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DIDEMNINS AND TUNICHLORIN: NOVEL NATURAL PRODUCTS FROM THE MARINE TUNICATE *TRIDIDEMNUM SOLIDUM*[1]

KENNETH L. RINEHART,* VIMAL KISHORE, KEITH C. BIBLE, RYUICHI SAKAI, DAVID W. SULLINS, and KAI-MING LI

Roger Adams Laboratory, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

ABSTRACT.—Didemnins A, B, and C, cyclic depsipeptides previously reported by our laboratory from the Caribbean tunicate *Tridemnum solidum*, have now been synthesized and, in the process, their structures have been revised to include the new amino acid (3S,4R,5S)-isostatine, $C_2H_5CH(CH_3)CHNH_2CHOHCH_2COOH$. All other isomers of isostatine have also been prepared.

Didemnin B is currently in Phase II clinical trials as an anticancer agent, and the antineoplastic activity, as well as the antiviral and immunosuppressive activities, of the didemnins are reviewed.

A highly unusual porphynoid has also been isolated from *T. solidum*. The compound has been named tunichlorin and is apparently the first nickel-containing chlorin (and only the second nickel-containing porphyrin-related compound) identified from a living organism.

Recent years have provided a remarkable number and variety of biologically active marine natural products. This explosion of pharmacologically interesting marine-derived substances has been stimulated not only by advances in chemical instrumentation but also by an extension of the sphere of collection of marine organisms for testing. The Gulf of California and the Caribbean have now been explored thoroughly, and an increasing number of reports are appearing of bioactive materials from marine species collected in the Western Pacific (Okinawa, Australia, New Zealand, Palau, etc.) as well (1,2).

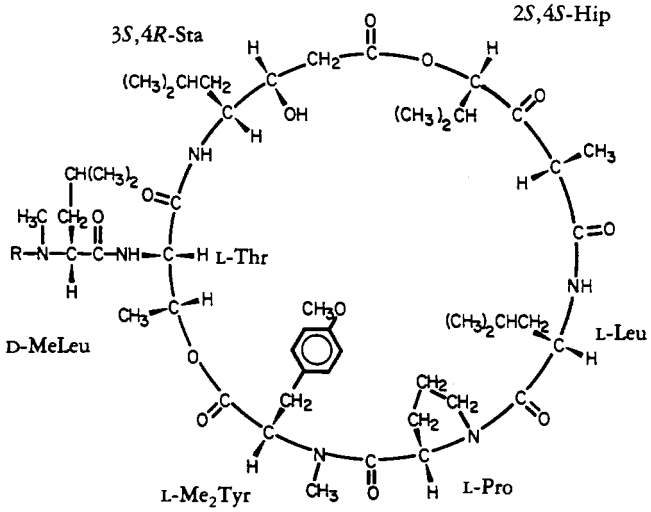
In addition to this horizontal expansion of the collecting sphere, a vertical extension has taken place. It is now possible to collect not only from intertidal areas by wading and snorkeling and from depths to 100 feet by scuba but also routinely from depths to 3000 feet by using manned submersibles and remotely controlled vehicles (3).

The original predictions of clinically useful compounds isolated from marine plants and animals (4) seem justified now that a number of such materials appear to be candidates for early clinical trials (5,6). At present, however, only one marine-derived compound is undergoing such testing (7); that compound, didemnin B, will be the main subject of this discussion.

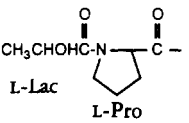
BIOLOGICAL ACTIVITY OF THE DIDEMNINS.—Didemnin B is one of a number of related compounds isolated from the Caribbean tunicate *Tridemnum solidum* Van Name (Didemnidae). The activity of this tunicate first came to our attention in 1978, during the National Science Foundation-sponsored expedition of the *R/V Alpha Helix* in the Western Caribbean (AHCE 1978) (8). The organism in question, a leathery compound tunicate overgrowing corals, sponges, gorgonians, algae, and so forth, proved to be strongly antiviral in on-site assays carried out by Dr. R.G. Hughes, Jr. (Roswell Park Memorial Institute, Buffalo, New York), whether it was collected in Panamanian, Colombian, Honduran, Belizean, or Mexican waters. As a result of the observations of potent activity in the field, larger quantities of the tunicate were collected for chemical studies to be carried out at the University of Illinois in Urbana.

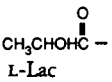
[1]Presented as a plenary lecture (by K.L.R.) at "The Search for New Drugs from Natural Sources" Symposium of the 28th Annual Meeting of the American Society of Pharmacognosy at the University of Rhode Island, Kingston, Rhode Island, July 19-22, 1987.

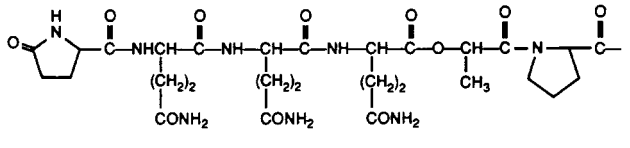
There, the antiviral materials in *T. solidum* were found to consist mainly of five components, didemnins A to E, whose structures were assigned as **1a-5a**, respectively (9, 10).

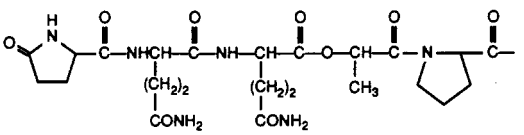


1a Didemnin A: R=H

2a Didemnin B: R= 

3a Didemnin C: R= 

4a Didemnin D: R= 

5a Didemnin E: R= 

The simplest of the five is didemnin A, in which the side chain is terminated by an *N*-methyl-D-leucine unit. While some of the units assigned to the cyclic depsipeptide were standard amino acids (L-Thr, L-Leu, L-Pro), others were methylated (D-MeLeu, *N,O*-dimethyl-L-tyrosine), and still others were unique [the γ-amino acid statine (Sta) and the β-keto acid hydroxyisovalerylpropionic acid (Hip)]. In the other didemnins shown, the *N*-methylleucine chain terminus is extended by substitution with lactic acid (didemnin C), lactyl-proline (didemnin B), or pyroglutamyl-(glutaminyl)$_n$-lactyl-proline (didemnins D and E).

A remarkable feature of the didemnins is the considerable variability in biological activity, depending upon the particular side chain found. For example, in quantitative testing at The Upjohn Company, Kalamazoo, Michigan, by Dr. H.E. Renis, didemnin B proved to be approximately ten times as active as didemnin A (8, 11-13). The in vitro tests against DNA viruses were followed by in vivo tests in a mouse vaginal *Herpes* model, where didemnin B again proved to be far more active than didemnin A (13-15). At optimal dosage didemnin B was able to save approximately 85% of the mice from what for them is otherwise a lethal infection (Figure 1). Similar results were obtained in tests against RNA viruses (16, and unpublished observations by Dr. P.G. Canonico, USAMRIID, Fort Detrick, Maryland), most dramatic being those against the lethal viruses for Rift Valley fever (RVF), Venezuelan equine encephalomyelitis (VEE), yellow fever (YF), sandfly fever (SF), and a Pichinde virus (PICH) (Figure 2), where didemnin B was effective for the most part at approximately 0.05 μg/ml; comparable effects using ribavirin required 250 μg/ml. Here, too, in vivo activity was demonstrated against RVF virus in mice, where 90% of the mice could be saved at the optimal dosage. Unfortunately for viral therapy, the drug is toxic, and slightly higher doses of didemnin B were lethal to the mice.

At the same time that the antiviral activity of the *T. solidum* extract was noted on shipboard, pronounced cytotoxicity was observed in the form of cell killing of monkey kidney (CV-1) cells (8). This activity, too, was confirmed as cytotoxicity to L1210 leukemia cells in testing by Dr. L.H. Li at Upjohn, and when the pure didemnins became available, they were tested (11, 13, 17, 18). As in its antiviral activity, didemnin B was the most active of the three original didemnins (A, B, and C), showing potent

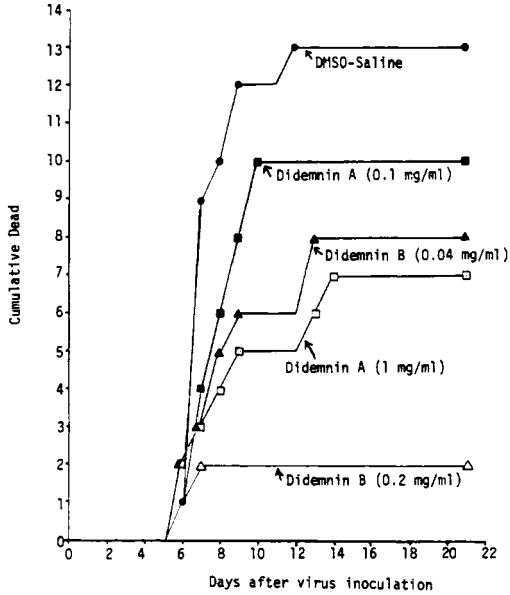


FIGURE 1. Protection of female mice from genital *Herpes simplex* virus, type 2 (HSV-2) infection by intravaginal treatment with didemnins A and B. Mice were inoculated with 9.0×10^4 PFU (plaque-forming units)/0.1 ml HSV-2 and treated intravaginally with 0.1 ml drug 3× per day for 3 days beginning 1 h after virus inoculation; 14 mice in each group (12).

