J. Med. Chem. **1996,** *39,* 2819−2834

Structure−Activity Relationships of the Didemnins[1,2]

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Received January 16, 1996⊗

Bioactivities of 42 didemnin congeners, either isolated from the marine tunicates *Trididemnun solidum* and *Aplidium albicans* or prepared synthetically and semisynthetically, have been compared. The growth inhibition of various murine and human tumor cells and plaque reduction of HSV-1 and VSV grown on cultured mammalian cells were used to assess cytotoxicity and antiviral activity. Biochemical assays for macromolecular synthesis (protein, DNA, and RNA) and enzyme inhibition (dihydrofolate reductase, thymidylate synthase, DNA polymerase, RNA polymerase, and topoisomerases I and II) were also performed to specify the mechanisms of action of each analogue. Immunosuppressive activity of the didemnins was determined using a mixed lymphocyte reaction (MLR) assay. These assays revealed that the native cyclic depsipeptide core is an essential structural requirement for most of the bioactivites of the didemnins, especially for cytotoxicities and antiviral activities. The linear side-chain portion of the peptide can be altered with a gain, in some cases, of bioactivities. In particular, dehydrodidemnin B, tested against several types of tumor cells and in *in vivo* studies in mice, as well as didemnin M, tested for the mixed lymphocyte reaction and graft vs host reaction in murine systems, showed remarkable gains in their *in vitro* and *in vivo* activities compared to didemnin B.

Introduction

The didemnins, marine organism-derived cyclic depsipeptides, were isolated from the Caribbean tunicate *Trididemnum solidum* by our group as antitumor and antiviral agents[3−5] and numerous biological studies on didemnins have been conducted.[6,7] In *in vitro* studies, didemnin B (DB, Chart 1, **1**), one of the most potent components, was cytotoxic to L1210 murine leukemia cells at very low concentrations and to CV-1 monkey kidney cells at much higher concentrations.[3] In *in vivo* studies, **1** was effective in P388 and B16 murine tumor models and in a Yoshida ascites model.[3,8] Compound **1** was the first marine-derived compound to be evaluated in phase I and phase II clinical trials by the National Cancer Institute. As an antitumor agent[9] it has shown complete or partial response in previously treated non-Hodgkins lymphomas.[10] Inhibition of protein synthesis and, to a lesser extent, DNA synthesis was earlier proposed as the mode of action for the cytotoxicity of the didemnins.[11a] Recently, Crews et al. reported purification of a didemnin-binding protein from a bovine brain homogenate which appeared to be identical to human translational enlongation factor-1α (EF-1α) by affinity chromatography using *N*-biotinylbis(ϵ-aminocaproyl)didemnin A as a ligand. Didemnin A binds to EF-1α in a GTP-dependent manner, i.e., it binds to the GTP−EF-1α complex, but does not inhibit EF-1α's GTPase activity.[11b] More recently, SirDeshpande and Toogood have reported that didemnin B inhibits protein synthesis by stabilizing aminoacyl-tRNA binding to the ribosomal A site, preventing translocation but not peptide bond formation.[11c] Most recently, didemnin B has been reported to induce apoptosis in human HL-60 cells at the most rapid rate yet recorded.[11d]

Both didemnins A (DA, Chart 1, **2**) and B (**1**) have been shown to be strong inhibitors of various viruses *in vitro*.[3,12] Compounds **1** and **2** also showed activity *in vitro* against strains of the lethal RNA viruses Venezuelan equine encephalomyelitis, yellow fever, sandfly fever, Rift Valley fever, and a Pichinde virus, for all of which no effective chemotherapeutic agents exist.[13] *In vivo*, **1** protected 90% of Rift Valley fever virus infected mice, although considerable toxicity to the host animal was observed.[5,13]

In vivo testing of **1** and **2** against herpes simplex virus-2 (HSV-2) in mice has shown some efficacy in topical administration, but intracranial administration against encephalitis HSV-1, subcutaneous administration against Semliki-Forest virus, and cutaneous application against HSV-1 failed because of narrow therapeutic indexes.[5,12] On the whole, the high toxicity of **1** and **2** precludes their use as antiviral agents.

Didemnin B (**1**) inhibited lymphocyte blastogenesis and the mixed lymphocyte reaction (MLR) *in vitro* in murine cells,[14] requiring lower concentrations than cyclosporin A tested in a human lymphocyte system.[15] Some efficacy was observed in the graft vs host (GVH) reaction in mice and allograft transplantation in a rat with an auxiliary heart graft after treatment with **1**.[14,16] These results suggest the potential of the didemnins as immunosuppressive agents.

While **1** has shown a remarkable spectrum of biological activities, each has been accompanied by considerable toxicity. Hence, modifications of the compound to

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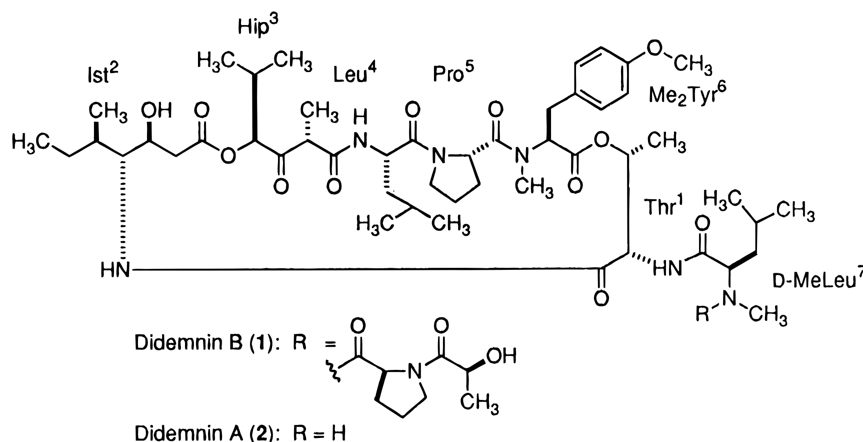
⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0022-2623(96)00048-9 CCC: $12.00 © 1996 American Chemical Society

Chart 1



increase a specific bioactivity while attenuating its general toxicity is a worthy endeavor.

We have previously reported some structural modifications and structure–activity relationships (SAR's) of didemnins.[7] These preliminary results revealed that simple acylation of the *N*-terminus of didemnin A enhanced activities dramatically.[3c,7] Several limited SAR studies of didemnins have been recently reported by others. Jouin and co-workers reported a total synthesis of nordidemnin B and four nordidemnin congeners and their *in vitro* and *in vivo* activities: mandelyl-Pro-, (*p*-hydroxyphenyl)propionyl-Pro-, and palmityl-Pro-nordidemnin A and [L-MeLeu[7]]nordidemnin B.[17] Kessler and co-workers reported the preparation and solution structure of [L-MeLeu[7]]didemnin B.[18] Most recently Joullié and co-workers reported synthesis and bioactivity of didemnin B congeners: [dehydro-L-Pro[8]]- and [*trans*-4-hydroxyPro[8]]DB, dehydro-L-Pro-DA and *trans*-4-hydroxyPro-DA as well as L-Pro-DA.[19] The biological results can be summarized as follows: mandelyl-Pro-norDA, (*p*-hydroxyphenyl)propionyl-Pro-nor DA, and [dehydroPro[8]] analogues showed comparable activity to **1**, but *trans*-4-hydroxyPro[8] analogues showed cytotoxicity somewhat weaker than DB. Palmityl-Pro-norDA and [L-MeLeu[7]] congeners had significantly diminished activity in the inhibition of tumor cell growth. The [dehydroPro[8]] analogues showed comparable antiproliferative activity in an *in vitro* immunosuppressive assay.[19] These studies demonstrated some SAR's of didemnins, but a more systematic study has been needed to determine the structural features responsible for their antitumor, antiviral, and immunosuppressive activities, which is essential information in studying the molecular level mechanism of action of the didemnins and in developing more selective drugs.

Over 60 didemnin analogues have been obtained during semisynthetic and synthetic studies of didemnins and the isolation of bioactive components from extracts of *T. solidum* and *Aplidium albicans*.[2] We describe herein preparation and bioactivities of 42 of these analogues, representing a great part of the SAR of didemnins, and discuss the structural requirements of these remarkable peptides.

Chemistry

Sequences and some physicochemical properties of all didemnin congeners described in this paper are listed in Table 1. Structures of unusual subunits are shown in Chart 2.

Natural Didemnins. A total of 15 didemnin analogues isolated by us from extracts of the Caribbean tunicate *T. solidum* have been characterized.[2,3,20] Of these, 11 congeners (**1**, **2**, **12**, **13**, **17**, **26**, and **38**–**42**) were evaluated in the present study.

Additionally, [pyruvyl[9]]DB (dehydrodidemnin B, **30**), recently isolated from the Mediterranean tunicate *A. albicans*, was also tested.[21]

The two most abundant didemnins, B (**1**) and A (**2**), are the lead compounds in this study. Didemnin A (**2**) has the basic structure common to many other congeners, consisting of a cyclic depsihexapeptide with a D-*N*-methylleucine (D-MeLeu) side chain attached to Thr[1]. Compound **2** (DA) was used as the starting material in the preparation of 20 congeners since its *N*-terminus, a free secondary amino group, offers a site to attach various acyl groups to the cyclic depsipeptide. Didemnin B (**1**), which has a Lac-Pro-acyl unit attached to the *N*-terminus of **2**, was also used as a starting molecule for some semisynthetic modifications.

Congeners with the Cyclic Depsipeptide Modified. 1. The Isostatine[2] Subunit. One of the most unusual subunits, isostatine (Ist), is an intriguing target site for modifications. During the total synthesis of the didemnins, all eight possible stereoisomers of Ist were synthesized,[22] and selected stereoisomers have been incorporated into the cyclic backbone of the peptide to observe the effects of the stereochemistry of Ist on bioactivities. Since Z-didemnin A (**3**) is known to possess more potent bioactivities than **2** itself,[1,6,7] bioactivities of three compounds varying in a single stereocenter—Z-[3*R*,4*R*,5*S*-Ist[2]]-, Z-[3*S*,4*S*,5*S*-Ist[2]]-, and Z-[3*S*,4*R*,5*R*-Ist[2]]didemnins A, compounds **4**, **5**, and **6**, respectively—were compared. These compounds were prepared following the method used in the total synthesis of **2**, which contains [3*S*,4*R*,5*S*-Ist[2]] (Scheme 1).[23]

The hydroxyl group at the C-3 position of Ist[2] was also modified. Z-Didemnin A (**3**) was acetylated, and hydrogenolysis of *N*-Z-*O*-acetyl[Ist[2]]didemnin A afforded *O*-acetyl[Ist[2]]didemnin A (**7**).

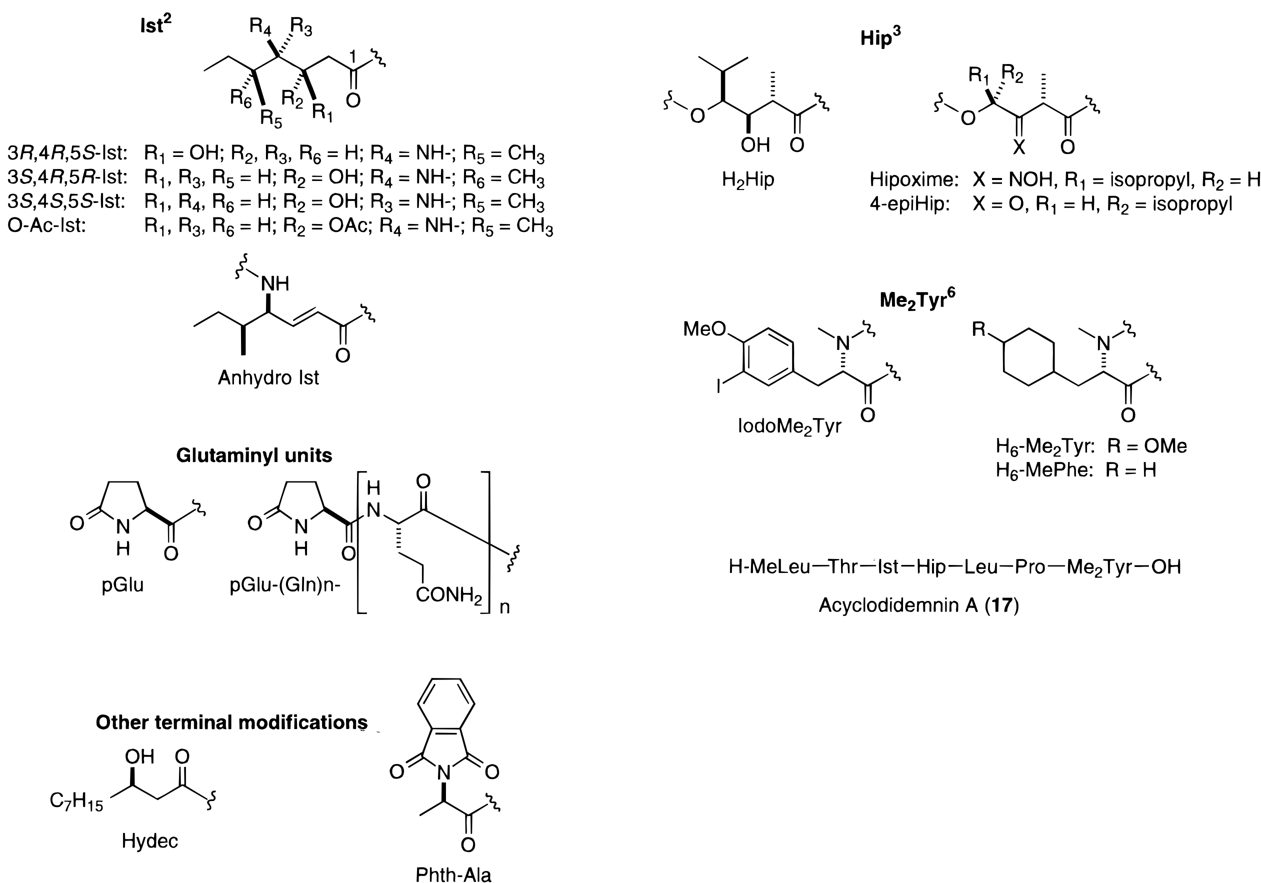
Treatment of **1** with phthalimide, diethyl azodicarboxylate (DEAD), and PPh_3 gave two products, **8** ([Phth-Ala[9]]DB) and an anhydro byproduct, **9** ([Anhydro-Ist[2]][Phthal-Ala[9]]DB), which has a *trans*-2,3-olefin in the Ist[2] unit.[20,24]

