EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent: 25.08.1999 Bulletin 1999/34

(51) Int Cl.ª: C07K 11/02, C07K 1/14, A61K 38/15

(21) Application number: 90303672.1

(22) Date of filing: 05.04.1990

(54) Novel cytotoxic cyclic depsipeptides from the tunicate trididemnum solidum
Zytotoxische zyklische Depsipeptide des Manteltierchen Trididemnum Solidum
Depsipeptides cytotoxiques cycliques à partir de tunicier trididemnum solidum

(84) Designated Contracting States: AT BE CH DE DK ES FR GB GR IT LI LU NL SE


(43) Date of publication of application: 24.10.1990 Bulletin 1990/43

(73) Proprietor: THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS Urbana, Illinois 61801 (US)

(72) Inventors:
- Rinehart, Kenneth L., Jr.
  Urbana, Illinois 61801 (US)
- Sakai, Ryuichi
  Urbana, Illinois 61801 (US)
- Stroh, Justin G.
  Pawcatuck, Connecticut 06379 (US)

(74) Representative: Ruffles, Graham Keith et al
MARKS & CLERK,
57-60 Lincoln's Inn Fields
London WC2A 3LS (GB)

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- PEPTIDES CHEMISTRY AND BIOLOGY, PROCEEDINGS OF THE TENTH AMERICAN PEPTIDE SYMPOSIUM, St. Louis, Missouri, 23rd - 28th May 1987, pages 626-631; K.L. RINEHART: "Didemin and its biological properties"

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Printed by Jouve, 75001 PARIS (FR)
Description

Background of the Invention

[0001] Didemmins, antitumor and antiviral cyclic decapeptides, were initially isolated in 1981 from the Caribbean tunicate Trididemnum solidum (Rinehart Jr., K.L., J.B. Gloer, J.C. Cook Jr., S.A. Milsak, T.A. Schall [1981] J. Am. Chem. Soc. 103:1857). So far, nine related peptides, didemmins A (3), B (4), C (5), nordidemmins A (6), and B (7), didemmins D (8), E (9), G (10), and methylene didemmin A (11) have been characterized (Rinehart Jr., K.L., J.B. Gloer, R.G. Hughes Jr., H.E. Renis, J.P. McGovern, E.B. Swynenberg, D.A. Stringfellow, S.L. Kuentzel, L.H. Li [1981] Science 212:933). Gloer, J.B. [1983] Ph.D. Dissertation, University of Illinois at Urbana-Champaign; Gutowski, R.E. [1984] M.Sc. Thesis, University of Illinois at Urbana-Champaign). See Figures 1, 1A, 1B, and 1C for the structural formulas of the bold face compound numbers referred to herein. Didemmin A (3) is the simplest and most abundant constituent, and it consists of five amino acid and two non-amino acid subunits. The structure of one of the non-amino acid subunits, (3S,4R,5S)-isostatine, was originally assigned as (3S,4R)-statine, but was later found to be incorrect, and it was revised during the synthetic study of 3 (Rinehart, K.L., V. Kishore, S. Nagarajan, R.J. Lake, J.B. Gloer, F.A. Bozich, K.-M. Li, R.E. Maleczka Jr., W.L. Totsen, M.H. G. Munro, D.W. Sullins, R. Sakai [1987] J. Am. Chem. Soc. 109:6846). Rinehart, K.L., V. Kishore, K.C. Bible, R. Sakai, D.W. Sullins, K.-M. Li [1988] J. Nat. Prod. 51:1; Sakai, R., First Year Paper I, University of Illinois at Urbana-Champaign). Of the didemmins isolated so far, 4 has shown the most potent biological activities, and strong antitumor efficacy has led this compound into Phase II clinical trials. Structurally, all didemmins except for nor- and methylene didemmins contain 3 as the basic skeleton, and the only differences between them are their side chains. Differences in the side chain or simple modifications in the ring functional groups, however, cause drastic changes in their biological properties (Gloer, 1983, supra). These interesting structure-activity relationships stimulated us to find new didemmins for further chemical and biological studies. See U.S. Patent No. 4,548,814, which discloses and claims processes for preparing didemmins A, B, and C, and nordidemmins A, B, and C. Also, see U.S. Patent No. 4,493,796, which discloses and claims didemmins A, B, C, D, and E.