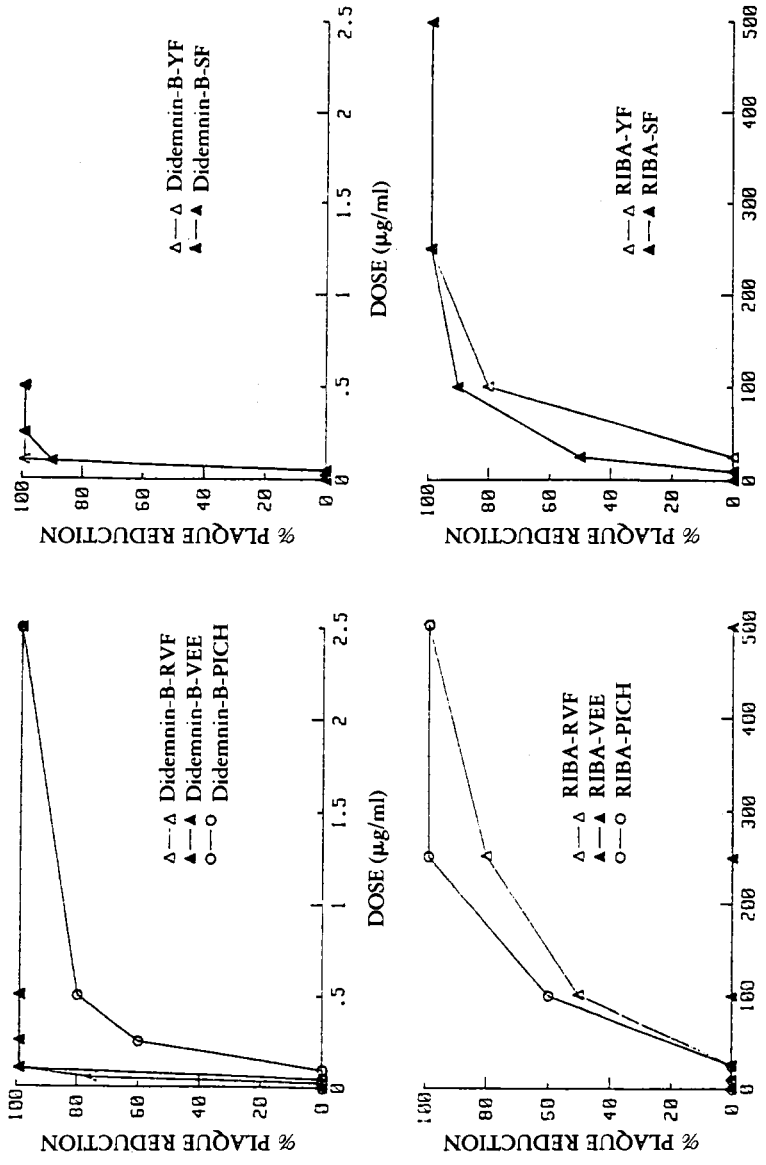


FIGURE 2. Comparison of in-vitro plaque-reduction activities of didemnine B and ribavirin (RIBA) vs. Rift Valley fever virus (RVF), Venezuelan equine encephalomyelitis virus (VEE), a Pichinde virus (PICH), yellow fever virus (YF), and sandfly fever virus (SF). Vero cells were used for RVFV, VEE, and PICH, while YF virus was assayed in MK_2 cells. Drug solutions (0.3 ml) of appropriate concentrations were added to 24-well tissue culture trays, followed immediately by 0.1 ml of virus [50-200 PFU (plaque-forming units)] (P.G. Canonico *et al.*, USAMRIID, Fort Detrick, MD, unpublished observations).

cytotoxicity, ID_{50} 0.002 μg/ml. When didemnins A and B were tested in vivo, didemnin B was found to extend the lifetime of P-388 leukemia-infected mice by as much as 100% in an appropriate dose regimen (Table 1). Didemnin B could also extend the lifetime of mice infected with B16 melanoma by up to 60% (T/C 160). These impressive activities were disclosed to the National Cancer Institute (NCI) where the activity of didemnin B had been discovered independently; a compound of unknown structure isolated by Dr. A.J. Weinheimer, at that time at the University of Oklahoma, was being tested by the NCI and later turned out to be identical with didemnin B.

TABLE 1. Antitumor Activities of Didemnin B in vivo.[a]

Schedule of administration	Dose (mg/kg/ injection)	P-388 Leukemia		B16 Melanoma	
		T/C[b] (%)	Body weight change (g)	T/C[c] (%)	Body weight change (g)
Days 1-9, ip	0.03			135	+0.8
	0.06	143	+0.1	144	−0.4
	0.25	138	−2.2	157	−3.3
	0.5	T[d]	−3.4	T	−4.5
	1	T	T		
Days 1,5,9, ip	0.03	125	+1.6		
	0.06	140	+1.2	139	+1.2
	0.25	157	−0.5	155	+0.3
	0.5	176	−1.7	146	−1.3
	1	199	−3.8	160	−3.2

[a]Tumor was inoculated (iv) at 10^6 cells/mouse.
[b]Median death of untreated animals inoculated (ip) with P-388 leukemia = 10.2 days.
[c]Median death of untreated animals inoculated (ip) with B16 melanoma = 19.4 days.
[d]Toxic (13,14).

Ultimately, didemnin B proved to be sufficiently active that the NCI decided to carry out clinical trials on the compound (7), employing a sample isolated in our laboratory from ca. 250 kg of tunicate. Toxicity studies in mice, rats, and dogs showed a number of different dose-related toxicities, but these were reversed on discontinuation of treatment. In Phase I clinical trials in humans, no unexpected toxic manifestations were observed, and the dose-limiting adverse effects of didemnin B were nausea in one study and nausea accompanied by reduced liver function in the other (19,20). Currently, didemnin B is at the beginning of Phase II clinical trials, which are expected to last at least one year.

A third impressive activity of didemnin B, immunosuppression, was discovered somewhat later by Drs. D.W. Montgomery and C.F. Zukoski at the University of Arizona, Tucson (21-23). In standard tests for immunosuppression of T- and B-cell mitogenesis and in the mixed lymphocyte reaction, didemnin B proved exceedingly potent in vitro (Table 2); it was perhaps a thousand times more active than the standard immunosuppressive agent cyclosporin A, although it was less selective in vitro than cyclosporin A. In an in vivo graft-versus-host (GVH) reaction involving the transfer of spleen cells from one mouse strain to another, didemnin B also showed inhibition of splenomegaly that was clearly dose-related (Figure 3). Moreover, preliminary studies of rat heart auxiliary grafts by Dr. L.E. Stevens at LDS Hospital, Salt Lake City, Utah, showed rejection of the heart grafts to be delayed by up to 80% when the rats were treated with didemnin B. Thus, there are at least three major activities of didemnin B that warrant further study, with work on the antitumor activity being most advanced.

TABLE 2. Comparison of Immunosuppressive Drug Potency in vitro.[a]

Assay	Azathioprine	Cyclosporin A	Steroids	Didemnin B[b]
T-cell mitogenesis	rats: 2-8 μg/ml baboon: 0.4-0.6 μg/ml human: 9 μg/ml	human: 0.28 μg/ml guinea pig: 0.046 μg/ml	human: 0.05-0.16 μg/ml	mice: 0.00005 μg/ml
B-cell mitogenesis	human: 1 μg/ml	no effect	none-increase	mice: much less than 0.0001 μg/ml
Mixed lymphocyte reaction	human: 3 μg/ml baboon: 2.9 μg/ml	human: 0.2-0.3 μg/ml	human: 0.12 μg/ml	mice: less than 0.00001 μg/ml
Protein synthesis	human: 85 μg/ml	untested	human: 5μg/ml	mice: 0.19 μg/ml
RNA synthesis	no effect	untested	yes, no IC_{50} reported	no inhibition

[a]Table provided by D.W. Montgomery, 1984. IC_{50} values derived from published reports of in vitro studies; where differing values were available, the lowest are listed.