Table 1. Structures and Some Physical Properties of Didemnins

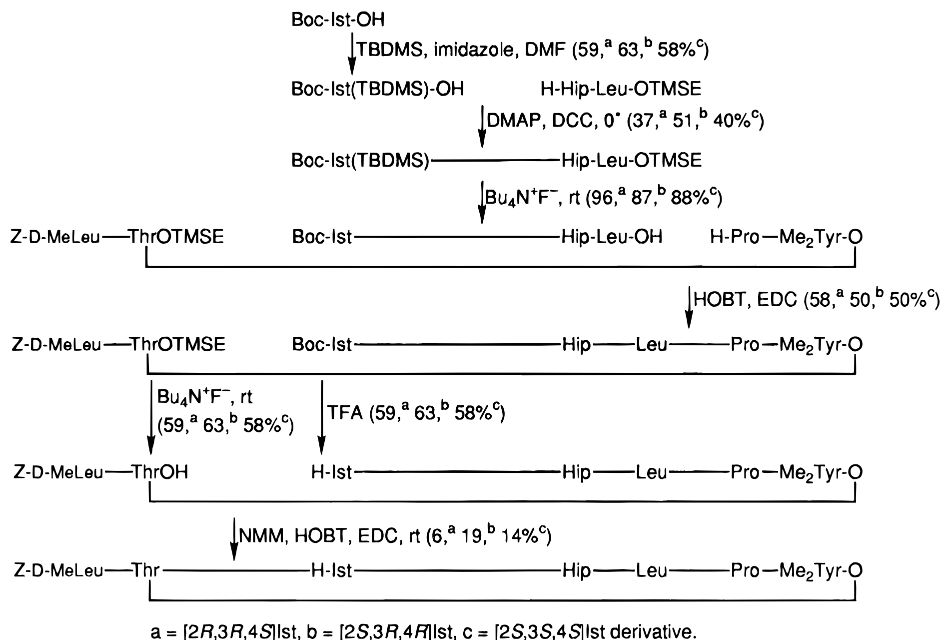
					modified sites (indicated by bold face)[a]						
					within the ring			linear peptide		glutaminyl	
didemnins	no.	formula[b]	HPLC t_R min (solvent)[c]	$[\alpha]_D$, deg, in $CHCl_3$ (temp, °C, concn)	Ist[2]	Hip[3]	Me_2Tyr^6	N[a] (**8**)	**9**	**10–15**	ref
didemnin A (DA) lead	**2**	$C_{49}H_{78}N_6O_{12}$	17.0 (E)	−136 (25, 7.50)[d]	Ist	Hip	Me_2Tyr	H			
didemnin B (DB) lead	**1**	$C_{57}H_{89}N_7O_{15}$	26.8 (A)	−78 (25, 6.91)[d]	Ist	Hip	Me_2Tyr	Pro			3a
Z-DA	**3**	$C_{57}H_{84}N_6O_{14}$	36.8 (A)	−98 (26, 0.96)	Ist	Hip	Me_2Tyr	Z			23
Z-[(3R,4R,5S)-Ist[2]]DA	**4**	$C_{57}H_{84}N_6O_{14}$	36.0 (A)	−14 (25, 0.16)	**3R,4R,5S-Ist**[e]	Hip	Me_2Tyr	Z			f
Z-[(3S,4R,5R)-Ist[2]]DA	**5**	$C_{57}H_{84}N_6O_{14}$	35.6 (A)	−102 (25, 0.11)	**3S,4R,5R-Ist**[e]	Hip	Me_2Tyr	Z			f
Z-[(3S,4S,5S)-Ist[2]]DA	**6**	$C_{57}H_{84}N_6O_{14}$	43.8 (A)	−28 (25, 0.11)	**3S,4S,5S-Ist**[e]	Hip	Me_2Tyr	Z			f
O-acetyl-DA	**7**	$C_{51}H_{80}N_6O_{13}$	24.4 (A)	−136 (24, 0.38)	**O-Acetyl-Ist**[e]	Hip	Me_2Tyr	H			f
[Phth-Ala[9]]DB	**8**	$C_{65}H_{92}N_8O_{16}$	31.4 (A)	−32 (25, 0.11)	Ist	Hip	Me_2Tyr	Pro	**Phth-Ala**[e]		20
[AnhydroIst[2]][Phth-Ala[9]]DB	**9**	$C_{65}H_{90}N_8O_{15}$	41.2 (A)	−20 (28, 0.07)	**AnhydroIst**[e]	Hip	Me_2Tyr	Pro	**Phth-Ala**[e]		20
[H_2-Hip[3]]DA	**10**	$C_{49}H_{80}N_6O_{12}$	27.0 (E)	−91 (28, 0.26)	Ist	**H_2-Hip**[d]	Me_2Tyr	H			f
[Hip[3] oxime]DB	**11**	$C_{57}H_{90}N_8O_{15}$	22.4 (A)	−82 (22, 0.24)	Ist	**Hip oxime**[d]	Me_2Tyr	Pro			f
[epi-Hip[3]]DA	**12**	$C_{49}H_{78}N_6O_{12}$	g	−100 (23, 0.13)	Ist	**epi-Hip**[d]	Me_2Tyr	H			20
[Tyr[6]]DB (didemnin N)	**13**	$C_{55}H_{85}N_7O_{15}$	18.0 (A)	54 (24, 0.13)	Ist	Hip	**Tyr**	Pro			20
[iodoMe_2Tyr^6]DB	**14**	$C_{57}H_{88}IN_7O_{15}$	26.5 (A)	−74 (22, 0.17)	Ist	Hip	**iodoMe_2Tyr**[d]	Pro			f
[H_6-Me_2Tyr^6]DB	**15**	$C_{57}H_{95}N_7O_{15}$	22.2 (B)	−29 (23, 0.41)	Ist	Hip	**H_6-Me_2Tyr**[d]	Pro			f
[H_6-NMePhe[6]]DB	**16**	$C_{57}H_{94}N_7O_{14}$	26.5 (B)	29 (24, 0.41)	Ist	Hip	**H_6-NMePhe**[d]	Pro			f
N^{α}-acetyl-DA	**18**	$C_{51}H_{80}N_6O_{13}$	22.2 (A)	−81 (24, 1.1)	Ist	Hip	Me_2Tyr	**n-CH_3CO-**			f
N^{α}-propionyl-DA	**19**	$C_{52}H_{82}N_6O_{13}$	25.2 (A)	−74 (24, 0.90)	Ist	Hip	Me_2Tyr	**n-CH_3CH_2CO-**			f
N^{α}-n-butyryl-DA	**20**	$C_{53}H_{84}N_6O_{13}$	23.8 (B)	−88 (24, 0.26)	Ist	Hip	Me_2Tyr	**n-$CH_3(CH_2)_2CO$-**			f
N^{α}-pentanoyl-DA	**21**	$C_{54}H_{86}N_6O_{13}$	26.8 (B)	−71 (24, 0.33)	Ist	Hip	Me_2Tyr	**n-$CH_3(CH_2)_3CO$-**			f
N^{α}-hexanoyl-DA	**22**	$C_{55}H_{88}N_6O_{13}$	29.6 (B)	−94 (24, 0.10)	Ist	Hip	Me_2Tyr	**n-$CH_3(CH_2)_4CO$-**			f
N^{α}-octanoyl-DA	**23**	$C_{57}H_{92}N_6O_{13}$	40.4 (B)	−71 (24, 0.32)	Ist	Hip	Me_2Tyr	**n-$CH_3(CH_2)_6CO$-**			f
N^{α}-dodecanoyl-DA	**24**	$C_{61}H_{100}N_6O_{13}$	30.6 (C)	−64 (24, 0.33)	Ist	Hip	Me_2Tyr	**n-$CH_3(CH_2)_{10}CO$-**			f
N^{α}-octadecanoyl-DA	**25**	$C_{67}H_{112}N_6O_{13}$	25.0 (D)	−73 (24, 0.17)	Ist	Hip	Me_2Tyr	**n-$CH_3(CH_2)_{16}CO$-**			f
didemnin G (N^{α}-formyl-DA)	**26**	$C_{50}H_{78}N_6O_{14}$	27.2 (A)	−94 (28, 0.10)	Ist	Hip	Me_2Tyr	**CHO**			4b
N^{α}-leucyl-DA	**27**	$C_{55}H_{89}N_7O_{13}$	25.6 (E)	−54 (28, 0.17)	Ist	Hip	Me_2Tyr	**Leu**	**H**		f
N^{α}-prolyl-DA	**28**	$C_{54}H_{85}N_7O_{13}$	26.6 (E)	−73 (28, 0.18)	Ist	Hip	Me_2Tyr	Pro	**H**		f
N^{α}-D-prolyl-DA	**29**	$C_{54}H_{85}N_7O_{13}$	25.2 (E)	−70 (25, 0.65)	Ist	Hip	Me_2Tyr	D-**Pro**	**H**		f
[pyruvyl[9]]DB (dehydrodimenin B)	**30**	$C_{57}H_{87}N_7O_{15}$	24.0/25.2 (A)[h]	−69 (28, 0.45)	Ist	Hip	Me_2Tyr	Pro	**pyruvyl**		21
[acetyl[9]]DB	**31**	$C_{56}H_{87}N_7O_{15}$	26.4 (A)	−85 (24, 1.6)	Ist	Hip	Me_2Tyr	Pro	**CH_3CO-**		f
[propionyl[9]]DB	**32**	$C_{57}H_{89}N_7O_{14}$	28.6 (A)	−77 (25, 2.9)	Ist	Hip	Me_2Tyr	Pro	**CH_3CH_2CO-**		f
[isobutyryl[9]]DB	**33**	$C_{58}H_{91}N_7O_{14}$	30.8 (A)	−90 (25, 0.15)	Ist	Hip	Me_2Tyr	Pro	**isobutyryl**		f
[isobutyryl[9]-D-Pro[8]]DB	**34**	$C_{58}H_{91}N_7O_{14}$	28.4 (A)	−69 (25, 0.89)	Ist	Hip	Me_2Tyr	D-**Pro**	**Isobutyryl**		f
[Ala[8]]DB	**35**	$C_{55}H_{87}N_7O_{15}$	21.4 (A)	−120 (26, 1.3)	Ist	Hip	Me_2Tyr	**Ala**	L-Lac		f
[D-Pro[8]]DB	**36**	$C_{57}H_{89}N_7O_{15}$	22.4 (A)	−66 (25, 1.2)	Ist	Hip	Me_2Tyr	D-**Pro**	L-Lac		f
O-pGlu-DB	**37**	$C_{62}H_{94}N_8O_{17}$	23.4 (A)	−79 (20, 1.4)	Ist	Hip	Me_2Tyr	Pro	L-Lac	**pGlu**[e]	f
didemnin M	**38**	$C_{67}H_{102}N_{10}O_{19}$	19.8 (A)	−68 (25, 1.1)	Ist	Hip	Me_2Tyr	Pro	L-Lac	**pGlu-Gln-**[e]	20
didemnin E	**39**	$C_{72}H_{110}N_{12}O_{21}$	18.0 (A)	−14 (25, 0.16)	Ist	Hip	Me_2Tyr	Pro	L-Lac	**pGlu-$(Gln)_2$-**[e]	4a
didemnin D	**40**	$C_{77}H_{118}N_{14}O_{23}$	19.0 (A)	−14 (25, 0.16)	Ist	Hip	Me_2Tyr	Pro	L-Lac	**pGlu-$(Gln)_3$-**[c]	4a
didemnin X	**41**	$C_{82}H_{131}N_1{}^3O_{23}$	28.4 (A)	−65 (20, 0.93)	Ist	Hip	Me_2Tyr	Pro	L-Lac	**Hydec-$(Gln)_3$-**[c]	20
didemnin Y	**42**	$C_{87}H_{139}N_{15}O_{25}$	25.4 (A)	−89 (20, 0.63)[i]	Ist	Hip	Me_2Tyr	Pro	L-Lac	**Hydec-$(Gln)_4$-**[c]	20
acyclodidemnin A[d]	**17**	$C_{49}H_{80}N_6O_{13}$	16.1 (E)	−70 (26, 0.06)							

[a] Structures of unusual subunits are shown in Chart 2. Superscripts indicate unit modified, substituted, or added. [b] Based on HRFAB data (Δ within 4 mDa). [c] Solvent systems (RP C-18 column): A = 7:1 MeOH−H_2O, B = 8:1 MeOH−H_2O, C = 12:1 MeOH−H_2O, D = MeOH, E = 7:1 MeOH−0.1 M NaCl. [d] CH_2Cl_2. [e] Structure is shown in Chart 2. [f] Present work. [g] R_f = 0.13 (15:1 $CHCl_3$−MeOH, silica gel, where R_f = 0.23 for DA (HPLC data not available). [h] Two peaks appeared due to conformers.[21] [i] Measured in 4:1 $CHCl_3$−MeOH.

Chart 2



Scheme 1



a = [2*R*,3*R*,4*S*]Ist, b = [2*S*,3*R*,4*R*]Ist, c = [2*S*,3*S*,4*S*]Ist derivative.

2. The Hip[3] Subunit. The Hip (hydroxyisovalerylpropionyl) subunit is also an important modification site of the didemnins. It has been proposed that the keto group of the Hip unit plays a major role in the bioactivities of didemnins.[4a] Treatment of **2** with $NaBH_4$ afforded the reduced Hip analogue, [(2*S*,3*R*,4*S*)-H_2Hip[3]]didemnin A (=[H_2Hip[3]]DA, **10**)[4a,20] as a major product.

Treatment of **1** with hydroxylamine gave [Hip[3] oxime]-didemnin B (**11**).

Epididemnin A_1 (=[4-epiHip[3]]DA, **12**) from *T. solidum* contains a (2*S*,4*R*)-hydroxyisovalerylpropionyl (Hip) residue in place of (2*S*,4*S*)-Hip in **1**.[20]

3. Me$_2$Tyr[6] Subunit. Didemnin N (=[Tyr[6]]DB, **13**), isolated from *T. solidum*, has tyrosine (Tyr) in place of the *N,O*-dimethyltyrosine (Me_2Tyr) of **1**.[20] Several chemical modifications of the Tyr unit were carried out (see Chart 2 for structures). Treatment of **1** with I_2/AgI afforded the derivative **14** with iodine *ortho* to the methoxyl (Chart 2). Catalytic hydrogenation of **1** (Pt/C

H_2) reduced the aromatic ring of Me_2Tyr to give [$H_6Me_2Tyr^6$]didemnin B (**15**) and [H_6-N-MePhe^6]-didemnin B (**16**).

