	0.0007



Cumulative Simplifications. Inhibitory activity (IC_{50}, nM ±SD) for VCAM-1 and ICAM-1 protein expression in a human microvascular cell line (HMEC-1)

compound	VCAM-1	ICAM-1	selectivity for VCAM-1	rel potency (VCAM-1)
1	1	24	24	1
33	6	152	25	0.16
34	43	1650	38	0.02

Figure 10.

changes predicted a 22-fold loss in activity and the potency of **31** is only 4- to 5-fold different from this naïve expectation. In contrast, incorporating the additional residue 7 change embodied in **32**, which now contained five simplifications in the structure, provided only a weakly active analogue that was 1400-fold less active than **1** and 25-fold less active than the cumulative changes would predict.

The second set (**33** and **34**) incorporated only two of the more significant, yet productive, substitutions. Both incorporate the remarkable residue 5 NMe-Phe simplification (2-fold reduction), and **33** incorporates the residue 7 Sar substitution (2-fold reduction) whereas **34** lacks the residue 7 *N*-methyl group (Ala substitution, 7-fold reduction). Thus, **33** contains two simplifications that minimize an impact on potency, while **34** incorporates one simplification that enhances VCAM-1/ICAM-1 selectivity albeit with a more significant impact on potency. The first of these analogues, **33**, exhibited potent VCAM-1 expression inhibition (IC_{50} = 6 nM) that was exactly in line with expectations based on the individual substitutions (2.6 × 2.3 = 5.98 vs 6) and it maintained the intrinsic VCAM-1/ICAM-1 selectivity. The second analogue, **34** (IC_{50} = 43 nM), was similarly in line with expectations based on the individual substitutions (6.8 × 2.3 = 16 vs 43) where it was only 2- to 3-fold less potent than the cumulative changes would predict. Moreover, the VCAM-1/ICAM-1 selectivity improved with **34**, albeit not as dramatically as with **7** (Ala substitution).

Conclusions

An assessment of the impact of each residue, each side chain, and each structural feature of **1** was established enlisting a complete library of HUN-7293 analogues composed of systematic single point changes in the structure prepared by parallel solution-phase synthesis. This generalizable library approach of chemical mutagenesis of the cyclic depsipeptide provides a detailed first level structure−activity study that subsequent optimization efforts can be confidently based on. One of the most striking conclusions of the study is that a simple alanine scan of the naturally occurring depsipeptide may provide a sense of the importance of each residue, but it provides an incomplete data set that is problematic to draw substantive conclusions from. A second surprising observation that was unanticipated given that 6 of the 7 residues of **1** contain nonstandard side chains or are modified by *N*-methylation is that each nonstandard residue or side chain could be simplified without substantially impacting potency (e.g. **8**, **11**/**12**, C_2^3-*epi*-**1**, **16b**/**23**, **28**/**29**, **7**/**30**) and, in some instances, with improvements in the VCAM-1/ICAM-1 selectivity. Many, if not most, of these latter active analogues would not have been easily anticipated based on the results of the alanine scan.

Although similar approaches have been disclosed to optimize or explore a single residue within a naturally occurring cyclic peptide, we are not aware of an instance where all residues were examined simultaneously. Moreover, a more common approach to initial SAR studies of such natural products has been systematic derivatization or degradation studies to establish the structural features important for activity. These efforts, like an alanine scan, provide an incomplete data set on which to base future studies.

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Supporting Information Available: Experimental data for the synthesis of **1** and **4**−**34**, full characterization data for **1**, and **4**−**34**, and all synthetic intermediates and ^{1}H NMR spectra for **1**, **3**−**34**, **37**, **42**, **45**, **47**, and **48** (PDF) are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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