[b]D.W. Montgomery and C.F. Zukoski, unpublished observations.

SYNTHESES OF DIDEMNINS AND THEIR ANALOGUES.—It has been clear from the beginning that the didemnin molecule provides examples of striking structure-activity relationships. In most cases, didemnin B is about ten times more active than didemnin A, the difference between the molecules lying in acylation of the *N*-methyl-D-leucine unit of didemnin A. A number of simpler examples of *N*-acyl didemnin A derivatives (*N*-acetyl, *N*-propionyl, *N*-prolyl, *N*-leucyl) have now been prepared, and these synthetic derivatives show generally enhanced antiviral activity and cytotoxicity relative to didemnin A. On the other hand, *N,O*-diacetyl didemnin A showed enhanced antiviral activity relative to cytotoxicity, and a similarly improved antiviral/cytotoxicity ratio was seen for the formaldehyde adduct, with a methylene bridging the nitrogens of the *N*-methyl-D-leucine and threonine units. Reduction of the keto group of the Hip unit reduces both antiviral activity and cytotoxicity dramatically.

In the samples of *T. solidum* studied by us and collected in the western Caribbean, didemnin A is the most abundant component, didemnin B is less abundant, and didemnin C is present in trace amounts. Consequently, we set the conversion of didemnin

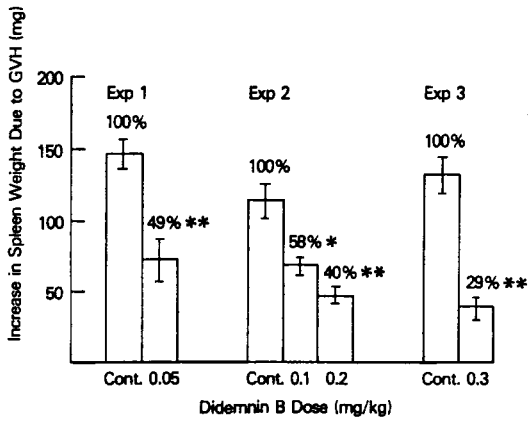
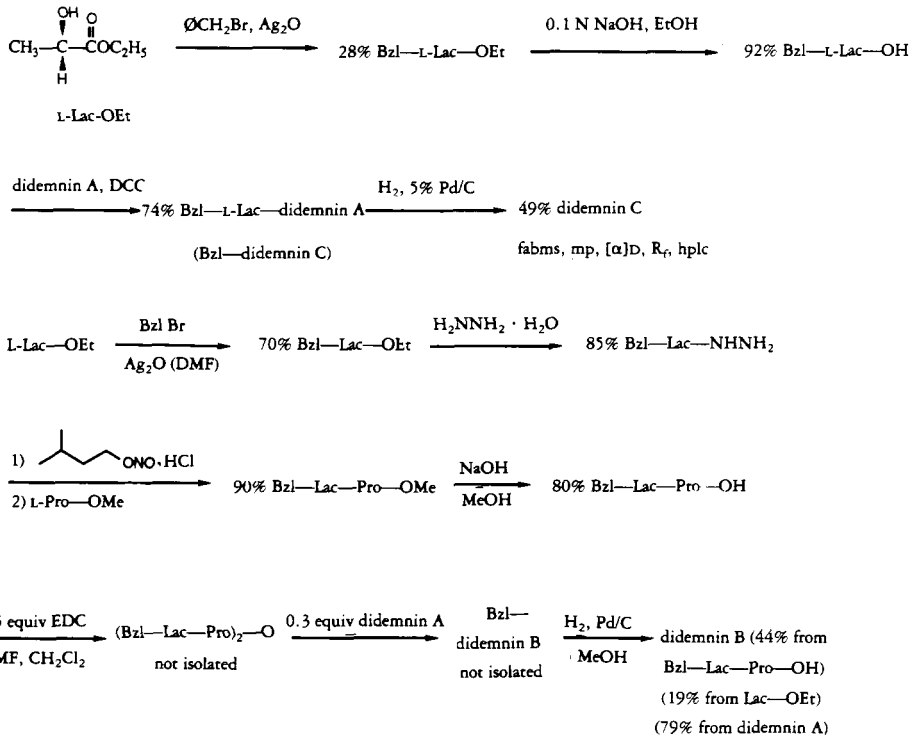


FIGURE 3. Effects of didemnin B on in-vivo graft-versus-host (GVH) reaction (D.W. Montgomery and C.F. Zukoski, University of Arizona, Tucson, unpublished observations).

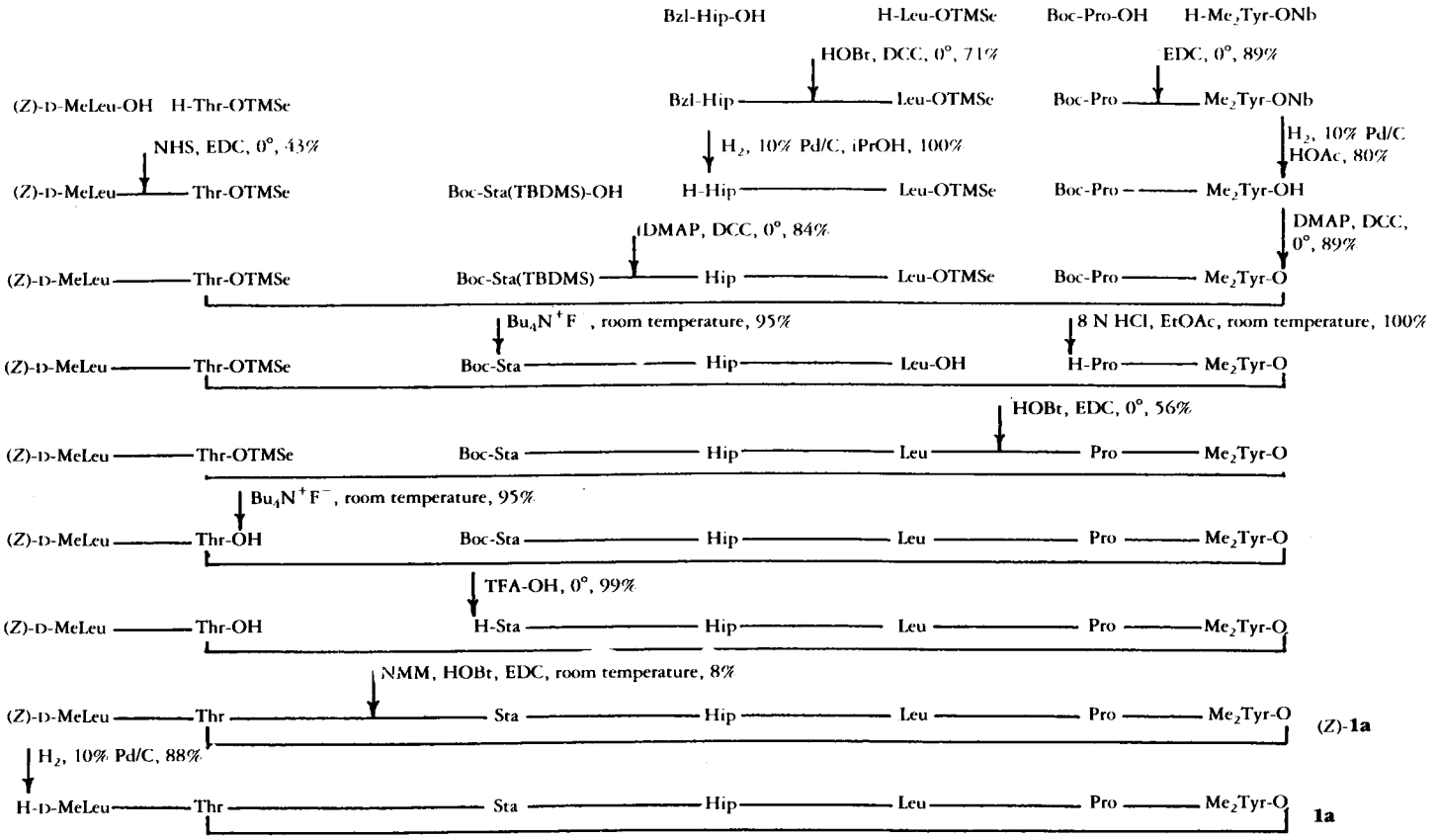
A to didemnin C and, especially, to didemnin B as major goals, and some four years ago semi-synthetic didemnins C and B were obtained in good yields by the routes shown in Scheme 1; the semi-synthetic products were identical with the natural materials in physical properties and bioactivities. Successful conversion of didemnin A to didemnin B increased the availability of didemnin B by approximately 100%, and the syntheses, involving L-lactic acid, established incidentally the stereochemistry of the lactic acid units in didemnins C and B.