4. Acyclodidemnin A. Acyclodidemnin A (**17**), isolated from *T. solidum*, is an acyclic derivative of **2** in which the ester linkage between [Thr^1] and [Me_2Tyr^6] has been hydrolyzed.[20]

Congeners with a Modified Linear Peptide Portion. 1. Acyl Derivatives of DA and DB. In an earlier study, *N*-acetyldidemnin A (**18**) was found to be more active than **2** itself, showing cytotoxicity to L1210 cells comparable to that of DB (**1**).[4a,5] The lipophilicity added to the molecule, and/or endcapping of the *N*-terminus to neutralize the peptide, presumably increases its cell permeability, resulting in more potent activity. In the present study various fatty acids were condensed with the *N*-terminus of **2** to evaluate the effects of lipophilicity and size of the acyl side chain on the activities. Compounds semisynthetically prepared include N^α-propionyldidemnin A (**19**), N^α-*n*-butyryldidemnin A (**20**), N^α-pentanoyldidemnin A (**21**), N^α-hexanoyldidemnin A (**22**), N^α-octanoyldidemnin A (**23**), N^α-dodecanoyldidemnin A (**24**), and N^α-octadecanoyldidemnin A (**25**).[2] Didemnin G (=N^α-formyl DA, **26**), an *N*-formyl derivative of **2**, was isolated from *T. solidum*.[4b]

2. Amino acid derivatives of **2** were also prepared to evaluate the effect of the eighth subunit on activities. Condensation of Z-L-Leu, Z-L-Pro, and Z-D-Pro with **2** followed by catalytic hydrogenation gave N^α-L-leucyldidemnin A (**27**),[3c] N^α-L-prolyldidemnin A (**28**),[3c] and N^α-D-prolyldidemnin A (**29**), respectively.

3. Didemnin B-type analogues which have two acyl units after the *N*-terminus of the didemnin A core were prepared to examine the structural factors contributing to didemnin B's potent bioactivities. The diacyl compounds acetyl-Pro-OH, propionyl-Pro-OH, isobutyryl-Pro-OH, isobutyryl-D-Pro-OH, *O*-benzyl-Lac-D-Pro-OH, and *O*-benzyl-Lac-L-Ala-OH were prepared and condensed with **2** by the mixed anhydride method. Deprotection and purification afforded the corresponding didemnin B-type analogues [acetyl^9]didemnin B (**31**), [propionyl^9]didemnin B (**32**), [isobutyryl^9]didemnin B (**33**), [isobutyryl^9-D-Pro^8]didemnin B (**34**), [Ala^8]didemnin B (**35**), and [D-Pro^8]didemnin B (**36**).

All the above compounds have the fundamental skeleton of didemnin A through D-MeLeu^7, but differ in the extended linear peptide portion of didemnin B.

4. Glutaminyldidemnins. *O*-pGlu-didemnin B (**37**) was prepared to evaluate the effect of the number of glutaminyl groups on the bioactivities. Didemnins M (**38**),[20,25] E (**39**), and D (**40**),[4a,7] which are naturally occurring, have *O*-[*p*Glu-(glutaminyl)$_n$-] peptide chains ($n = 1-3$) acylating the hydroxyl group of the Lac unit of **1**. Didemnins X (**41**) and Y (**42**), also isolated from *T. solidum*, have an (*R*)-3-hydroxydecanoyl terminus after the oligo-Gln peptide chains.[7,20]

Biological Results

Cytotoxicity. A simple screening procedure described by Bergeron et al.[26a] was used for the cytotoxicity assays using P388 (suspension culture of a lymphoid neoplasm from the DBA/2 mouse), A549 (monolayer culture of a human lung carcinoma), HT-29 (monolayer culture of a human colon carcinoma), and MEL-29 (monolayer culture of human melanoma) cell lines. Cytotoxicity data of all didemnin congeners are summarized in Table 2. An obvious point is the similarity in IC_{50} values of each congener vs the four cell lines, i.e., a lack of selectivity to these cell lines.

Table 2. Cytotoxicity[a] and Inhibition of Macromolecule Synthesis[b] by Didemnin Congeners

compd no.	cytotoxicity, IC_{50} (ng/mL)				inhibition of macromolecule synthesis IC_{50} (mg/mL)		
	P388	A549	HT-29	MEL-28	protein	DNA	RNA
30	0.2	0.2	0.5	0.5	0.5	0.1	>1
31	0.2	0.2	0.5	0.2	0.1	0.2	1
32	0.2	0.2	0.5	0.2	0.1	0.1	>1
33	0.2	0.2	0.5	0.5	0.05	0.1	1
19	0.5	0.5	0.5	0.5	0.07	0.1	1
20	0.5	0.5	0.5	0.5	0.08	0.1	1
21	0.5	0.5	0.5	0.5	0.04	0.1	1
14	0.5	1	1	1	0.1	0.4	>1
16	1	1	1	1	0.07	0.1	>1
18	1	1	1	1	0.06	0.1	1
22	1	1	2	2.5	0.09	0.1	1
26	2	2	2	2	0.1	0.3	>1
27	2	2	2	2	0.5	0.1	>1
28	2	2	2	2	0.3	1	NA[c]
35	2	2	2	2	0.1	0.2	>1
37	2	2	2	2	0.5	>1	NA
40	2	2	2	2	0.4	NA	NA
1	2	2	2	2	0.1	0.4	>1
8	2	2	2	2	0.2	1	1
38	2	2	2	2	0.4	1	NA
39	2	2	2.5	2	>1	NA	NA
41	2	2	2.5	2	>1	NA	NA
42	2	2	2.5	2	>1	>1	NA
3	2.5	2.5	2.5	2.5	1	0.5	>1
15	5	5	5	5	0.1	0.3	1
34	5	5	5	5	0.2	0.5	NA
9	3	3	10	20	1	>1	NA
5	5	5	10	20	0.3	1	NA
2	10	10	10	10	0.4	1	NA
11	10	10	20	10	0.5	1	>1
23	20	20	5	5	0.5	1	NA
29	10	10	20	20	0.6	>1	NA
36	20	20	20	20	0.4	1	NA
13	50	50	100	50	1	1	NA
17	200	200	200	200	NA	NA	NA
7	200	200	200	200	>1	NA	NA
4	200	200	200	200	NA	NA	NA
10	200	200	200	200	>1	>1	NA
24	500	500	500	500	NA	>1	NA
25	500	500	1000	1000	NA	NA	NA
6	500	500	1000	1000	NA	NA	NA
12	2000	2000	2000	2000	>1	>1	NA

[a] P388 = murine lymphoma. A549 = human lung carcinoma. HT-29 = human colon carcinoma. MEL-28 = human melanoma. [b] P388 cells were used. [c] NA = not active.

Wide differences were observed, however, in the relative cytotoxicities of the didemnins tested, over a 10000-fold difference. The two lead compounds were taken to be DA and DB. We had earlier noted[7] that didemnin A, the only basic didemnin (with a free secondary amine group), was considerably less cytotoxic than didemnin B, which is now in clinical trials. This is true, but neither stands as an extreme of the series.

The most cytotoxic of the didemnins were compounds **30**–**33**, containing less polar short acyl groups (C_2–C_4) instead of the lactic acid of DB. These compounds all had IC_{50}'s of 0.2 ng/mL. Only slightly less active was the class consisting of **19**–**21**, nonpolar short-chain *N*-acyl (C_3–C_5) derivatives of didemnin A, with IC_{50} = 0.5 ng/mL.

At the other extreme were didemnin A analogues containing stereoisomers or reduced analogues of Ist^2

Table 3. *In Vivo* Antitumor Activities of Dehydrodidemnin B ([Pyruvyl[9]]didemnin B, **30**) in Mice[a]

dose[b] (mg/kg/day)	body wt change (g)	day of death	median survival (day)	T/C[c] (%)	alive
		P388[d]			
					(day 23)
320	-3.3	4, 5, 6, 6, 6, 9	6.0	60	0
160	-3.2	10, 19, 21, 21, 22, 22	21.0	210	0
80	-1.4	13, 19, 19, 20, 20, 20	19.5	195	0
		B16[e]			
					(day 24)
160	-1.3	15, 31, 32, 32, 36, 38, 38, 39, 41, 42	37	218	9
80	-0.5	30, 31, 31, 32, 32, 32, 32, 33, 34, 35	32	188	10
40	0.1	29, 29, 29, 30, 30, 30, 31, 31, 32, 32	30	178	10

dose (mg/kg/day)	body wt change (g)	NP[g] (day 14)	median tumor vol (mm^3)	T/C[h] (%)
		Lewis Lung[f]		
160	−3.7	5	0	0.00
80	−4.5	2	163	0.13
40	−2.6	0	473	0.37

[a] Assays were carried out by Athur D. Little Inc. [b] Schedule 1−9 days ip. [c] Treated/control; significant activity, >125%. [d] P388 murine lymphoma. Median survival of control mice, 10.0 days. [e] B16 melanoma. Median survival of control mice, 17.0 days. [f] Lewis lung carcinoma, median tumor volume of control mice 1372 mm^3. [g] NP = number of nonpalpable tumors on day 14. [h] Significant activity: T/C < 0.40.

or Hip[3] (**4**, **6**, **10**, **12**), together with long-chain acyl (C_{12}−C_{18}) derivatives of didemnin A (**24**, **25**). It is of interest that *N*-acyl derivatives of didemnin A are among the most and the least cytotoxic didemnins, depending on chain length. It is also of interest that some (but not all) steric modifications in the Ist[2]-Hip[3] portion of the ring can have profoundly negative effects on the cytotoxicity. Thus, the epimers of Ist at C-3 or C-4 are much less active, but the C-5 epimer, stereoisomeric in the alkyl group, is like DA. The hydroxyl group of Ist[2] may be involved in H-bonding since the derivative (**7**) containing an *O*-acetylisostatine[2] is much less active. Even more dramatic is the analogue containing the C-4 epimer of Hip in DA (**12**), which is nearly inactive. The keto group in Hip[3] is also important, since the DA analogue with dihydro Hip[3] (**10**) is much less active. [Hip[3] oxime]DB (**11**), however, is less cytotoxic by only 5-fold, a far less significant loss than for the reduced analogue **10**, suggesting that the sp^2 carbon at C-3 of Hip in **11** may maintain didemnin's backbone conformation and is essential.

The results from Table 2 show that analogues which are site-modified within the depsipeptide ring backbone, the cyclic structure itself, tend to lose their cytotoxicity. Acyclodidemnin A (**17**) showed much weaker cytotoxicity than its cyclic counterpart **2**, indicating the importance of the ring. Generally, analogues which have modified stereocenters or functional groups directly attached to the cyclic peptide backbone showed significant losses of their bioactivities. The *N*-methyl in Me_2Tyr may be important for the conformation of DA, since iododidemnin (**14**) and reduced didemnins **15** and **16**, which retain the *N*-methyl group, showed bioactivities comparable to those of the parent compounds, while the Me_2Tyr analogue didemnin N ([Tyr[6]]DB, **13**), lacking the methyls, was much less active. These data indicate the inherent importance of the cyclic backbone structure of the depsipeptide.

On the other hand, modifications in the linear peptide portion of the didemnins in some cases resulted in increased cytotoxicities. Of the acyl derivatives of **2** (**18**−**25**), short-chain N^{α} acyl groups generally enhanced cytotoxicity, but longer chain acyl groups (>C_8) decreased activities substantially. Although the *in vitro* cytotoxicity of *N*-acetyldidemnin A (**18**) is comparable to that of **1**, compound **18** was inactive against P388 leukemia *in vivo* in the range 0.08−8 mg/kg.

L-Amino acid-substituted didemnins A, **27** and **28**, were approximately as active as **1**, while D-amino acid-substituted **29** was less cytotoxic.

Didemnin B-type analogues **30**−**33**, which have a ninth subunit more hydrophobic than that of **1** (Lac), generally were more active than **1**. Among these analogues, naturally occurring dehydrodidemnin B (**30**) was tested *in vivo* (Table 3). Compound **30** exhibited potent *in vivo* antitumor activities in mice implanted with B16 melanoma or P388 leukemia, at lower doses than **1**. Most remarkably, **30** showed effectiveness against the Lewis lung carcinoma system, for which **1** failed to show any activity.[21] On the other hand, compound **33** was inactive against P388 leukemia *in vivo* in the range 0.8−50 mg/kg.

[D-Pro[8]] analogues of didemnin B, **34** and **36**, showed substantially weaker cytotoxicity compared to the corresponding [L-Pro[8]] compounds, **33** and **1**, respectively, but [L-Ala[8]]didemnin B (**35**) was as active as **1**.

Glutaminyl didemnins, **37**−**42**, showed potent cytotoxicity, but a clear correlation of cytotoxicities with the number of Gln's in the side chain was not observed.

Antiviral Activity. The plaque reduction method was used for antiviral assays.[26b] The RNA virus vesicular stomatitis virus (VSV) was grown on baby hamster kidney cells (BHK) and the DNA virus *Herpes simplex* virus type I (HSV-1) was grown on CV-1 monkey kidney cells. Antiviral activities for some analogues are listed in Table 4. None of the didemnins significantly inhibited proliferation of the human immunodeficiency virus (HIV) in CEM cells (data not shown).

Cytotoxicity to the tumor cells and antiviral activities of didemnins usually varied in a parallel fashion. It should be noted that in the antiviral assays most didemnins tested gave good virus inhibition accompanied by toxicity to the host cells as marked by LS (light staining) or PT (partial toxicity) in Table 4. Like the cytotoxicity, the antiviral activity vs HSV (a DNA virus) and VSV (an RNA virus) was strong for short-chain acyl analogues of DB (**30**−**34**), and for *N*-Ac-DA (**18**, the most

active of all). On the other extreme acycloDA (**17**) and epi-HipDA (**12**) were inactive. Surprisingly, *N*-Pro-DA (**28**), [Ala^8]DB, and [D-Pro^8]DB (**35** and **36**) were quite active against VSV and glutaminyl didemnins (**37**, **39**, **41**) were quite active. Didemnins which showed noticeable RNA virus (VSV) activity generally showed inhibition of RNA synthesis (Table 2). This explains the fact that antiviral activity in the plaque reduction assay is always accompanied by cytotoxicity to the host cells.

Inhibition of Macromolecular Synthesis. Inhibitions of protein, DNA, and RNA synthesis were evaluated by measuring cellular incorporation of tritiated precursors into P388 cells (Table 2).[27] Didemnins B (**1**) and A (**2**) have been previously shown to inhibit macromolecular synthesis.[11,12] In the present study, most congeners which showed potent cytotoxicities and antiviral activities were strong inhibitors of protein and, to a lesser extent, DNA synthesis. The strongest inhibitors of protein synthesis were short-chain acyl derivatives of DA (**18**–**22**). Similar acyl analogues of DB (**30**–**33**) also were among the most active inhibitors. Inhibition of DNA synthesis was generally at slightly higher concentrations than protein synthesis, while RNA synthesis was not as routinely inhibited. Short-chain (up to C_6) *N*-acyl DA's and some other analogues with relatively hydrophobic side chains showed weak inhibition of RNA synthesis. These data suggest that cytotoxicity and antiviral activity of the didemnins may be attributed to a combination of the inhibition of macromolecular syntheses, especially protein and DNA, though the differential between macromolecular synthesis (μg/mL) and cytotoxicity (ng/mL) is still great. The spread of activity (SAR) within cytotoxicity is also much greater.