Brief Summary of the Invention

[0002] An extract of the marine tunicate Trididemnum solidum (Subphylum Urochordatea) was chromatographed as shown in Scheme 1 to allow isolation of the new compounds, didemmins X (1) and Y (2). Their structures were elucidated by chemical and spectral methods, largely fast atom bombardment mass spectrometry (FABMS) and MS/MS studies, and are shown in Figure 2. The absolute stereochemistry of the β-hydroxydecanoyl group was determined by comparison with synthetic material. As shown in Table 1, didemmins X and Y inhibit the growth of L1210 mouse leukemia cells in vitro.

| Table 1. Three-day L1210 cell growth inhibition assay (at 37°C). |
|----------------------|-----------------|-----------------|-----------------|-----------------|
|                     | ID<sub>50</sub> | ID<sub>90</sub> | ID<sub>50</sub> | ID<sub>90</sub> |
| Didemmin            | ug/mL           | ug/mL           | ug/mL           | ug/mL           |
| X                   | 0.004           | 0.017           | 0.0048          | 0.017           |
| Y                   | 0.0064          | 0.021           | 0.0048          | 0.02            |
| D                   | 0.0034          | 0.015           | 0.0042          | 0.016           |
| E                   | 0.0011          | 0.0076          | 0.0008          | 0.0056          |
| B                   | 0.0078          | 0.058           | 0.0038          | 0.015           |
| A                   |                 |                 |                 |                 |

[0003] Thus, these compounds can be used to treat neoplastic diseases in animals and humans. Further, since these novel compounds are active against DNA and RNA viruses, they can be used to treat infections in humans, animals, and plants that are caused by such viruses. Acid addition salts and acyl derivatives of the novel didemmins can be made and used for the same biological purposes as the parent compounds.

Brief Description of the Drawings

[0004] Figures 1, 1A, 1B, and 1C show the structural formulas of the bold-faced compound numbers.

[0005] Figure 2 shows 1H NMR data of didemmins X, E, Y, and D.
Figure 3 shows \(^1\)H NMR data of the methanolyisis product of didemnin X, denoted "12", and didemnin B.

Figure 4 shows chiral gas chromatographic data of didemmins X, Y, and E.

Figure 5 shows \(^1\)H NMR data of 3-[(+)-10-camphorsulfonyl]decanoic acid methyl ester. (A) 3-(R) synthetic (18a), (B) 3-(R) naturals (14b), and (C) 3-(S) synthetic (18b), derivatives.

Detailed Description of the Invention

The Organism

The organism from which didemmins (X and Y) are extracted is a colonial marine tunicate of the family Didemnidae, Trididemnum solidum. This is in the suborder Aplousobranchia of the order Enterohera of the class Ascidiaecae of the subphylum Urophoridae of the phylum Chorda. The animals can be readily obtained by scuba techniques at depths of 10 to 100 feet where they encrust rocks, sponges, gorgonians, etc., in colony sizes up to 3 feet in diameter and 1/2 inch in thickness. Depending on location, they are green-white to purple-white to brown-white to orange-white.

Specific locations from which these organisms have been obtained are as follows:

1. Southwest Cay, Lighthouse Reef, Belize, 17° 11.8' N by 87° 36.5' W at a depth of 50 to 100 feet;
2. Rada el Cove, Isla San Andrés, Colombia, 12° 31' 46' N by 81° 44' 5' W at 25 to 33 feet;
3. Palancar Reef, Isla de Cozumel, Mexico, 20° 18.2' N by 87° 2.5' W at 60 to 100 feet;
4. On the west side of the southern tip of Turneffe Island, Belize, 17° 11.3' N by 87° 55.6' W at 50 to 75 feet;
5. Punta Oeste, Coco's Hole Harbor, Isla Roatan, Honduras, 16° 15' 5' N by 86° 36' W at 10 to 70 feet;
6. On the leeward side of the westernmost Holandes Cay, Isla San Blas, Panama, 9° 35.6' N by 78° 47' W at 60 feet.

Isolation and Purification of Didemmins X and Y

A variety of methods can be used to isolate and purify the didemmins and nordidemmins from samples of the tunicate organism, for example, solvent extraction, partition chromatography, silica gel chromatography, liquid-liquid distribution in a Craig apparatus, adsorption on resins, and crystallization from solvents.