SCHEME 1. Routes to semi-synthetic didemnins B and C (24; 1984 International Chemical Congress of the Pacific Basin Societies, Honolulu, HI, December 16-21; Paper 10E01).

Having completed the partial syntheses of didemnins B and C, we turned to a total synthesis of didemnin A, which would also constitute a total synthesis of didemnins B and C. The synthesis of didemnin A (Scheme 2) (24) was designed to introduce the rarest and most expensive subunits, statine and hydroxyisovalerylpropionic acid (Hip), as late as possible in the synthesis. Of course those two units themselves had to be synthesized, statine by established procedures (25,26) and Hip by acylation of a propionic acid derivative; the latter was completed by Dr. S. Nagarajan some three years ago in a preparation that also established the stereochemistry of that compound (10,27). In addition to statine and Hip, two simpler subunits had to be prepared, both by known procedures, *N*-methyl-D-leucine (28) and *N,O*-dimethyltyrosine (29).

A second consideration in the synthesis was the strong tendency of Hip to cyclize to the corresponding tetronic acid. It can, however, be stabilized as a β-keto amide, and it was, therefore, converted immediately after its introduction in the synthesis to an amide (Scheme 2). Because the β-keto amide enolizes readily, the stereochemistry at the 2-position of Hip was impossible to predetermine, and both stereoisomers, the Hip and 2-*epi*-Hip derivatives, were carried forward in the synthesis. The plan was to separate the two stereoisomers at the end, after cyclization.



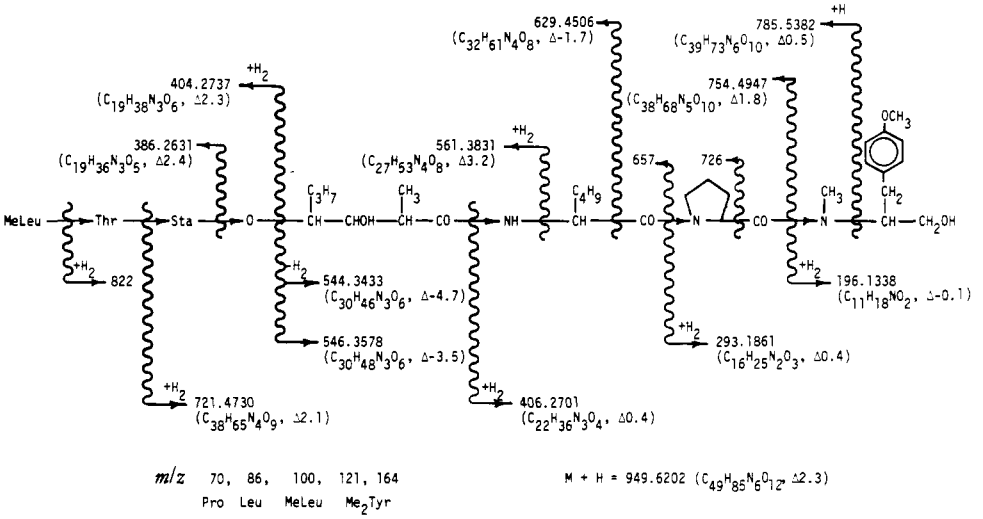
SCHEME 2. Synthetic route to **1a**.

Abbreviations: Bzl, benzyl; TMSe, β-trimethylsilylethyl; Boc, *t*-butyloxycarbonyl; Nb, *p*-nitrobenzyl; *Z*, benzyloxycarbonyl; TBDMS, *t*-butyldimethylsilyl; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; DMAP, 4-dimethylaminopyridine.

The final product of the coupling was to be benzyloxycarbonyldidemnin A, (Z)-did-A, which was then to be hydrogenolyzed to didemnin A. Natural didemnin A was converted to natural (Z)-did-A, and initial comparisons of the two products at that stage indicated that the synthetic (Z)-did-A thus prepared and natural (Z)-did-A were the same; both gave identical fab mass spectra, and their retention times on a C-18 reversed-phase column were identical; that is, they coeluted. In addition to the coeluting isomer, a second isomer, presumed to be the 2-*epi*-Hip isomer, was observed at a somewhat longer retention time.

The first indication that the synthetic material was not, in fact, identical with the natural material arose from a cd study carried out by M.D. Prairie at Upjohn when the coeluting isomer showed cd behavior somewhat different from that of the natural material. More importantly, in our hands normal phase hplc demonstrated that the two synthetic purported (Z)-did-A isomers both had retention times different from that of natural (Z)-did-A, and the ^{1}H-nmr spectra, though complicated, also showed slight differences. Thus, the material synthesized according to Scheme 2 was not authentic natural didemnin A.

REINVESTIGATION OF THE STRUCTURE OF DIDEMNIN A.—Faced with this lack of identity, one can presume either that the synthesis had gone awry or that the structure assignment of the natural material was incorrect. We ascertained first that the conversion of natural didemnin A to (Z)-didemnin A was not in question because natural (Z)-didemnin A could be reconverted to identical (natural) didemnin A on hydrogenolysis. We then reinvestigated the structure originally assigned to didemnin A. That the sequence of subunits was not in question was demonstrated readily by the fabms of a material obtained by borohydride reduction of didemnin A (Scheme 3). That spectrum gave the fragments expected at every position to confirm clearly the correctness of the sequence.



SCHEME 3. Mass spectral fragmentations (+fabms) of hexahydrodidemnin A (10).

Attention was then directed to identification of the individual subunits. Because Hip had been synthesized and compared with authentic cyclized Hip, the subunits in question were the amino acids. These had been identified by Dr. J.B. Gloer (14) by gc-ms on a packed OV-17 column (Figure 4), and each of the subunits had been shown to coelute with authentic samples. Moreover, on a chiral column the stereochemistry of proline, leucine, and threonine had been established clearly as L, while *N*-methyl-D-

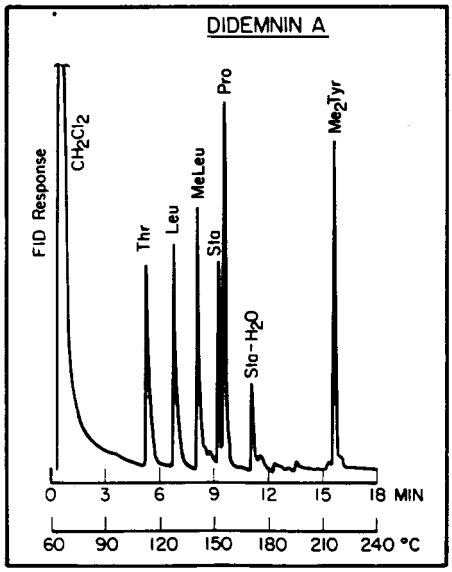


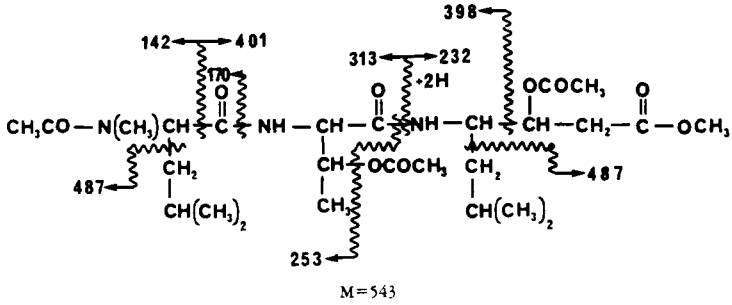
FIGURE 4. Gc traces of derivatives of amino acids from hydrolysis of didemnin A (14).

leucine and *N,O*-dimethyl-L-tyrosine derivatives had been isolated and their rotations compared with those of authentic samples. In the case of statine, authentic 3*S*,4*R* and 3*R*,4*R* samples both gave relatively complex patterns due to dehydration, but the 3*S*,4*R* sample had a pattern on the packed column that matched far more closely that observed for the natural statine from didemnin A.