Enzyme Inhibition Assays. The didemnins were also assayed for inhibiton of the enzymes DNA and RNA polymerases,[28] topoisomerases I and II,[29] dihydrofolate reductase,[30] thymidylate synthase,[31] and adenosine deaminase.[32] None of the compounds tested significantly inhibited these enzymes. (Data not shown.)

Immunosuppressive Activities. **1. Two-Way Mixed Lymphocyte Response (MLR) Assay.** The MLR is a cell-mediated immune response induced by co-culturing two sources of murine splenocytes. In the present case the overall immunomodulatory properties of the didemnins and didemnin analogues were evaluated using a bidirectional MLR derived from murine splenocytes of genetically dissimilar strains of mice. Table 5 summarizes the variable biological activities of 42 didemnins in suppressing an immune response in this *in vitro* assay system.

The results show that all of the compounds suppress the immune reaction, but over a wide range of potencies. Of these, didemin M (**38**) showed the strongest inhibition of the immune response, with an IC_{50} value of 0.76 pM (1.0 pg/mL). Other pyroglutamyl didemnins—*O*-pGlu-DB (**37**) and didemnin E (**39**)—are the second and third most active (IC_{50} 5.3 and 8.4 pM, respectively). In fact, all four of the pyroglutamyl compounds (**37**–**40**) show strong suppression of the MLR. Although the N^{α}-acyl-DA class of nine compounds was distributed over the entire range of potencies, two of the short-chain members, N^{α}-Ac-DA (**18**) and N^{α}-hexanoyl-DA (**22**), were particularly effective, with IC_{50}'s of 0.027 nM (0.027 ng/mL) and 0.021 nM (0.022 ng/mL), respectively, for **18** and **22**, and N^{α}-propionyl-, butyryl- and pentanoyl-DA's were all in the top half. Short-chain [acyl^9] analogues of DB were also quite active.

The native compounds didemnins A and B were in the middle ranking of all compounds tested, whereas most site-modified classifications (chiefly Ist^2, Hip^3) and long-chain acyl derivatives of DA were in the lower third, containing less potent compounds, with N^{α}-octadecanoyl-DA (**25**) having the largest IC_{50} at 5110 nM (6040 ng/mL).

2. Lymphocyte Viability Assay. All of the 42 compounds were evaluated for cytotoxicity to one of the lymphocyte populations in the MLR (i.e. Balb/c splenocytes). The purpose of the lymphocyte viability assay (LcV) is to measure metabolic activity in lymphocytes after they have been exposed to the compounds for the duration of and under conditions equivalent to the MLR. The inverse of the data yields cytotoxicity information about the compounds on murine lymphocytes. The rationale of using resting cells, in this case unstimulated lymphocytes, to measure cytotoxicity has a strategic importance. In the MLR the heterogeneous cell populations are predominantly resting cells when the assay begins. Transformation (i.e. induction) is initiated upon costimulation by differing lymphocyte populations, but cellular proliferation (i.e. lymphoblasts, cells in mitosis) occurs between days 2 and 4 in the murine MLR.[33] Therefore, the first-stage cytotoxic effects of compounds in the assay are most likely to occur early, before lymphoblasts develop.

The results show that cytotoxicity of the compounds to lymphocytes occurs over a narrower range of concentrations (3 logs vs 7 logs) as well as at much lower potencies (0.074–11.0 μM) than their immune inhibitory effects (Table 5), thus creating a large therapeutic index, in most cases.

For unexplained reasons, the greatest cytotoxicity was observed for [$H_6Me_2Tyr^6$]DB (**15**) and N^{α}-butyryl-DA (**20**) with LC_{50} values of 0.074 μM (0.08 μg/mL) and 0.099 μM (0.10 μg/mL), respectively. The remaining compounds have much lower cytotoxicities.

3. Lymphocyte Noncytotoxic Immunosuppression. A ratio of cytotoxicity-to-inhibitory effects of the didemnins and their analogues (i.e., a therapeutic index) is also shown in Table 5. This index identifies those compounds which may inhibit an *in vitro* cell-mediated immune response by noncytotoxic means.

Five compounds showed very large ratios (more than 10^5). These are the most active compounds (in the same order) in the mixed lymphocyte reaction—the pyroglutamyl-substituted didemnins B and the short-chain acyl-substituted didemnins A. Similarly, at the bottom of the list is N^{α}-octadecanoyldidemnin A (**25**), with a ratio of 2, which correlates well with its lowest ranking in the MLR (IC_{50} 5.110 nM) and moderate cytotoxicity (>8.50 μM). *O*-pGlu-DB (**37**) is the only synthetic analogue of didemnin B represented in this group, with a noncytotoxic inhibition ratio of 1 500 000.

The parent compounds, didemnins A and B, showed similar midrange ratios between 10 000 and 15 000, with site-modified analogues of each distributed about equally above and below each parent compound.

4. Lymphoblast Viability Assay. The didemnins were also evaluated for cytotoxicity to a blastogenic form of the lymphocytes. Concanavalin A, the T-cell mitogen,

Table 4. Antiviral Activities of Some Analogues of Didemnins

	VSV/BHK				HSV/CV-1		
compd	mg/mL	cytotoxicity[a]	antiviral activity[b]	compd	mg/mL	cytotoxicity[a]	antiviral activity[b]
18	1	0, PT	+++	**18**	10	10, LS	+++
	0.5	0, PT	++		3	10, LS	+++
	0.2	0, PT	++		1	8, LS	+++
	0.1	0, PT	++		0.3	0, LS	++
1	1	12, PT	+++	**37**	10	0, LS	+++
	0.1	12, PT	+		1	0, LS	+++
	0.01	0			0.1	0	−
	0.001	0	−				
32	1	0, PT	+++	**39**	10	0, LS	+++
	0.1	0, PT	+		1	0, LS	++
	0.01	0, PT	±		0.1	0	−
	0.001	0, PT	±				
31	1	0, PT	+++	**41**	10	0, LS	+++
	0.1	0, PT	+		1	0, LS	++
	0.01	0, PT	−		0.1	0	−
	0.001	0	−				
33	1	0, PT	+++	**34**	10	0, LS	+++
	0.1	0, PT	+		3	0, LS	++
	0.01	0, PT	−		1	0, LS	+
	0.001	0, PT	−		0.3	0	±
					0.1	0	−
28	1	0, PT	+++	**36**	10	0, LS	++
	0.5	0, PT	++		3	0	+
	0.2	0, PT	+		1	0	−
	0.1	0, PT	±				
34	1	0, PT	++	**28**	10	0, LS	+++
	0.1	0, PT	+		3	0, LS	+
	0.01	0	−		1	0	±
					0.3	0	−
39	1	T	−	**30**	1	0, LS	+++
	0.1	10, PT	+		0.1	0	±
	0.01	0	−		0.01	0	−
					0.001	0	−
41	1	T		**1**	1	0, LS	+++
	0.1	10, PT	+		0.1	0	±
	0.01	0			0.01	0	−
					0.001	0	−
37	1	T		**31**	1	0, LS	+++
	0.1	8, PT	+		0.1	0	±
	0.01	6	−		0.01	0	−
					0.001	0	−
2	1	0, PT	+	**32**	1	0, LS	+++
	0.5	0, PT	+		0.1	9	±
	0.2	0	±		0.01	0	−
	0.1	0	−		0.001	0	−
30	1	0, PT	+++	**33**	1	0, LS	+++
	0.1	0, PT			0.1	0	±
	0.01	0	−		0.01	0	−
	0.001	0	−		0.001	0	−
35	1	0, PT	++	**35**	10	0, LS	+++
	0.1	0, PT	±		3	0	±
	0.01	0	0		0.1	0	−
36	1	0, PT	++	**2**	10	12	+++
	0.1	0, PT			5	8	±
	0.01	0, PT	−		2	5	−
					1	5	−
29	1	0, PT	+	**27**	10	8, LS	+++
	0.5	0, PT			3	8, LS	±
	0.2	0, PT			1	5	−
	0.1	0	−		0.3	0, LS	−
13	1	0, PT		**38**	10	0, LS	+++
	0.1	0, PT	−		1	0	−
	10	0, PT	−		0.1	0	−
7	10	0, PT		**29**	10	0, LS	++
	5	0, PT			3	5	±
	2	0, PT	−		1	0	±
	1	0, PT	−		0.3	0	−
12	10	0, PT		**17**	10	0	±
	5	0	−		1	0	−
	2	0	−		0.1	0	−
27	1	0, PT	−	**7**	10	11	−
	0.5	0, PT	−		5	8	−
	0.2	0, PT	−		1	5	−
	0.1	0, PT	−		1	5	−

Table 4 (Continued)

	VSV/BHK				HSV/CV-1		
compd	mg/mL	cytotoxicity[a]	antiviral activity[b]	compd	mg/mL	cytotoxicity[a]	antiviral activity[b]
38	1	T	–	**12**	10	0	–
	0.1	8, PT	–		5	5	–
	0.01	0	–		2	2	–
17	10	5	–	**13**	1	0	–
	1	0	–		0.1	0	–
	0.1	0	–		0.01	0	–

[a] 0 (least toxic) to 16 (toxic); PT = partial toxicity; LS = light staining, indicating some toxicity. [b] +++ = complete inhibition, ++ = strong inhibition, + = moderate inhibition, ± = marginal inhibition, – = no inhibition.

Table 5. *In Vitro* Immunosuppressive Activities of Didemnins[a]

		two-way mixed lymphocyte reaction (MLR)	cytotoxicity to lymphocytes		cytotoxicity to lymphoblasts	
compd no.	compd name	suppression MLR, IC_{50} nM (ng/mL)	LcV,[b] LC_{50} μM (μg/mL)	ratio LcV (LC_{50})/ MLR (IC_{50})	LbV,[c] LC_{50} μM	ratio LbV (LC_{50})/ MLR (IC_{50})
38	didemnin M	0.00076 (0.001)	>7.41 (>10)	>10000000	2.5E–4	326
37	*O*-pGlu-DB	0.0053 (0.007)	>8.20 (>10)	>1500000	7.9E–5	15
39	didemnin E	0.0084 (0.012)	>6.77 (>10)	>800000	1.4E–3	169
22	N^{α}-hexanoyl-*DA*	0.021(0.022)	9.60 (>10)	>460000	1.1E–4	5
18	N^{α}-acetyl-DA	0.027 (0.027)	>10.0 (>10)	>370000	3.9E–4	14
33	[isobutyryl^{9}]DB	0.037 (0.041)	2.24 (2.40)	60000	2.04E–4	6
40	didemnin D	0.12 (0.20)	>6.22 (>10)	>50000	8.5E–4	7
31	[acetyl^{9}]DB	0.21 (0.22)	2.80 (3.10)	14000	1.6E–4	1
32	[propionyl^{9}]DB	0.23 (0.26)	1.98 (2.20)	8500	1.84E–4	1
9	[anhydroist^{2}][Phth-Ala^{9}]DB	0.25 (0.30)	>8.2 (>10)	33000	1.9E–3	7
5	*Z*-[(3*S*,4*R*,5*R*)-Ist^{2}]DA	0.28 (0.30)	>9.3 (>10)	33000	1.2E–2	43
20	N^{α}-butyryl-DA	0.30 (0.30)	0.099 (0.1)	300	7.4E–5	<1
35	[Ala^{8}]DB	0.31 (0.33)	>9.20 (>10)	>30000	7.17E–4	2
19	N^{α}-propionyl-DA	0.37 (0.37)	6.50 (6.50)	18000	6.8E–5	<1
30	[pyruvyl^{9}]DB (DDB)	0.38 (0.43)	2.60 (2.90)	6800	7.9E–5	<1
8	[Phth-Ala^{9}]DB	0.39 (0.49)	>8.1 (>10)	21000	8.1E–4	2
1	DB	0.42 (0.46)	6.34 (7.00)	15000	6.0E–4	1
21	N^{α}-pentanoyl-DA	0.48 (0.48)	9.70 (>10)	20000	5.9E–5	<1
41	didemnin X	0.50 (0.83)	6.01 (10.0)	>2000	3.1E–3	6
42	didemnin Y	0.50 (0.89)	>5.57 (>10)	>11000	1.1E–3	2
16	[H_6-*N*MePhe^{6}]	0.52 (0.57)	>9.20 (>10)	>18000	7.3E–4	1
17	acyclodidemnin A	0.57 (0.54)	>10.4 (>10)	>18000	0.24	436
34	[isobutyryl^{9}-D-Pro^{8}]DB	0.60 (0.67)	5.60 (6.20)	9000	4.81E–3	8
14	[iodoMe_2Tyr^{6}]DB	0.66 (0.81)	>8.10 (>10)	>12000	8.2E–4	1
15	[H_6-Me_2Tyr^{6}]DB	0.72 (0.81)	0.074 (0.080)	100	1.6E–3	2
26	N^{α}-formyl-DA	0.72 (0.70)	>10.0 (>10)	>14000	1.1E–3	2
27	N^{α}-leucyl-DA	0.74 (0.78)	>9.50 (>10)	>13000	3.8E–3	5
36	[D-Pro^{8}]DB	0.83 (0.92)	>9.00 (>10)	11000	0.013	15
11	[Hip^{3} oxime]DB	0.85 (0.96)	>8.9 (>10)	10000	1.7E–3	2
28	N^{α}-prolyl-DA	0.96 (1.00)	>9.60 (>10)	>10000	5.1E–3	5
2	DA	0.98 (0.93)	>11.0 (>10)[d]	11000	0.015	15
3	Z-DA	1.02 (1.10)	7.50 (8.10)	7400	1.9E–3	2
23	N^{α}-octanoyl-DA	5.10 (5.50)	>9.35 (>10)	1800	0.021	4
29	N^{α}-D-prolyl-DA	11.7 (12.2)	>9.60 (>10)	>8000	0.022	2
4	*Z*-[(3*R*,4*R*,5*S*)-Ist^{2}]DA	18.0 (19.0)	9.00 (9.70)	500	0.22	12
10	[H_2-Hip^{3}]DA	30.5 (29.0)	>10.6 (>10)	350	0.46	16
13	didemnin N	34.2 (37.0)	>9.20 (>10)	>270	2.1E–1	1
12	[*epi*-Hip^{3}]DA	86.0 (81.0)	>10.6 (>10)	120	5.1E–2	1
7	*O*-acetyl-DA	100 (100)	>10.0 (>10)	100	7.7E–2	1
24	N^{α}-dodecanoyl-DA	106 (119)	>8.90 (>10)	>84	0.16	2
6	*Z*-[(3*S*,4*S*,5*S*)-Ist^{2}]DA	270 (290)	>9.3 (>10)	34	2.6	9
25	N^{α}-octadecanoyl-DA	5110 (6040)	>8.50 (>10)	>2	2.7	1

[a] Arranged in order of IC50's (most active to least active) in MLR. [b] LcV = Lymphocyte viability. [c] LbV = Lymphoblast viability. E–4 is the same as 10^{-4}. [d] Highest concentration 10 μg/mL.

was used to induce cellular proliferation of the splenocytes. Since cellular proliferation occurs between days 2 and 4 in the murine MLR, there may be secondary cytotoxic effects of the compounds occurring later as lymphoblasts develop. This form of the cell is more fragile and therefore usually more susceptible to cytotoxicity.