In a reinvestigation of the retention times of the *N*-trifluoroacetyl butyl ester derivatives of the component amino acids using a chiral capillary column, the L-threonine, *N*-methyl-D-leucine, L-leucine, L-proline, and *N,O*-dimethyl-L-tyrosine subunits were again shown to coelute with authentic samples, but the "statine" derived from hydrolysis of didemnin A did not coelute with any of the four authentic stereoisomers of statine. Consequently, the structure assigned "statine" appeared to be incorrect.

The original assignment was based on the gc-ms fragmentation pattern of the statine derivative [gc-ms *m/z* 423 (M, ci), 310 (ci), 236 (ei), 208 (ei), 182 (ei)] (8), and the location of the hydroxyl and amino groups appeared to be certain, although fragmentation was lacking in the alkyl group. We turned then to a "statine"-containing tripeptide obtained by base hydrolysis of didemnin A followed by acetylation. This tripeptide derivative was shown to contain methylleucine, threonine, and "statine" and had been used earlier in the sequence assignment of didemnin A, as shown in Scheme 4, where the mass spectral fragmentation is indicated. Careful examination of the ^{1}H-nmr spectrum of this tripeptide derivative showed that the methyl region (Figure 5) contained not only the expected doublets but also a triplet at 0.928 ppm, indicating an ethyl group in the molecule that could be associated only with the "statine" component. Thus, it appeared that didemnin A contained not a "statine" unit but an "isostatine" unit, derived biogenetically from isoleucine plus acetate, rather than from leucine plus acetate.[2]

[2]These results assigning the isostatine unit were reported at the 10th Peptide Symposium (St. Louis, MO, May 23-29, 1987, Paper LTh24), where a French group described their similar conclusion about the structure of a didemnin isolated from *Tridemnum cyanophorum* (B. Castro, P. Jouin, A. Cavé, M. Dufour, B. Banaigs, and C. Francisco, Paper P-292).



SCHEME 4. Mass spectral fragmentation (cims) of the tripeptide (MeLeu→Thr→"Sta"), derivative obtained from didemnin A (J.B. Gloer, "Structures of the Didemnins," Ph.D. Thesis, Urbana, IL: University of Illinois, 1983).

The isostatine structure, of course, has three asymmetric centers and, thus, exists as one of eight stereoisomers rather than as one of four stereoisomers, as in statine. We proceeded then to synthesize each of the eight stereoisomers, starting from L-isoleucine, D-isoleucine, L-alloisoleucine, and D-alloisoleucine, as shown in Scheme 5. The eight stereoisomers were then separated and derivatized, and their retention times on gc (three gc peaks from each stereoisomer, due to dehydration) were compared with those of the isostatine isomer isolated from hydrolysis of didemnin A. Only one of the eight isomers showed both the retention times and relative gc peak intensities of the natural material (Table 3); that isomer was derived synthetically from D-alloisoleucine. The stereochemistry at C-4 and C-5 of the natural isostatine was, therefore, established as *R* and *S*, respectively.

Study of a γ-lactam by-product (Scheme 6) of the derivatization of the correct synthetic isostatine isomer allowed assignment of the stereochemistry at C-3. Its ^{1}H-nmr spectrum showed a very small coupling constant between H-3 and H-4 (ca. 2 Hz, Scheme 6), which can be the case only if the two hydrogens are *trans* to one another. Assignment of the *trans* configuration, then, shows the absolute configuration of C-3 to be *S*, completing the structural reassignment of didemnins A through E as **1b-5b**. With the new structural information in hand, the total synthesis of didemnin A was repeated (Scheme 7), employing the appropriate isostatine isomer to give synthetic (*Z*)-didemnin A, which proved to be identical with natural (*Z*)-didemnin A in all respects. Similarly, synthetic didemnin A and natural didemnin A were shown to be identical.

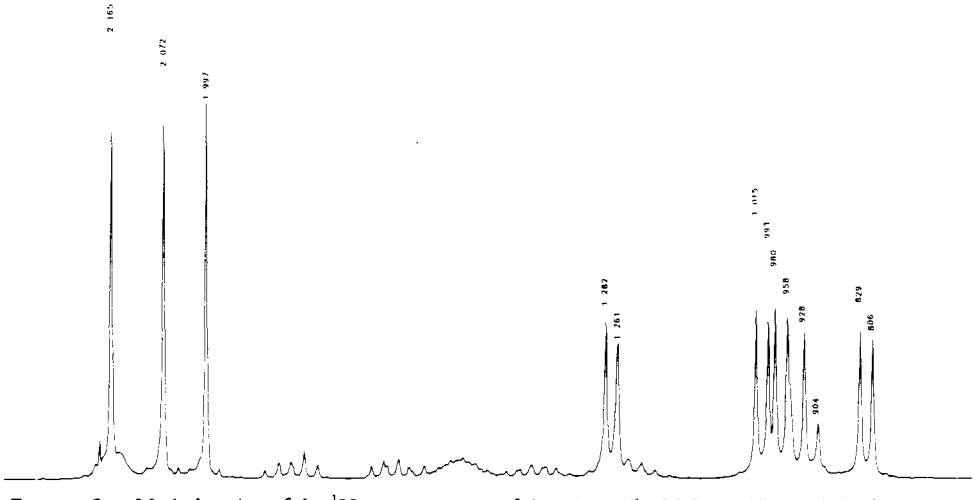
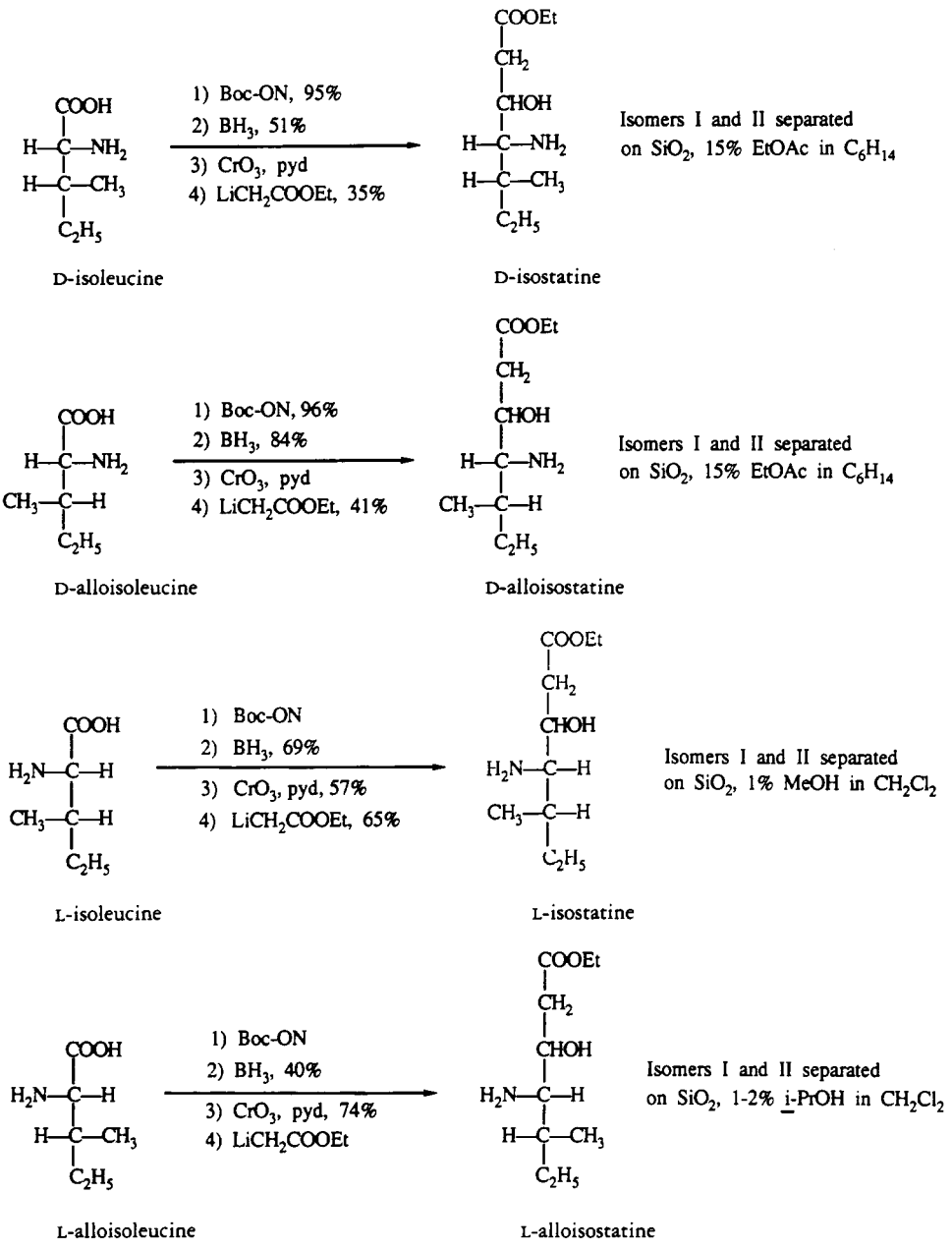


FIGURE 5. Methyl region of the ^{1}H-nmr spectrum of the tripeptide (MeLeu→Thr→"Sta") derivative.



SCHEME 5. Synthetic routes to the eight stereoisomers of isostatine.

In addition to natural (*Z*)-didemnin A, the synthesis did indeed yield (*Z*)-(2-*epi*-Hip)-didemnin A as a second isomer. Interestingly, this isomer, when isolated and reisolated by hplc and reinjected, gave both natural (*Z*)-didemnin A and (*Z*)-(2-*epi*-Hip)-didemnin A on rechromatography, regardless of how many times the (*Z*)-(2-*epi*-Hip)-didemnin A was isolated. Thus, it is clear that, in some solvents at least, (*Z*)-(2-*epi*-Hip)-didemnin A is converted via enolization to the preferred isomer, natural (*Z*)-didemnin A.

With the synthesis of (*Z*)-didemnin A and its conversion to natural didemnin A, the syntheses of didemnins A, B, and C are complete. Moreover, by using the synthetic

TABLE 3. Retention Times of the Eight Stereoisomers of Statine and of the Isomer Present in the Hydrolyzate of Natural Didemnin A.

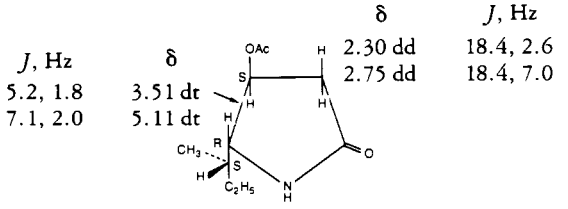
Isomer	Retention times[a] (min)		
D-Isostatine B	14.0,	19.5,	21.6
D-Isostatine A	14.0,	19.5,	21.6
L-Isostatine B	14.0,	19.4,	21.3
L-Isostatine A	14.0,	19.5,	21.4
D-Alloisostatine A	14.6,	18.6,	21.2
D-Alloisostatine B	14.7,	18.6,	21.3
L-Alloisostatine A	14.6,	18.4,	21.0
L-Alloisostatine B	14.6,	18.4,	21.0
Didemnin A hydrolyzate	14.6,	18.6,	21.2

[a] ≡, nearly exclusive gc peak; ≡, most intense; =, next; —, least intense.

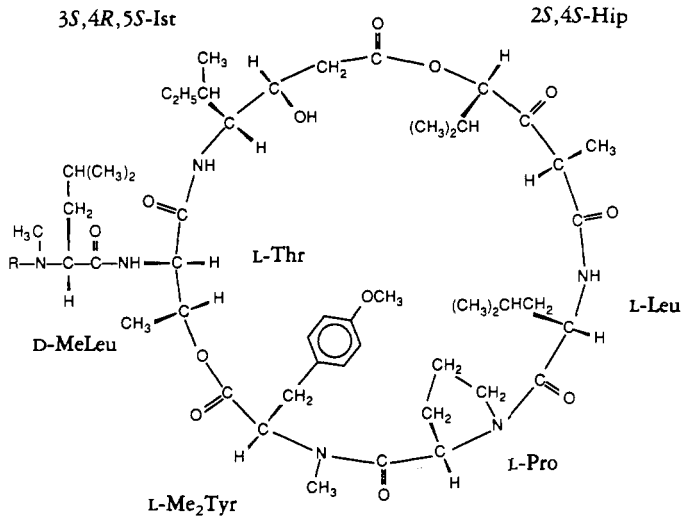
route described here, the other isomers of isostatine can be incorporated effectively into isomeric didemnin A, allowing further study of the structure-activity relationship.

STRUCTURE OF TUNICHLORIN, A NICKEL-CONTAINING PORPHYNOID.—In addition to the didemnins, extracts of the tunicate *T. solidum* contain a number of other compounds, including several pigments. One of the pigments has been identified as zeaxanthin and another, tentatively, as antheraxanthin diacetate (30). The most interesting, however, is a blue-green pigment obtained in very small amounts (ca. 10^{-5}%) from the homogenized tunicate (31,32). Although the pigment itself, tunichlorin, is relatively unstable and difficult to purify, methanolysis of the crude tunichlorin-containing material in 0.07 N *p*-toluenesulfonic acid gives a derivative, dimethyltunichlorin (Scheme 8), which has been the principal object of structural studies.

High resolution fabms indicated the methylated material to be a dimethyl derivative (M+H, 623.2147) of tunichlorin itself (M+H, 595.1829). The electronic spectra of tunichlorin and dimethyltunichlorin are nearly identical and quite characteristic of porphynoids. More specifically, they are characteristic of porphynoids in which one of the pyrrole rings has been reduced to a dihydropyrrole, since the uv and visible absorption peaks are of comparable intensity [λ max (CH_2Cl_2) 416 nm, ε 42,000; λ max 641,



SCHEME 6. γ-Lactam by-product of the derivatization of the correct synthetic isostatine isomer and ^{1}H-nmr chemical shifts.



1b Didemnin A: R=H

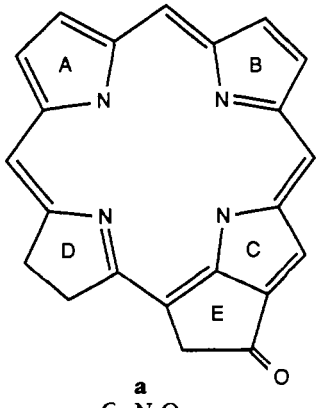
2b Didemnin B: R=CH_3CHOHC-N(L-Pro)-C(=O)- (L-Lac)

3b Didemnin C: R=$CH_3CHOHC(=O)$- (L-Lac)

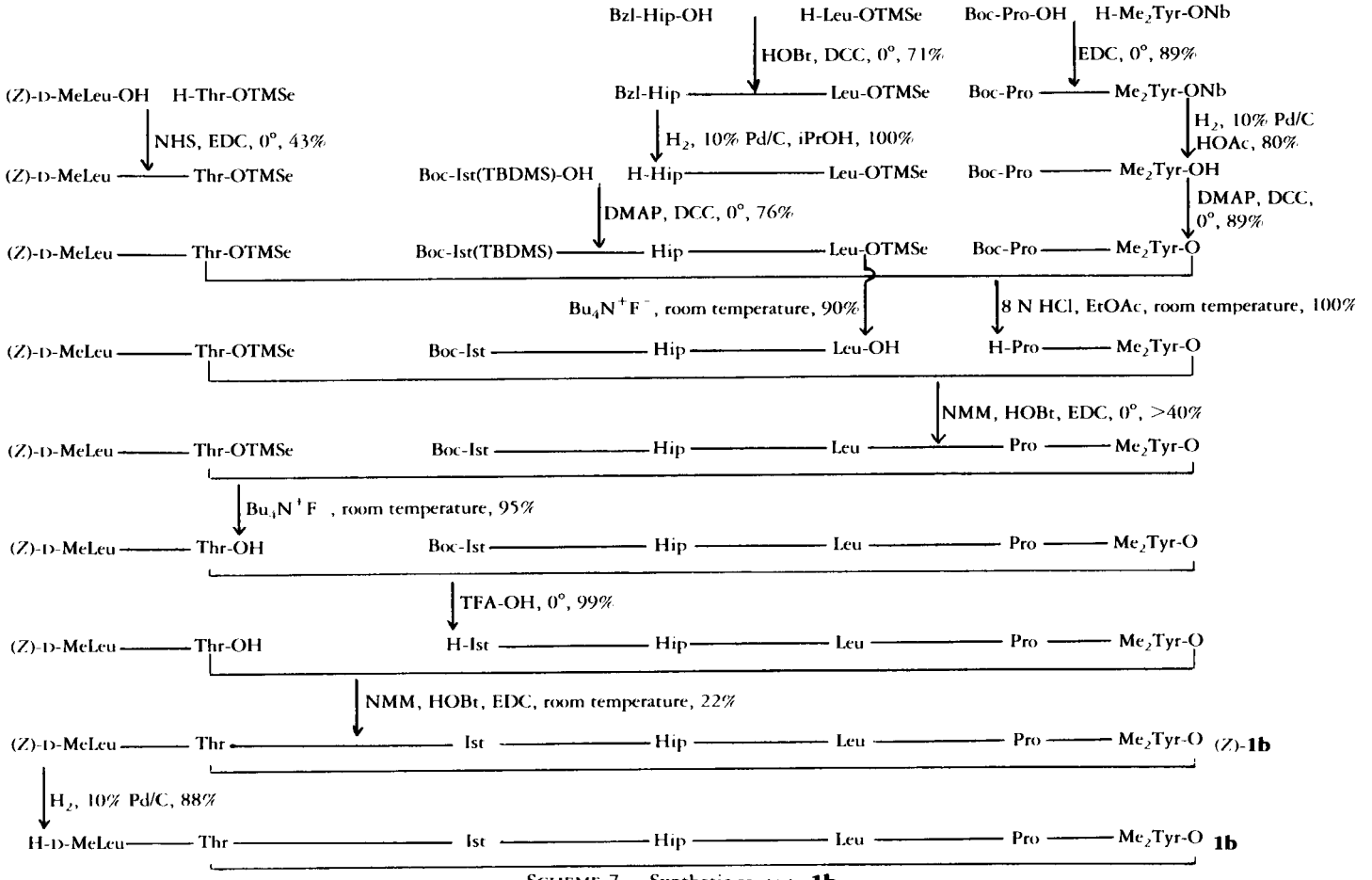
4b Didemnin D: R=L-pGlu—L-Gln—L-Gln—L-Gln—L-Lac—L-Pro—