The results show that the cytotoxicity of the compounds to lymphoblasts occurs over a much wider range of concentrations than was observed toward lymphocytes (Table 5). Not unexpectedly, much lower cytotoxic concentrations (down to 7.9E-5 μM) were generally observed compared to lymphocytes.

The order of cytotoxicity to lymphoblasts is generally, but not exactly, like that seen for cytotoxicity to lymphocytes. One exception seems to be for *O*-pGlu-DB (**37**), which ranked higher in cytotoxicity to lymphoblasts than to lymphocytes. This change in rank accounts for a marked difference in the ratios of noncytotoxic immunosuppression discussed below.

5. Lymphoblast Noncytotoxic Immunosuppression. Some important differences between lymphocyte

Table 6. Immunosuppression of Didemnins on Graft vs Host Reaction (GVHR) Assay[a]

compound	dose[b] (mg/kg/day)	body wt[c] day 0 (g ± SD)	body wt day 5 (g ± SD)	body wt change day 5 (g)	alive day 8 (%)	mean group spleen wt normalized to day 8 body wt	indexed[d]
didemnin B (**1**)	0.16	17.0 ± 0.9	15.6 ± 1.6	-1.4	100	4.0	1.24
	0.016	17.2 ± 0.7	18.2 ± 0.7	1.0	100	4.2	1.33
	0.0016	17.4 ± 0.8	18.4 ± 0.8	1.0	100	6.1	1.91
didemnin M (**38**)	0.16	16.6 ± 0.8	16.4 ± 1.0	1.0	100	4.9	1.54
	0.016	16.6 ± 0.5	17.8 ± 0.7	-0.2	100	4.4	1.38
	0.0016	15.8 ± 1.3	17.2 ± 1.2	1.4	100	4.1	1.27
O-pGlu-DB (**37**)	0.16	15.8 ± 1.2	15.2 ± 1.2	-0.6	100	4.2	1.32
	0.016	16.4 ± 0.8	17.2 ± 1.5	0.8	100	5.6	1.76
	0.0016	17.0 ± 1.5	17.4 ± 1.7	0.4	100	5.2	1.63
positive control[e]		16.8 ± 1.0	18.0 ± 1.3	1.2	100	5.4	1.70
syngenetic[f]		16.6 ± 0.5	18.0 ± 1.3	1.4	100	3.2	1.00
cyclophosphamide	200	16.3 ± 0.4	13.8 ± 0.4	-2.5	100	0.4	0.14

[a] Balb/c-to-CB6F1 GVHR model. See the Experimental Section for detail. [b] Schedule qd 1−7, 0.5 mL of solution/mouse. [c] Mice were weighed on days 0, 5, 8. [d] Index = spleen wt treated/spleen wt syngenetic injection. >1.3 (50% suppression of positive control is considered to be significant suppression). [e] Vehicle only. [f] Syngenetic (CB6F1−CB6F1) injection.

and lymphoblast assays are observed. First, the toxicity/suppression ratios are smaller in the lymphoblast assay, indicating that the inhibition of the immune response at later stages in the reaction would be most affected by cytotoxicity to proliferating immune cells. However, the exception to this, consistent with a judgment of noncytotoxic immunosuppression, occurs for acycloDA (**17**), didemnin M (**38**), and didemnin E (**39**). The ratios are larger as a group to distinguish them as compounds that might rely on other means to inhibit the immune reaction than via cytotoxicity involving lymphoblasts. Didemnins M and E have this distinction whether the cytotoxicity data are for lymphocytes or lymphoblasts.

6. Graft vs Host Reaction (GVHR). Three representative compounds—didemnin M (**38**), *O*-pGlu-DB (**37**), and didemnin B (**1**)—showing high (**37**, **38**) or moderate (**1**) *in vitro* immunosuppressive activity were subsequently evaluated in a multidose assay (0.16, 0.016, and 0.0016 mg/kg per injection; qd 1−7) for their *in vivo* immunosuppressive effects on the GVHR splenomegaly assay.

The results show didemnin M (**38**) optimally suppressed the allogeneically induced splenomegaly response in CB6F1 mice grafted with Balb/c splenocytes at 1.6 μg/kg/inj by 61% compared to control (Table 6). Higher doses (16 and 160 μg/kg) were less effective, but not toxic. Didemnin B (**2**) and *O*-pGlu-DB (**37**) were equally effective but at optimal dosages of 160 μg/kg per injection, showing 66% and 54% suppression, respectively. These were the highest doses for each and neither was toxic to the animals. Lower doses were less effective for *O*-pGlu-DB. Didemnin B showed a 53% suppression at the next lower dose of 16 μg/kg per injection but was not effective at the lowest dose (1.6 μg/kg per injection). The relationship of these *in vivo* effects correlates well with their *in vitro* data.

Discussion

The present study has defined SAR's for the didemnins regarding three important bioactivities: cytotoxicity, immunosuppression, and antiviral activity. The data in Tables 2 and 4 suggest that the cytotoxicity and antiviral activity of all congeners may be due to inhibition of macromolecular synthesis, i.e. protein, DNA, and RNA. Inhibition of DNA and RNA synthesis appears to be particularly important in antiviral activity, since the compounds may interfere with the assembly of viral DNA or RNA in the infected cells.

Structurally, the original cyclic depsipeptide backbone has been shown to be essential for all three bioactivities, except acyclodidemnin A (**17**) for immunosuppression. All other modifications within the cyclic peptide portion resulted in diminished bioactivities. Stereochemical and functional changes in the peptide backbone led to significant losses in bioactivities, suggesting that the peptide conformation maintained by the original stereocenters and functional groups is indispensable. AnhydroIst analogue **9** and Hip oxime analogue **11** were the only analogues with bioactivity comparable to their counterparts, **8** and **1**, respectively. These compounds are presumably capable of maintaining the original backbone conformation.

The alkyl and aryl side chains of Ist and Me_2Tyr, respectively, could be modified without drastic loss of cytotoxicity or antiviral activity. The latter case was somewhat surprising, since the aryl group of didemnin B was proposed by Hossain et al. to be one possible binding site to the receptors.[34] In the MLR assay, however, the Me_2Tyr unit demonstrated some importance as all analogues site modified at this subunit showed lower potency in MLR than their counterparts.

In contrast to the cyclic peptide portion, modification by acyl groups extending from the *N*-terminus of the MeLeu unit led to gains in bioactivities. Short-chain acyl (C2−C6) and L-amino acid derivatives of **2** were comparably active to **1** *in vitro*. *In vivo* testing of **18**, however, showed that at least some of those modifications are not appropriate to give significant efficacy.[3c] In the didemnin B-type compounds, terminal (unit 9) acyl groups less polar than and similar in size to Lac gained *in vitro* and *in vivo* antitumor activity. Interestingly, amino acid derivatives of **2** and didemnin B-type analogues which contained [D-Pro^8] showed much lower activities than their counterparts. NMR studies of these [D-Pro^8] analogues showed that they consist of several conformers in solution ($CDCl_3$, Figure 1). This suggests that [Pro^8] regulates the orientation of the peptide side chain, by a β-turn structure according to the suggestions of Kessler et al.,[35] which may affect the entire peptide shape.

The immunology data suggest that the immunosuppressive activity of didemnins is mediated mostly by cytotoxicity to lymphoblasts occurring at a later stage

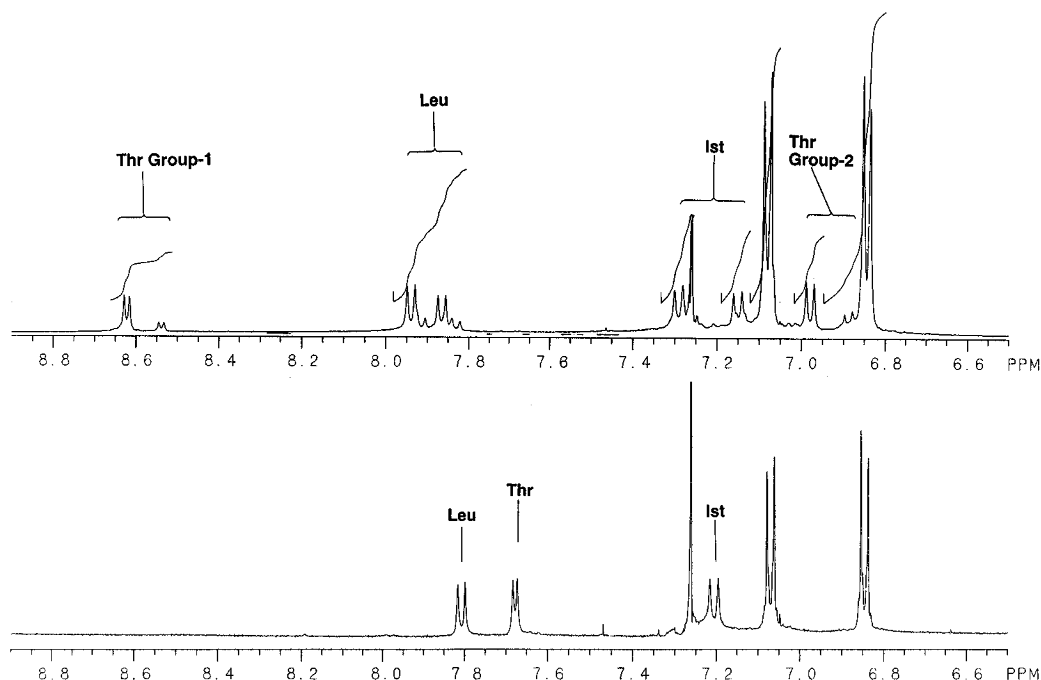


Figure 1. ^{1}H NMR spectra (500 MHz in $CDCl_3$) of the amide proton regions for didemnin B (**1**, below) and [D-Pro[8]]DB (**36**, above). In the latter compound, NH's appeared as multiple signals, suggesting that [D-Pro[8]]DB exists as several stable conformers in solution. It is noteworthy that the amide proton for the Thr subunit of **36** gave two distinct chemical shifts, group 1 and group 2. The assignments are based on COSY data.

of activation in the cell-mediated immune response, except for some glutaminyl didemnins, **37**–**39**, and acyclo-DA (**17**), as indicated by the relatively constant therapeutic index utilizing cytotoxicity data to proliferating lymphoblasts versus resting lymphocytes. These results indicate that the immunosuppressive activity of didemnins is, in most cases, a result of antiproliferation of stimulated T-cells, as demonstrated for **1** by others.[36] In the cases of **37**–**39** and **17**, however, some unique or specific mechanisms of action such as those seen in cyclosporin A,[37] FK 506, or rapamycin[38] may be involved in the immunosuppressive actions (e.g. noncytotoxic immunosuppression during early stage events involving T-cell activation).

More detailed biochemical study on the cellular mechanism for the antiproliferative activity of the abovementioned didemnin congeners should result in further understanding of their interesting immunosuppressive activity.

Experimental Section

General. Optical rotations were measured using a 5-cm cell (1 mL). NMR spectra (200, 300, and 500 MHz, ^{1}H) were obtained using either deuteriochloroform ($CDCl_3$), deuteriomethanol (CD_3OD), or a mixture of both as solvents and internal standard [7.26 (^{1}H) and 77.0 (^{13}C) ppm for $CDCl_3$, 3.30 (^{1}H) and 49.0 (^{13}C) ppm for CD_3OD or a mixture of CD_3OD–$CDCl_3$]. FABMS spectra and HRFABMS data were obtained using magic bullet as a matrix. All solvents for reactions were distilled over appropriate drying agents prior to use. RPHPLC was perfomed by using a semipreparative C-18 silica gel column with UV detection at 254 nm and a flow rate of 1 mL/min. Solvent systems are indicated in each case.

Bioassays. Cytotoxicity Measurement. Cytotoxicities were measured using the procedure described previously.[25] Briefly, cells were maintained in logarithmic phase of growth in a medium comprised of the following: Eagle's minimum essential medium (MEM) with Earle's balanced salts [with L-Gln (2.0 mM) and nonessential amino acids, without Na_2CO_3] supplemented by 10% fetal calf serum, Na_2CO_3 (10^{-2} M), penicillin G, and streptomycin sulfate. P388 cells (10^4 cells/16-mm well) and A-549, HT-29, Mel-28 cells (2×10^4 cells/16-mm well) were seeded in 1 mL of the above medium with each concentration of samples. All assays were carried out in duplicate. After 3 days of incubation (37 °C, 98% humidity, 10% CO_2) cells were visually counted against control wells to determine IC_{50}.

DNA, RNA, and Protein Synthesis. These assays were carried out by following the method published previously, with slight modification.[27] Inhibitions of DNA, RNA, and protein synthesis were determined by measuring cellular incorporation of [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, and DL-[4,5-^{3}H]leucine, respectively, into P388 cells. Cells were cultured in MEM with 5% newborn calf serum (3×10^5 cells/mL). Compounds were added with DMSO (1% final DMSO concentration) to testing concentrations. These cells were added to the mixture of each tritiated precursor (1–3 μCi/mL), the corresponding thymidine, uridine, or leucine (4 mM) was incubated (37 °C, 5% CO_2 in air) and then collected on a Durapore filter (0.45 μm, Millipore) by vacuum filtration, and the macromolecules were precipitated by addition of 5% trichloroacetic acid (TCA). The filter was washed by 5% TCA three times and then transferred to scintillation vials. Scintillation cocktail Biogreen-2 was added to the vial, and radioactivity was counted by a liquid scintillation counter.