5b Didemnin E: R=L-pGlu—L-Gln—L-Gln—L-Lac—L-Pro—

ε 43,000]. In addition, split absorption is found in the Soret region (λ max 389 nm, ε 34,000; λ max 416, ε 42,000), which is characteristic of porphynoids containing a cyclopentanone E ring. Thus, the general ring structure **a**, containing $C_{22}N_4O$, can be deduced for the porphynoid. The cyclopentanone carbonyl is also evident in the ^{13}C-

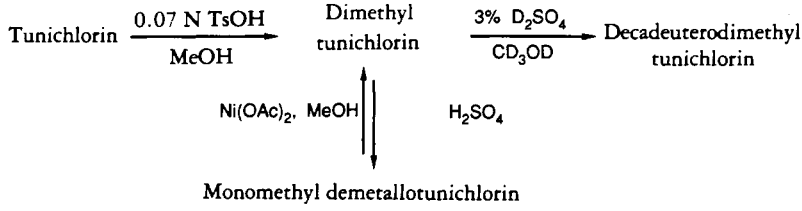


a
$C_{22}N_4O$



SCHEME 7. Synthetic route to **1b**.

Abbreviations: Bzl, benzyl; TMSe, β-trimethylsilylethyl; Boc, *t*-butyloxycarbonyl; Nb, *p*-nitrobenzyl; *Z*, benzyloxycarbonyl; TBDMS, *t*-butyldimethylsilyl; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; DMAP, 4-dimethylaminopyridine.



SCHEME 8. Conversion of tunichlorin to derivatives.

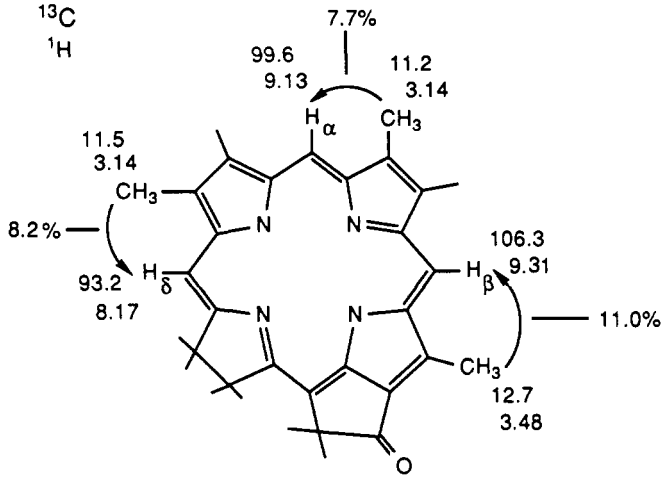
nmr spectrum; other groups indicated by that spectrum are shown in Table 4. These units amount to a total formula of $C_{34}H_{36}N_4O_4$ or 564 mass units. Because the molecular weight of dimethyltunichlorin was indicated to be 622, 58 mass units remains unaccounted for.

It was assumed that the remaining 58 mass units was due to a metal, and, indeed, dimethyltunichlorin could be converted by treatment with concentrated H_2SO_4 to monomethyl demetallotunichlorin (Scheme 8). With an atomic weight of 58 or 59, nickel and cobalt were the possible metals, with nickel far more likely. Evidence for nickel in dimethyltunichlorin was provided by hrms, which agreed with nickel for tunichlorin itself (Δ 2.6 mmu) and for dimethyltunichlorin (Δ 2.1 mmu) and by atomic absorption spectroscopy, which indicated 8.1% nickel (calcd 8.9%). Numerous nickel-containing porphynoids have been obtained from shale oil, but none has been identified from living organisms, with the exception of F430, a highly modified cofactor produced by a methanogen (33).

In placing the remaining groups of tunichlorin (Table 4) on the chlorin skeleton, we noted first that two of the bridgehead aromatic hydrogens are exchanged in CD_3OD-D_2SO_4 (along with the α-hydrogens in the cyclopentanone ring and others, see **8**), a reaction characteristic of H-α and H-δ in the chlorin nucleus. Those two aromatic hydrogens and the third one (H-β) could all be identified by nOe as being adjacent to methyl substituents on the pyrrole rings, as shown in Scheme 9. Other substituents identified included a methoxymethyl unit, which was converted to a hydroxymethyl by hydrolysis in concentrated H_2SO_4 (Scheme 10) and to a trideuteromethoxymethyl group by deuteromethanolysis. A carbomethoxy group also underwent exchange in the deuteromethanolysis. An aromatic ethyl group and aliphatic $>CHCH_3$ and $>CHCH_2CH_2-$ groups were identified by coupling constants and by 2D nmr and decoupling experiments.

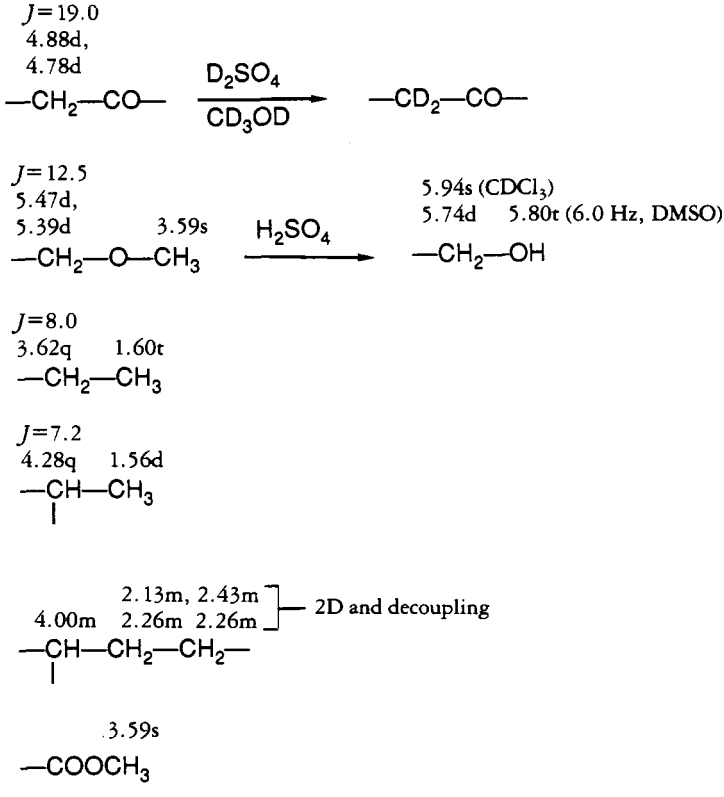
TABLE 4. ^{13}C-nmr Assignments for Dimethyl tunichlorin.

Group	No.	Formula
>C=O	1	CO
—COO—	1	CO_2
CH_3O—	2	$C_2H_6O_2$
—OCH_2—	1	CH_2O
=C<	15	C_{15}
=CH—	3	C_3H_3
—CH—	2	C_2H_2
—CH_2—	4	C_4H_8
—CH_3	5	C_5H_{15}

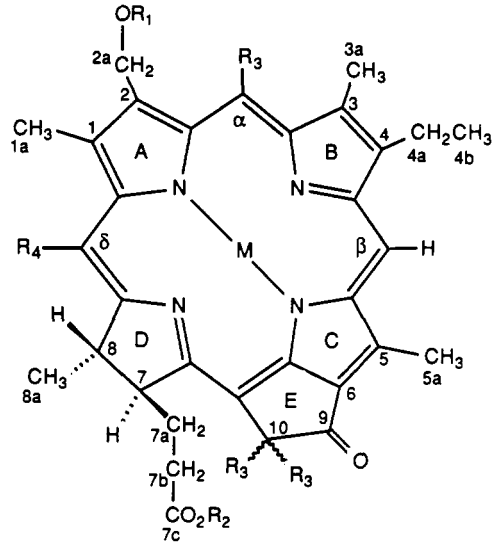


SCHEME 9. Nuclear Overhauser enhancements for partial structure of tunichlorin.

The logical position for the $>CHCH_3$ and the $>CHCH_2CH_2-$ units is that shown in Scheme 11; this was confirmed by nOe correlation of the $-CH_2CH_2-$ unit with the $CH_3CH<$ methine hydrogen, which in turn was correlated by nOe with the aromatic δ-hydrogen at 8.17 ppm. The aromatic α-hydrogen was similarly correlated by nOe studies with the methoxymethyl group, leaving only one position for the aromatic



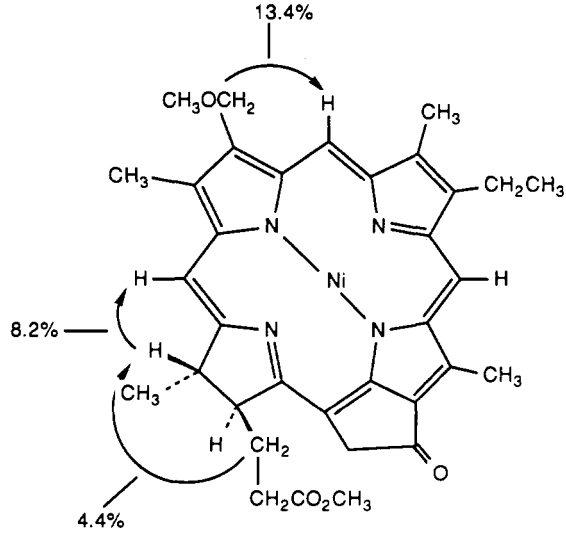
SCHEME 10. Functional groups and their ^{1}H-nmr absorptions in dimethyltunichlorin.



	R_1	R_2	R_3	R_4	M
6	H	H	H	H	Ni
7	Me	Me	H	H	Ni
8	CD_3	CD_3	D	D	Ni
9	H	Me	H	H	H_2
10	CD_3	CD_3	H	D	Ni
11	Me	Me	H	H	Cu

ethyl, at C-4. Similarly, the only position allowed for the carbomethoxy group is as the terminus of the aliphatic ethylene unit. The structure of dimethyltunichlorin is, thus, defined as that shown in Scheme 11. The structures of tunichlorin [**6**], dimethyltunichlorin [**7**], and related compounds [**8-11**] are shown here, including a copper analogue [**11**] obtained by addition of copper acetate to monomethyl demetallotunichlorin [**9**].

Dimethyltunichlorin itself is not the only chlorophyll-like pigment obtained from the tunicate. As shown in Table 5, the most abundant pigment by far is pheophytin a, a



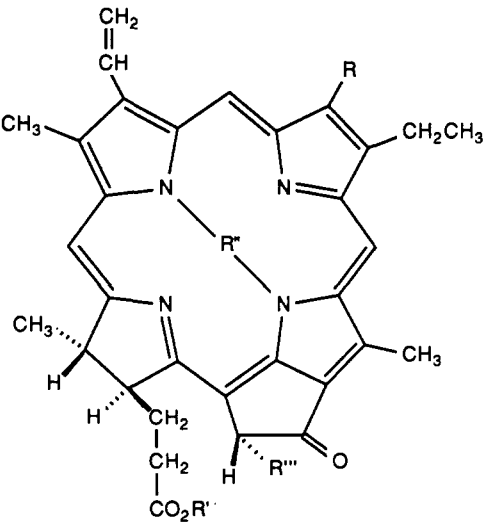
SCHEME 11. Dimethyltunichlorin and nOe data.

TABLE 5. Chlorophyll-like Pigments Obtained from *Tridemnum solidum*.

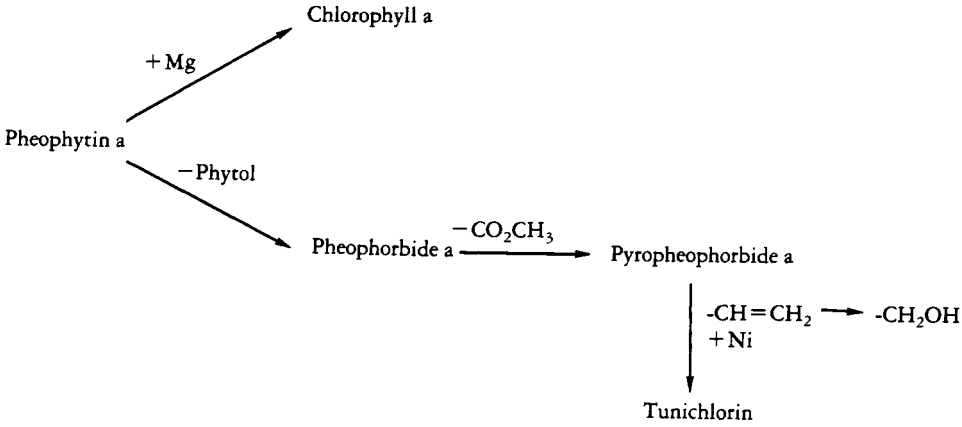
Compound	Tunicate wet wt (%)	Relative abundance
Pheophytin a	0.127	10,073
10-OH Pheophytin a	4.71×10^{-3}	368
Pyropheophytin a (?)	3.79×10^{-3}	324
Tunichlorin	8.96×10^{-6}	1
Pheophorbide a	2.80×10^{-6}	0.313
10-OH Pheophorbide a (?)	5.60×10^{-7}	0.063
Tunichlorin C-7/C-8 Isomer (?)	1.12×10^{-7}	0.0125
Chlorophyll a	variable	variable

demetallo analog of chlorophyll a. Chlorophyll a is regularly found in blue-green algae as the only chlorophyll, whereas prochlorons found commensally with other tunicates contain both chlorophylls a [**12**] and b [**13**].

The differences between tunichlorin [**6**] and chlorophyll a [**12**] are the lack of a C-10 carbomethoxy group in tunichlorin, the replacement of the C-2 vinyl group by a hydroxymethyl in tunichlorin, the lack of a phytol ester in tunichlorin, and the replacement of magnesium by nickel. It is not known at this time whether tunichlorin is produced by the tunicate or by the cyanobacterium growing commensally with it. A reasonable hypothesis for its biogenesis (Scheme 12) is that pheophytin a [**14**] is produced by the cyanobacterium and converted to chlorophyll a, but that it is also converted to pheophorbide a [**15**] and pyropheophorbide a [**16**], presumably still by the blue-green alga, and that the tunicate converts pyropheophorbide a to tunichlorin. Regardless of the biogenetic pathway, tunichlorin remains a most unusual natural product and provides additional interest in the tunicate *T. solidum*.



12 Chlorophyll a: R=Me, R′=phytyl, R″=Mg, R‴=$COOCH_3$
13 Chlorophyll b: R=CHO, R′=phytyl, R″=Mg, R‴=$COOCH_3$
14 Pheophytin a: R=Me, R′=phytyl, R″=H, H, R‴=$COOCH_3$
15 Pheophorbide a: R=Me, R′=H, R″=H, H, R‴=$COOCH_3$
16 Pyropheophorbide a: R=Me, R′=H, R″=H, H, R‴=H



SCHEME 12. Proposed biogenetic route to tunichlorin.

ACKNOWLEDGMENTS

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