DNA Polymerase Assay. Each compound in DMSO (1% final DMSO concentration) was added to a mixture (final volume 50 μL) comprised of *Escherichia coli* DNA polymerase-1 in 50 mM Tris-HCl (pH 7.5), $MgCl_2$ (10 mM), dithiothreitol (1 mM), bovine serum albumin (30 μg/mL), activated calf thymus DNA (40 μg/mL), dGTP, dTTP, dCTP (35 μM, each), dATP (17 μM), and 1 μCi of [8-^{3}H(N)]dATP (10–25 Ci/mmol). The reaction was quenched by adding TCA (10%) and sodium pyrophosphate (1.0 M). The macromolecule precipitate was collected, and the radioactivity was counted as described above.

RNA Polymerase Assay. Each compound in DMSO (1% final DMSO concentration) was added to a mixture (final volume 50 μL) comprised of *E. coli* RNA polymerase in 40 mM Tris-HCl (pH 7.5), $MgCl_2$ (10 mM), KCl (50 mM), dithiothreitol (1 mM), bovine serum albumin (100 μg/mL), activated calf thymus DNA (40 μg/mL), GTP, UTP, CTP (70 μM, each), ATP (35 μM), and 1 μCi of [2,8-^{3}H]ATP (25–40 Ci/mmol). The

reaction was quenched, and the radioactivity of the macromolecule was counted as above.

Preparation of Splenocyte Cell Suspensions for Primary Culture. Separate splenocyte cell suspensions were prepared by homogenization of spleens from 6−12 week old female C57Bl/6 (H-2^b) and BALB/c (H-2^d) mice in cold (4 °C) tissue culture medium [TCM: RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM HEPES, 1% antimycotic solution (i.e. 100 units/mL penicillin, 0.25 μg/mL amphotericin-B, 100 μg/mL streptomycin), 25 μg/mL gentamicin, and 2% L-glutamine]. The homogenate was centrifuged at 500*g* for 5 min at 4 °C. The resulting pellet was resuspended in cold TCM at 10 mL/spleen and evaluated for viability by hemacytometer using the trypan blue exclusion method. The cell concentration was adjusted to 2.5×10^6 cells/mL for each splenocyte population.

Mixed Lymphocyte Reaction (MLR).[39] Each compound was dissolved in absolute ethanol (abs. EtOH) and diluted 16-fold (from 10 μg/mL to 3.33×10^{-6} μg/mL). Duplicate volumes (10 μL) were added into wells of a 96-well microtiter plate and then evaporated to dryness at room temperature. Splenocytes derived from Balb/c and C57Bl/6 mice were prepared as described above, and 100 μL of each cell suspension was added to each well. Wells containing 200 μL of media alone served as nonspecific control wells.

Assay plates were incubated in a 5% CO_2 humidified incubator at 37 °C for 96 h and then pulsed overnight (about 15 h) with 1 μCi of [^{3}H]thymidine (20 Ci/mmol) per well and finally filtered to recover tritiated-thymidine incorporated into newly synthesized DNA.

The MLR data were calculated as a percentage of immune cell proliferative activity relative to control, and an IC_{50} value was interpolated for each test compound.

Lymphocyte Viability (LcV) Assay.[40] Test compounds were prepared in two sets as described above. Splenocytes were prepared as described previously from one murine strain (Balb/c), and a volume of 200 μL of the cell suspension was added to one set of test compounds and control wells.

Assay plates were incubated as in the MLR and then pulsed overnight with 75 μL per well MTT−thiazolyl blue solution (150 μg). The plates were decanted, and the resulting insoluble formazan crystals were dissolved in 200 μL of isopropyl alcohol. Absorbance at 570 nm was measured.

The LcV data were calculated as a percentage of basal metabolic activity, or percent viability, relative to BALB/c control, and an LC_{50} value was interpolated for each test compound.

Lymphoblast Viability (LbV) Assay. The cytotoxicity of didemnins and didemnin analogues on lymphoblasts was evaluated using a modification of the above LcV procedure. Mitogen-induced lymphoblastic proliferation was initiated by preincubation of splenocytes, as prepared above, with 1.0 μg/mL of concanavalin A (Con A) in a 5% CO_2 humidified incubator at 37 °C for 30 min.[41] Test compounds were prepared as described above. Preincubated Con A splenocytes were added in a volume of 200 μL to each well. Incubation, processing, and data calculation were the same as described above for the LcV assay.

Graft vs Host GVH Reaction. Three didemnins (didemnin B, didemnin M, and pGlu-didemnin B) were evaluated in a modified Simonsen splenomegaly assay.[42] Briefly, an F1 hybrid host animal is grafted with immunocompetent spleen cells from the parent strain. The index used to measure the success of the GVHR is splenomegaly (increased spleen weight due to cellular proliferation of grafted lymphocytes). An index > 1.3 (graft index) is considered to be a successful graft rejection of the recipient animal. Immunosuppression is considered as a reduction of the graft index.

On day 0, $CB6F_1$ female mice, 4 weeks of age, were grafted by intraperitoneal (ip) injection of 50×10^6 splenocytes from BALB/c female mice in high-glucose (4500 mg/mL) Dulbecco's modified Eagles medium. A syngeneic control group ($CB6F_1$-to-$CB6F_1$) was similarly prepared and served as the negative control. Grafted mice were divided into treatment groups containing six mice each. Groups were injected ip with test compound (dissolved in vehicle; 1% abs. EtOH in sterile phosphate-buffered saline) at one of three dose levels (0.16, 0.016, and 0.0016 mg/kg per injection) in a multidose assay, cyclophosphamide (200 mg/kg per injection), or vehicle on days 1−7 (qd 1−7). On day 8 all groups were sacrificed, spleens were excised, and a graft index was calculated for each group by the following formula:

$$\text{graft index} = \frac{[(\text{spleen wt of test group})/(\text{body wt of test group}) \times 100]}{(\text{spleen wt of syngeneic group})/(\text{body wt of syngeneic group})}$$

Preparation of Didemnin Congeners. Native Didemnins. All native didemnins were isolated from the extract of *T. solidum,* except for dehydrodidemnin B (**30**), which was isolated from *Aplidium albicans*.[21] Isolation and structure determination of these peptides have been described elsewhere.[3,20,21]

Boc-(3*R*,4*R*,5*S*)-Ist-OH. Boc-(3*R*,4*R*,5*S*)-Ist-OEt[22] (130 mg, 0.43 mmol) was treated with KOH (1N, 0.5 mL) in dioxane (1.0 mL) and H_2O (0.5 mL) at room temperature for 1 h. An oily product (98 mg, 83%) was afforded after the usual workup: $[\alpha]^{25}_D$ +33° (*c* 0.13, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.88 (1H, d, J = 10.0 Hz), 4.22 (1H, m), 3.32 (1H, m), 2.6−2.5 (2H, m), 1.45 (9H, s); HRFABMS calcd for $C_{13}H_{26}NO_5$ M_r 276.1811 (M + H), found 276.1816.

Boc-(3*S*,4*R*,5*R*)-Ist-OH. Boc-(3*S*,4*R*,5*R*)-Ist-OEt[22] was treated as above to give an oil (84%): $[\alpha]^{25}_D$ −1.2° (*c* 0.27, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.50 (1H, d, J = 9.8 Hz), 4.06 (1H, m), 3.60 (1H, m), 2.6−2.4 (2H, m), 1.45 (9H, s); HRFABMS found 276.1804.

Boc-(3*S*,4*S*,5*S*)-Ist-OH. Boc-(3*S*,4*S*,5*S*)-Ist-OEt was treated as above to give an oil (84%): $[\alpha]^{25}_D$ −41.3° (*c* 0.29, $CHCl_3$); ^{1}H NMR[22] (200 MHz, $CDCl_3$) δ 4.90 (1H, d, J = 10.0 Hz), 4.27 (1H, m), 3.22 (1H, m), 2.7−2.4 (2H, m), 1.45 (9H, s); HRFABMS found 276.1804.

Boc-(3*R*,4*R*,5*S*)-Ist(TBDMS)-OH. Boc-(3*R*,4*R*,5*S*)-Ist-OH (97 mg, 0.35 mmol) was treated with *tert*-butyldimethylsilyl (TBDMS) chloride (0.16 g, 0.45 mmol) in the presence of imidazole (145 mg, 2.1 mmol) in DMF (1.1 mL) under N_2 for 20 h. The product was chromatographed (SiO_2, hexane−EtOAc, 1:4) to give an oil (80 mg, 59%): $[\alpha]^{25}_D$ +21° (*c* 0.29, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.72 (1H, d, J = 9.0 Hz), 4.33 (1H, m), 3.37 (1H, m), 2.7−2.4 (2H, m), 1.46 (9H, s), 0.90 (9H, s), 0.09 and 0.07 (6H, 2:1 singlets); HRFABMS calcd for $C_{19}H_{40}NO_5Si$ M_r 390.2676 (M + H), found 390.2662.

Boc-(3*S*,4*R*,5*R*)-Ist(TBDMS)-OH. The above treatment of Boc-(3*S*,4*R*,5*R*)-Ist-OH gave an oil (63%): $[\alpha]^{25}_D$ −0.74° (*c* 0.41, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.64 and 4.50 (0.5 H each, d, J = 9.0 Hz), 4.17 (1H, m), 3.70 and 3.30 (0.5H each, m), 2.6−2.4 (2H, m), 1.46 and 1.43 (9H, s), 0.88 (9H, brs), 0.11 and 0.02 (6H, 4:1singlets); HRFABMS found 390.2680.

Boc-(3*S*,4*S*,5*S*)-Ist (TBDMS)-OH. The above treatment of Boc-(3*S*,4*S*,5*S*)-Ist-OH gave an oil (58%): $[\alpha]^{25}_D$ −30.3° (*c* 0.37, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.75 (1 H, d, J = 9.0 Hz), 4.37 (1H, m), 3.30 (1H, m), 2.6−2.4 (2H, m), 1.46 (9H, s), 0.90 (9H, brs), 0.12 (6H, s); HRFABMS found 390.2676.

Boc-(3*R*,4*R*,5*S*)-Ist(TBDMS)-Hip-Leu-OTMSE. A solution of DCC (1,3-dicyclohexylcarbodiimide, 44 mg, 0.22 mmol) in CH_2Cl_2 (1 mL) was added to a stirred solution of Hip-Leu-OTMSE[23] (74 mg, 0.19 mmol), Boc-(3*R*,4*R*,5*S*)-Ist(TBDMS)-OH (74 mg, 0.19 mmol), and DMAP ((*N*,*N*-dimethylamino)-pyridine, 23 mg, 0.19 mmol) in CH_2Cl_2 (3 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h then at room temperature for 12 h. The product was chromatographed (SiO_2, EtOAc−hexane 9:1) to give a viscous oil (54 mg, 37%): $[\alpha]^{25}_D$ −7.5° (*c* 0.28, $CHCl_3$); HRFABMS calcd for $C_{30}H_{75}N_2O_9Si$ M_r 759.5011 (M + H), found 759.5035.

Boc-(3*S*,4*R*,5*R*)-Ist(TBDMS)-Hip-Leu-OTMSE (51%): $[\alpha]^{25}_D$ −19° (*c* 0.34, $CHCl_3$); HRFABMS found 759.4998.

Boc-(3*S*,4*S*,5*S*)-Ist(TBDMS)-Hip-Leu-OTMSE (40%): $[\alpha]^{25}_D$ −27°(*c* 0.33, $CHCl_3$); HRFABMS found 759.5011.

Boc-(3*S*,4*S*,5*S*)-Ist-Hip-Leu-OH. A solution of Boc-(3*R*,4*R*,5*S*)-Ist(TBDMS)-Hip-Leu-OTMSE (65 mg, 0.086 mmol) in THF (0.6 mL) was stirred with tetrabutylammonium fluoride (TBAF) solution (1 M, 0.195 μL in THF) at room temperature for 3 days. Water was added to the product after

removal of the organic solvent. Crude oily material was obtained after the usual workup which was separated (SiO_2, $CHCl_3$–MeOH, 9:1) to give an oil (45 mg, 96%): $[\alpha]^{24}_D$ −9.8° (*c* 0.31, $CHCl_3$); HRFABMS calcd for $C_{27}H_{49}N_2O_9$, M_r 545.3438 (M + H), found 545.3444.

Boc-(3*S*,4*R*,5*R*)-Ist-Hip-Leu-OH (87%): $[\alpha]^{24}_D$ −11° (*c* 0.25, $CHCl_3$); HRFABMS found 545.3444.

Boc-(3*S*,4*S*,5*S*)-Ist-Hip-Leu-OH (88%): $[\alpha]^{24}_D$ −21° (*c* 0.25, $CHCl_3$); HRFABMS found 545.3432.

Z-D-MeLeu-Thr-{*O*-[Boc-(3*R*,4*R*,5*S*)Ist-*O*-Hip-Leu-Pro-Me$_2$Tyr]}-*O*TMSe. A solution of Boc-(3*S*,4*R*,5*R*)-Ist-Hip-Leu-OH (30 mg, 0.055 mmol) and *N*-hydroxybenzotriazole (HOBT, 13.5 mg, 0.1 mmol) in THF (0.4 mL) was added to a solution of Z-D-MeLeu-Thr-*[O*-(Pro-Me$_2$Tyr)]-OH·HCl (40 mg, 0.050 mmol) in DMF (0.3 mL) in the presence of *N*-methylmorpholine (NMM, 4 μL). A solution of EDC [1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, 11.5 mg, 0.056 mmol] in THF (0.3 mL) was added at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 12 h. The products were purified by RPHPLC (MeOH–H_2O, 9:1). Two products (epimers at the α-position of Hip, identical FABMS data) were combined (yield 41.5 mg, 58%): $[\alpha]^{25}_D$ −0.9° (*c* 0.89, $CHCl_3$); HRFABMS calcd for $C_{67}H_{107}N_6O_{17}Si$ M_r 1295.7462 (M + H), found 1295.7460.

Z-D-MeLeu-Thr-{*O*-[Boc-(3*S*,4*R*,5*R*)Ist-*O*-Hip-Leu-Pro-Me$_2$Tyr]}-*O*TMSe (50%): $[\alpha]^{25}_D$ +1.9° (*c* 0.89, $CHCl_3$); HRFABMS calcd for $C_{67}H_{107}N_6O_{17}Si$ M_r 1295.7462 (M + H), found 1295.7471.

Z-D-MeLeu-Thr-{*O*-[Boc-(3*S*,4*S*,5*S*)Ist-*O*-Hip-Leu-Pro-Me$_2$Tyr]}-*O*TMSe (50%): $[\alpha]^{25}_D$ −9° (*c* 0.19, $CHCl_3$); HRFABMS calcd for $C_{67}H_{107}N_6O_{17}Si$ M_r 1295.7462 (M + H), found 1295.7460.

Z-[(3*R*,4*R*,5*S*)Ist^2]didemnin A (4). A stirred solution of Z-D-MeLeu-Thr-{*O*-[Boc-(3*R*,4*R*,5*S*)Ist-*O*-Hip-Leu-Pro-Me$_2$Tyr]}-*O*TMSe (40 mg, 0.031 mmol) in THF (0.2 mL) was treated with tetrabutylammonium fluoride (1 M in THF, 0.12 mL) for 16 h. A viscous oil (35 mg, FABMS *m/z* 1195) was obtained after the usual workup which showed one spot on TLC (SiO_2). To this product (in CH_2Cl_2, 0.2 mL) was added TFA (0.25 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. Excess TFA was removed *in vacuo* to give the ninhydrin-positive product (36 mg, *m/z* 1096 by FABMS, TLC one spot). NMM (4 μL) was added to a solution (CH_2Cl_2, 1 mL) of the above product at 0 °C, followed by CH_2Cl_2 (15 mL). To a stirred solution of HOBT (14.1 mg, 0.10 mmol) and EDC (14.2 mg, 0.070 mmol) in DMF (0.5 mL) and CH_2Cl_2 (60 mL) was slowly added the above mixture over 6 h at 0 °C. The reaction mixture was stirred at room temperature for 4 days and then concentrated. The EtOAc soluble portion was washed, and the product (27 mg) was separated by RPHPLC (MeOH–H_2O, 85:15) to give a solid (2.3 mg, 6%). This product, a mixture of Z-didemnin A and its epimer at the α-position of the Hip residue, was separated by HPLC (silica gel, EtOAc–hexane, 3:2). The first fraction and the second fraction gave Z-[(3*R*,4*R*,5*S*)Ist^2]didemnin A (**4**) and Z-[(3*R*,4*R*,5*S*)Ist^2,α-*epi*-Hip^3]didemnin A, respectively.[23] Compound **4**: colorless solid; $[\alpha]^{25}_D$ −14° (*c* 0.16, $CHCl_3$); HRFABMS calcd for $C_{57}H_{85}N_6O_{14}$ M_r 1077.6124 (M + H), found 1077.6115.

Z-[(3*S*,4*R*,5*R*)-Ist^2]didemnin A (5): yield 19%; colorless solid; $[\alpha]^{25}_D$ −102° (*c* 0.11, $CHCl_3$); HRFABMS found 1077.6124.

Z-[(3*S*,4*S*,5*S*)-Ist^2]didemnin A (6): yield 14%; colorless solid; $[\alpha]^{25}_D$ −28° (*c* 0.11, $CHCl_3$); HRFABMS found 1077.6124.

***O*-Acetyldidemnin A (7).** To a solution of **2** (55.2 mg, 0.059 mmol) in benzene (0.8 mL) was added benzyl chloroformate (50 mL, 6 equiv) and Et_3N (10 μL). The mixture was stirred at room temperature for 24 h, and then the solvents were removed by N_2. The resulting solid was separated (silica gel, EtOAc) to give *N*-Z-didemnin A. The product was treated with pyridine (0.5 mL) and acetic anhydride (0.5 mL) for 12 h at room temperature to give *N*-Z-*O*-acetyldidemnin A (63 mg, 92%): ^{1}H NMR ($CDCl_3$, 300 MHz);[1] HRFABMS calcd for $C_{59}H_{87}N_6O_{15}$ M_r 1119.6229 (M + H), found 1119.6217.

A mixture of *N*-Z-*O*-acetyldidemnin A (40 mg 0.035 mol) and Pt/C (10%, 40 mg) in 2-propanol (1 mL) and acetic acid (10 mL) was stirred in a H_2 atmosphere for 2.5 h at room temperature. The product was filtered through a short (4 g) silica gel column with EtOAc–2-propanol (4:1). The residue was purified by HPLC (silica gel, EtOAc) to give **7** (6 mg, 17%): $[\alpha]^{24}_D$ −136° (*c* 0.38, $CHCl_3$); white powder; IR (film) 3319, 1734, 1653, 1635 cm^{-1}; ^{1}H NMR (300 MHz);[1] FABMS *m/z* 986 (M + H); HRFABMS calcd for $C_{51}H_{81}N_6O_{13}$ M_r 985.5861 (M + H), found 985.5871.

[(2*S*,3*R*,4*S*)-H$_2$Hip^3]Didemnin A (10). A solution of $NaBH_4$ (3.50 mg, 0.095 mmol) in THF–H_2O (1:1, 2 mL) was added dropwise to a solution of **2** (79.4 mg, 0.084 mmol) in THF (2 mL) at 0 °C. The mixture was stirred at 0 °C for 50 min and the temperature was elevated to room temperature over 2 h; HCl (1 N, 90 μL) was added, and the product was extracted with CH_2Cl_2. The organic layer was concentrated to give a solid (79.2 mg) which was chromatographed (silica gel, $CHCl_3$–MeOH, 6:1) to give nearly pure **10** (53.4 mg, 67%). A portion was purified by RPHPLC (MeOH–NaCl (0.4 M), 7:1): HRFABMS calcd for $C_{49}H_{81}N_6O_{12}$ M_r 945.5912 (M + H), found 945.5934.

[Hip^3 oxime]Didemnin B (11). To a solution of **1** (25.1 mg, 22.6 mmol) in CH_3OH (1 mL) was added NH_2OH–HCl (57.3 mg, 825 mmol) followed by $(C_2H_5)_3N$ (115 mL, 825 mmol). The solution was stirred at room temperature for 1 week and concentrated (N_2). The residue was chromatographed as above to give **11** (11.9 mg, 47%): ^{1}H NMR ($CDCl_3$, 500 MHz)[1] δ 7.70 (1H, d, J = 9.7 Hz), 7.67 (1H, d, J = 5.2 Hz), 7.40 (1H, d, J = 10.0 Hz), 6.09 (1H, d, J = 5.4 Hz, Hip-α); HRFABMS calcd for $C_{57}H_{91}N_8O_{15}$ M_r 1127.6604 (M + H), found 1127.6619.

Iododidemnin B (14). To a solution of **1** (5.5 mg, 0.005 mmol in CH_2Cl_2 0.2 mL) was added CF_3CO_2Ag (8.8 mg, 0.04 mmol) followed, dropwise, by a solution of I_2 (10 mg, 0.04 mmol in CH_2Cl_2, 0.2 mL). The suspension was stirred overnight at room temperature. Excess reagents were removed (filtration, Na_2SO_3 wash). The solvent was removed, and the crude product was purified by HPLC (C-18, MeOH–H_2O, 7:1) to give **14** (5.0 mg, 82%): ^{1}H NMR ($CDCl_3$)[1] δ 7.51 (1H, s), 7.15 (1H, d, J = 8.0 Hz), 6.77 (1H, d, J = 8.0 Hz), 3.87 (3H, s), 2.59 (3H, s, *N*-CH_3); HRFABMS calcd for $C_{57}H_{89}IN_7O_{15}$ M_r 1238.5461 (M + H), found 1238.5458.

[H$_6$Me$_2$Tyr^6]Didemnin B (15) and [H$_6$-*N*-MePhe^6]-Didemnin B (16). A mixture of **1** (16.3 mg, 0.015 mmol), Pt/C (10%, 38.3 mg), and TFA (20 μL) in CH_3OH (5 mL) was stirred under H_2 for 4 h at room temperature. The mixture was filtered through a C-18 Sep-pak with MeOH and concentrated to give a solid (33.1 mg), which was separated by HPLC (C-18, CH_3OH–0.4 M NaCl, 7:1) to give **15** (3.3 mg, 20%) as a white powder: $[\alpha]^{23}_D$ −29° (*c* 0.41, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz);[1] HRFABMS calcd for $C_{57}H_{96}N_7O_{15}$ M_r 1118.6964 (M + H), found 1118.7001.

Fraction 2 yielded **16** (5.2 mg, 32%) as a white solid: $[\alpha]^{24}_D$ −29° (*c* 0.41, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz);[1] HRFABMS calcd for $C_{56}H_{94}N_7O_{14}$ M_r 1088.6859 (M + H), found 1088.6902.

***N*$^\alpha$-Acetyldidemnin A (18)** was prepared from **2** as described:[3c] HRFABMS calcd for $C_{51}H_{81}N_6O_{13}$ M_r 985.5862 (M + H), found 985.5880.

***N*$^\alpha$-Propionyldidemnin A (19)** was prepared from **2** as described:[3c] HRFABMS calcd for $C_{52}H_{83}N_6O_{13}$ M_r 999.6018 (M + H), found 999.5985.

***N*$^\alpha$-*n*-Butyryldidemnin A (20).** To a solution of **2** (30 mg, 31 mmol) in dry CH_2Cl_2 was added *n*-butyric anhydride (10 mg, 0.63 mmol) at 0 °C followed by a catalytic amount (2 mg) of DMAP. The mixture was left at 0 °C for 48 h. EtOAc and aqueous $NaHCO_3$ were added, and the organic layer was dried (Na_2SO_4), concentrated, and separated to give **20** (27 mg, 86%): colorless solid, HRFABMS calcd for $C_{53}H_{84}N_6O_{13}$ M_r 1013.6185 (M + H), found 1013.6175.

***N*$^\alpha$-Acyl[pentanoyl (21), hexanoyl (22), octanoyl (23), dodecanoyl (24), hexadecanoyl (25)]didemnins. General Method.** EDC (0.31 mmol) was added at 10 °C to a stirred solution of the free acid (0.63 mmol) in CH_2Cl_2 (2 mL). The mixture was allowed to react for 1.5 h at 10 °C, **2** (0.31 mmol) was added to the solution, and the reaction mixture was stirred for 2 h at 10 °C and then left at −20 °C for 20 h. The mixed anhydride (prepared as above, 0.31 mmol) was added, and the reaction mixture was allowed to stand at 0 °C for 24 h. Solvent was evaporated, and the product after usual workup was

purified (SiO_2, $CHCl_3$−MeOH 3−5%) to give the correponding N^{α}-acyldidemnins.

N^{α}-Pentanoyldidemnin A (21): 90%; HRFABMS calcd for $C_{54}H_{87}N_6O_{13}$ M_r 1027.6331 (M + H), found 1027.6306.

N^{α}-Hexanoyldidemnin A (22): 90%; HRFABMS calcd for $C_{55}H_{89}N_6O_{13}$ M_r 1041.6488 (M + H), found 1041.6477.

N^{α}-Octanoyldidemnin A (23): 90%; HRFABMS calcd for $C_{57}H_{93}N_6O_{13}$ M_r 1069.6791 (M + H), found 1069.6785.

N^{α}-Dodecanoyldidemnin A (24): 89%; HRFABMS calcd for $C_{61}H_{101}N_6O_{13}$ M_r 1125.7427 (M + H), found 1125.7401.

N^{α}-Octadecanoyldidemnin A (25): 89%; HRFABMS calcd for $C_{67}H_{113}N_6O_{13}$ (M + H), found 1181.8040.

***N*-(D-Pro)didemnin A (29).** DCC (24.4 mg, 0.12 mmol) was added to a solution of Z-D-proline (Pro) (59 mg, 0.24 mmol) in CH_2Cl_2 (0.5 mL) at 5 °C. The mixture was stirred at 5 °C for 2 h. Compound **2** (75.4 mg, 0.08 mmol) in CH_2Cl_2 (0.5 mL) was added, and the mixture was allowed to stand for 8 h at 10 °C and then was concentrated. The residue was suspended in cold EtOAc, filtered, and concentrated *in vacuo* to an oil which was separated (silica gel, EtOAc−CH_2Cl_2, 7:3) to give *N*-(Z-D-Pro)didemnin A as a white powder (83.9 mg, 0.071 mmol, 89%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.9* (1H, m), 7.2−7.4 (5H, m), 7.05 (2H, d, *J* = 8.4 Hz), 6.82 (2H, d, *J* = 8.4 Hz), 3.76 (3H, s), 2.88*, 2.89*, 2.77* (s), 2.55*, 2.52* (s) (*peaks were observed as pairs); HRFABMS calcd for $C_{62}H_{92}N_7O_{15}$ M_r 1174.6651 (M + H), found 1174.6663.

A mixture of *N*-(Z-D-prolyl)didemnin A (80 mg, 0.077 mmol) and Pd/C (10%, 36 mg) in MeOH (2 mL) was stirred vigorously in a hydrogen atmosphere for 2 h at room temperature, filtered through a C-18 Sep-pak column with MeOH, and concentrated to a white powder (65.6 mg). A portion (17.0 mg) of the powder was separated by HPLC to give pure **29** (11.9 mg, 57%): white powder; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.97−7.3 (br NH's) 7.07 (2H, d, *J* = 8.4 Hz), 6.83 (2H, d, *J* = 8.4 Hz), 3.79 (3H, s), 2.92 (3H, brs), 2.53 (3H, s); HRFABMS calcd for $C_{54}H_{86}N_7O_{13}$ M_r 1140.6284 (M + H), found 1140.6285.

***N*-(L-Pro)didemnin A (28).**[3c] Z-L-Pro was coupled to **2** and deprotected as above to give a white powder (27% overall): ^{1}H NMR ($CDCl_3$, 300 MHz)[1] δ 8.03 (1H, d, *J* = 8.7 Hz), 7.73 (1H, br d, *J* = 9.6 Hz), 7.33 (1H, br s), 7.06 (2H, d, *J* = 8.4 Hz), 6.84 (2H, d, *J* = 8.4 Hz), 3.88 (3H, s), 3.02 (3H, s), 2.53 (3H, s); HRFABMS found 1040.6277.

***N*-(L-Leu)didemnin A (27).** A sample of **27** prepared previously[3c] was repurified by RPHPLC (MeOH−NaCl (0.4 M), 7:1).

[Acetyl^9]didemnin B (31). To a suspension of L-Pro (247 mg, 2.15 mmol) in pyridine (1 mL) was added acetic anhydride (1 mL), and the mixture was stirred at room temperature for 5 min. The solvent was removed *in vacuo*, and the resulting oil was partitioned between EtOAc and HCl (1 N). The organic layer was dried over Na_2SO_4 and concentrated, and the resulting solid was recrystallized from EtOAc to give *N*-Ac-L-Pro (167 mg, 44%), a white powder: mp 108 °C [lit. 115 °C (from $CHCl_3$)[43]]; $[\alpha]^{25}_D$ −171° (*c* 0.91, $CHCl_3$). Anal. ($C_7H_{11}NO_3$) C, H, N.

N-Ac-L-Pro (31 mg, 0.197 mmol) was treated with DCC (20.3 mg, 0.099 mmol) in CH_2Cl_2 (0.1 mL) for 4 h at 10 °C. A solution of **2** (62 mg, 0.068 mmol) in CH_2Cl_2−DMF (6:4, 1 mL) was added to the mixture at 5 °C. The mixture was allowed to stand at 5 °C for 12 h, filtered, and then concentrated *in vacuo*. The resulting solid was separated (silica gel, EtOAc−2-propanol, 10:1) to give **31** (63 mg, 0.058 mmol, 88%) as a white powder: ^{1}H NMR ($CDCl_3$, 500 MHz);[1] HRFABMS calcd for $C_{56}H_{88}N_7O_{14}$ M_r 1082.6389 (M + H), found 1082.6396.

[Propionyl^9]didemnin B (32). Propionic anhydride (2.60 g, 0.02 mol) was added to a suspension of L-Pro (1.15 g, 0.01 mol) in pyridine (2 mL). The mixture was stirred for 30 min at room temperature, solvent was removed *in vacuo*, and the product was recrystallized from EtOAc to give *N*-propionyl-L-Pro (1.60 g, 96%): colorless needles; mp 98−99 °C; $[\alpha]^{25}_D$ −186° (*c* 1.7, $CHCl_3$); ^{1}H NMR (360 MHz).[1] Anal. ($C_8H_{13}NO_3$) C, H, N.

N-Propionyl-L-Pro was coupled with **2** (26.4 mg, 0.028 mmol) as in the synthesis of **31** to give [propionyl^9]didemnin B (**32**) (28.7 mg, 93%) as a white powder: ^{1}H NMR ($CDCl_3$, 500 MHz);[1] FABMS *m/z* 1097 (M + H), 281; HRFABMS calcd for $C_{57}H_{90}N_7O_{14}$ M_r 1096.6556 (M + H), found 1096.6572.

[Isobutyryl^9]didemnin B (33) and [Isobutyryl^9,D-Pro^8]-didemnin B (34). L-Pro (1.15 g, 0.01 mol) was treated with isobutyric anhydride in a procedure like the *N*-propionylproline synthesis to give *N*-isobutyrylproline (1.8 g, 96%): fine crystals; mp 80−82 °C; $[\alpha]^{25}_D$ −8.7° (*c* 1.56, $CHCl_3$); ^{1}H NMR.[1] Anal. ($C_9H_{15}NO_3$) C, H, N.

N-Isobutyrylproline was coupled with **2** (26.4 mg, 0.028 mmol) using the method described earlier for the preparation of **31**. The product was separated (silica gel, EtOAc) to give **33** as the first fraction (8.7 mg, 28%), a white powder: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.11 (1H, d, *J* = 5.5 Hz), 7.89 (1H, d, *J* = 9.0 Hz), 7.21 (1H, d, *J* = 10.0 Hz), 7.06 (2H, d, *J* = 8.5 Hz), 6.84 (2H, d, *J* = 8.5 Hz), 3.79 (3H, s), 3.12 (3H, s), 2.54 (3H, s); FABMS *m/z* 1112 (M + H), 295; HRFABMS calcd for $C_{58}H_{92}N_7O_{14}$ M_r 1110.6302 (M + H), found 1110.6737.

The second fraction gave **34** (13 mg, 42%): colorless needles: mp 162−164 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.15 (1H, d, *J* = 6.0 Hz), 7.92*, 7.87* (1H, d, *J* = 9.0 Hz), 7.26*, 7.11* (1H, d, *J* = 10.0 Hz), 7.06 (1H, d, *J* = 9.0 Hz), 6.94 ($^1/_4$H, d, *J* = 9.0 Hz), 6.84 (2H, d, *J* = 8.5 Hz), 3.78 (3H, s), 2.86 (3H, s), 2.54, 2.53 (3H, s) (*peaks observed as pairs); FABMS *m/z* 1110 (M + H), 295; HRFABMS calcd for $C_{58}H_{92}N_7O_{14}$ M_r 1110.6302 (M + H), found 1110.6726.

***O*-Benzyl-L-lactyl-L-Ala Methyl Ester.** A mixture of *O*-benzyl-L-lactic acid[23] (57.7 mg, 0.35 mmol), L-alanine methyl ester hydrochloride (50.0 mg, 0.36 mmol), *N*-hydroxysuccinimide (82 mg, 0.70 mmol), and NMM (35 mg, 0.35 mmol) in CH_2Cl_2−DMF (6:4, 2 mL) was stirred at −10 °C. A solution of DCC (100 mg, 0.49 mmol) and DMAP (2 mg) in CH_2Cl_2−DMF (6:4, 2 mL) was added to the mixture, which was allowed to warm from −10 °C to 4 °C over 2 h and then stood at 4 °C for 30 h. The reaction mixture was concentrated *in vacuo*, and the resulting product was suspended in cold EtOAc, filtered, and separated (silica gel, EtOAc) to give *O*-benzyl-L-lactyl-L-Ala methyl ester as a light yellow oil (81.7 mg, 94%, HPLC data): $[\alpha]^{20}_D$ −20° (*c* 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz);[1] ^{13}C NMR ($CDCl_3$,75 MHz);[1] HRFABMS calcd for $C_{14}H_{19}NO_4$ M_r 266.1392 (M + H), found 266.1398.

***O*-Benzyl-L-lactyl-L-Ala.** Aqueous KOH (1 N, 300 μL) was added to a solution of *O*-benzyl-L-lactyl-L-Ala methyl ester (62 mg, 0.25 mmol) in dioxane. The mixture was stirred for 12 h at room temperature, HCl (1 N, 300 μL) was added, and the solvent was removed *in vacuo*. The residue was suspended in CH_2Cl_2, filtered, and concentrated *in vacuo* to give *O*-benzyl-L-lactyl-L-Ala as a light yellow oil (56.9 mg, 98%): $[\alpha]^{26}_D$ −18.8° (*c* 2.11, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz);[1] HRFABMS calcd for $C_{13}H_{17}NO_4$ M_r 252.1236 (M + H), found 252.1238.

***O*-Benzyl[L-Ala^8]didemnin B.** A solution of *O*-benzyl-L-lactyl-L-Ala (22.6 mg, 0.095 mmol) and *N*-hydroxysuccinimide (13.1 mg, 1.2 mmol) in CH_2Cl_2 (1 mL) was added to a solution of DCC (21.5 mg, 0.10 mmol) in CH_2Cl_2 (0.5 mL) at −10 °C. The mixture, which became a heterogeneous emulsion, was stirred for 1 h at −10 °C. Compound **2** (81.4 mg, 0.086 mmol) and NMM (9.0 mg, 0.088 mmol) were added, and the reaction mixture stood at −10 °C for 3 h and then at 4 °C for 24 h. A catalytic amount of DMAP (1 mg) was added to the mixture, and the reaction stood for 16 h at 4 °C and then was concentrated. The residue was suspended in EtOAc, filtered, and concentrated *in vacuo* to give an oil, which was chromatographed on a silica gel column, eluting with $CHCl_3$−MeOH (15:1), to give *O*-benzyl[L-Ala^8]didemnin B (62 mg, 62%) as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$);[1] FABMS *m/z* 1176 (M + H), 361; HRFABMS calcd for $C_{62}H_{94}N_7O_{15}$ M_r 1176.6808 (M + H), found 1176.6814.

[L-Ala^8]Didemnin B (35). A mixture of *O*-benzyl[L-Ala^8]-didemnin B (53.2 mg, 0.045 mmol) and Pd/C (10%, 50 mg) in MeOH (2 mL, containing 100 μL of acetic acid) was vigorously stirred in an H_2 atmosphere for 3 h at room temperature. To the mixture was added 10 mg of $NaHCO_3$, and the reaction mixture was filtered through a C-18 Sep-pak column with MeOH and concentrated to give **35** (TLC, one spot; 47.6 mg, 97%). A portion of **35** was further purified for bioassay by HPLC (C-18, MeOH−H_2O, 7:1): IR (film) 3330, 1734, 1637, 1248 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz);[1] ^{13}C NMR ($CDCl_3$, 300

MHz);[1] FABMS *m/z* 1087 (M + H), 271; HRFABMS calcd for $C_{55}H_{88}N_7O_{15}$ M_r 1086.6338 (M + H), found 1086.6359.

***O*-Benzyl-L-lactyl-D-Pro Methyl Ester.** A mixture of *O*-benzyl-L-lactic acid (194.4 mg, 1.08 mmol) and DCC (111.2 mg, 0.54 mmol) in CH_2Cl_2 (1 mL) was stirred at 0 °C for 30 min. D-Pro-OMe·HCl (53.1 mg, 0.32 mmol) and NMM (33.0 mg, 0.33 mmol) in DMF (~1 mL) were added, and the mixture stood at 4 °C for 9 h. The product was filtered, concentrated *in vacuo*, suspended in cold EtOAc, filtered, and concentrated *in vacuo* to an oil. The crude product was separated (silica gel, EtOAc) to give *O*-benzyl-L-lactyl-D-Pro methyl ester (47.6 mg, 44%) as a colorless oil: $[\alpha]^{26}{}_D$ +1.42° (*c* 1.83, $CHCl_3$); IR (film) 1736, 1639, 1450, 1200, 1170 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz);[1] ^{13}C NMR ($CDCl_3$, 300 MHz);[1] HRFABMS calcd for $C_{16}H_{22}NO_4$ M_r 292.1549 (M + H), found 292.1550.

***O*-Benzyl-L-lactyl-D-Pro.** A solution of *O*-benzyl-L-lactyl-D-Pro methyl ester (124.7 mg) in dioxane (1 mL) and aqueous KOH (1 N, 0.5 mL) stood at room temperature for 20 h. The mixture was concentrated to give an aqueous emulsion, which was acidified to pH 2 (HCl), extracted with CH_2Cl_2, dried (Na_2SO_3), and evaporated to give an oil (117.9 mg, 100%): IR (film) 3400–2500 br, 1736, 1640 cm^{-1}; HRFABMS calcd for $C_{15}H_{20}NO_4$ M_r 278.1392 (M + H), found 278.1394.

***O*-Benzyl[D-Pro^8]didemnin B.** A mixture of *O*-benzyl-L-lactyl-D-Pro (52.4 mg, 0.19 mmol) and DCC (19.6 mg, 0.095 mmol) in CH_2Cl_2 (1 mL) was stirred at 0 °C for 2 h. A solution of **2** (59.7 mg, 0.063 mmol) in CH_2Cl_2 (1 mL) was added, and the reaction mixture was allowed to stand at 0 °C for 12 h and concentrated *in vacuo*. The residue was suspended in EtOAc and filtered, and the product was separated (silica gel, EtOAc) to give *O*-benzyl[D-Pro^8]didemnin B (61.9 mg, 83% based on unreacted **2**) as a white solid: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.94* (1H, d × 2, 6.9, 6.3), 7.22–7.42 (5H, m), 7.13 (1H, d, J = 9.9 Hz), 7.07 (2H, d, J = 8.7 Hz), 6.95 (1H, d, J = 9.0 Hz), 3.79 (3H, s), 2.95 and 2.88* (3H, s), 2.56 and 2.55* (3H, s) (*appearing as pairs of signals due to conformers); HRFABMS calcd for $C_{64}H_{96}N_7O_{15}$ M_r 1202.6664 (M + H), found 1202.6671.

[D-Pro^8]Didemnin B (36). A mixture of *O*-benzyl[D-Pro^8]-didemnin B (43.1 mg, 0.036 mmol) and Pd/C (10%, 40 mg) in MeOH (2 mL) and acetic acid (20 mL) was stirred under hydrogen for 2.5 h at room temperature. To the mixture was added 10 mg of $NaHCO_3$, and the product was filtered and concentrated *in vacuo* to a glass which was purified by HPLC (C-18, MeOH–H_2O, 7:1) to give pure **36** (39 mg, 97%) as a white powder: IR (film) 3420, 3330, 1732, 1635, 1539, 1248, 1176 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz, mixture of conformers, Figure 1) δ 8.62:8.57 (3:1, 1/3H, d's, J = 6.5 Hz), 7.93, 7.92, 7.86, 7.83 (5:1:4:2, 1H, d's, J = 9.5 Hz), 7.29:7.14 (1:1, 1H, d's, J = 10.0 Hz), 7.07 (2H, d, J = 8.5 Hz), 6.97:6.85 (1:4, 2/1H, J = 9.5 Hz), 6.84 (2H, d, J = 8.5 Hz), 5.19:5.16 (2:3, 1H, d's, J = 3.5 Hz, Hip H-4), 3.79 (3H, s, Me_2Tyr-OCH_3), 2.94:2.93:2.89:2.88 (5:2:4:1, 3H, singlets), 2.56:2.54 (2:3, 3H, singlets); HR-FABMS calcd for $C_{57}H_{90}N_7O_{15}$ M_r 1112.6491 (M + H), found 1112.6493.

***O*-Pyroglutamyldidemnin B (37).** A mixture of **1** (230 mg, 0.21 mmol) L-pyroglutamic acid (134 mg, 1.04 mmol) in DMF (5 mL), DCC (206 mg, 1.00 mmol), and DMAP (6 mg) was stirred for 20 h at room temperature. Water (50 mL) and CH_2Cl_2 (50 mL × 3) were added to the reaction mixture, and the organic layer was concentrated *in vacuo*. The resulting solid was separated (silica gel column, EtOAc–2-propanol,10:1) to give recovered **1** (77 mg, 33%) and **37** (135 mg, 53% conversion) as a white powder: IR (film) 3390, 1730, 1651, 1252 cm^{-1}; ^1H NMR (300 MHz);[1] FABMS *m/z* 1224 (M + H), 1113, 447, 275, 195; HRFABMS calcd for $C_{62}H_{95}N_8O_{17}$ M_r 1223.6384 (M + H), found 1223.6365.

Acknowledgment. This work was supported in part by grants from the National Institute of Allergy and Infectious Diseases (AI04769 to K.L.R.) and the National Institute of General Medical Sciences (GM27029 to K.L.R.). *In vivo* testing was carried out by Mr. Joseph Swiniarski, A. D. Little and Co., Boston, MA.

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(1) Data in this paper are taken in part from the following: Sakai, R. Biologically Active Compounds from Tunicates and a Sponge. Ph.D. Thesis, University of Illinois, Urbana, 1991.

(2) Preparation and some bioactivity of these and some other congeners were listed in the following: Rinehart, K. L., Jr. Pharmaceutical Compositions Containing Didemnins. U.S. Patent 5,294,603, March 15, 1994; *Chem. Abstr.* **1994**, *121*, 887. Either the threonine or the isostatine unit was assigned as unit[1] in our previous publications. In this paper, we use [Thr^1].

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JM960048G