Experimental Section

Infrared (IR) spectra were recorded on an IBM IR/32 FTIR. Optical rotations were measured with a Jasco DIP 360 digital polarimeter with a sodium lamp (589 nm) using a 5 cm (0.9 ml) cell. NMR spectra were obtained with a General Electric QE-300 instrument (300 MHz for \(^1\)H, 75 MHz for \(^13\)C). Chemical shifts are reported in ppm referenced to the chloroform peak at delta 7.26 ppm for \(^1\)H and 77.0 ppm for \(^13\)C, unless otherwise noted.

High- and low-resolution (HR and LR) fast atom bombardment (FAB) mass spectra were measured by L.S. Rong on a VG Analytical ZAB High-resolution mass spectrometry (ESI) mass spectra were measured by Dr. R.M. Milberg with a Finnigan MAT CH-5 DF spectrometer and a Finnigan MAT 731 instrument equipped with a multichannel signal analyzer. Melting points were determined on a Reichert microscope melting point apparatus and were not corrected. Gravity columns were prepared with commercial grade (Alfa large pore 58 micron) silica gel or NS (Nihon Seimitsu Kagaku, Tokyo; polystyrene divinyl benzene copolymer). High performance liquid chromatography (HPLC) was performed on a system containing an Altex model 110A pump, a Waters Associates R-401 differential refractometer, and a Beckman 153 UV detector. An Altex Ultrasphere silica (25 cm x 0.4 cm, 5 m particle size) and Alltech Spherisorb C-18, phenyl, amino, or cyano columns (25 cm x 1 cm, 5 or 10 m particle size) were used. A PC Inc. Ito multi-layer coil separator-extractor was used for centrifugal counter current chromatography (CCC). Gas chromatography (GC) analyses were carried out by using a Varian Model 3700 GC and an Alltech Associate Inc., Chiralcel-Val II capillary column (25 m x 0.32 mm) at a flow rate of 1.2 mL/min with a programmed oven temperature [90°(4°C/min)-180°C].

During the large-scale isolation of 4, a large amount of polar fraction A was produced (Scheme I) (Gutowsky supra). From this fraction, didemmin D (8) and E (9), along with the novel tunicolin pigments (Finehart, Kishore, Bible et al. [1988] supra), have been isolated.

In the isolation of these new peptides, efficient solvent partition and centrifugal counter current chromatography (CCC) were used extensively to avoid potential loss and decomposition of polar components. A portion of fraction A (9 g) was separated between the upper and the lower phases of ethyl acetate:heptane/methanol/water (7:4:4:3). FABMS of the two phases indicated that the polar peptides were concentrated almost exclusively in the lower phase.

The lower phase from the solvent partition was then separated by CCC with toluene/ethyl acetate/methanol/water (6:7:4:4:3) as the solvent system. The lower phase was used as a mobile phase to give crude didemmin D (8), E (9), Y (2), and X (1). 169 mg, 416 mg, 120 mg, and 248 mg, respectively, in order of elution. The crude peptides were purified by successive NS gel column chromatography, reversed phase, and normal phase HPLC to give the pure peptides.
The molecular weight of 1 was deduced as C_{22}H_{29}N_{3}O_{23} from HRFABMS. 1H and 13C NMR spectra of 1 and 2 were poorly resolved probably due to poor solubility and conformational inhomogeneity in the solution at room temperature. However, the complete spectral patterns of 1 and 2 were very similar to those of 4, 8, and 9 (Figures 1, 1A, 1B, and 1C), implying that 1 and 2 have the same basic skeleton as the other didemmins. Partial methanalysis of 1 gave 12 and 13 as the two major products. HRFABMS of 12 showed a molecular formula of C_{27}H_{33}N_{3}O_{23}, the same as that of 4. 1H NMR and optical rotation data of 12 were identical with those of authentic 4 (Figure 3). Compound 13 has very poor solubility in common solvents except for DMSO and DMF, and 1H NMR signals of 13 in DMSO or DMF are very broad at room temperature. The molecular formula of 13 was deduced from HRFABMS data as C_{26}H_{46}N_{6}O_{9}, suggesting that 13 must be a methyl ester of the side chain of 1. LRFABMS data showed fragmentation ions at m/z 555.3, 427.3, 299.2, and 188.2, and HRFABMS of each fragment ion showed that 13 contains three glutamyl units and a terminal C-10 compound (Scheme II).

A comparison of chiral GC data of the hydrolyzates of 1 and 9 showed the amino acid composition of 1 to be exactly that of 9, including the absolute stereochemistry (Figure 4).

Vigorous hydrolysis of 13 with 3 N hydrochloric acid gave a lipophilic compound 14. The molecular formula of C_{16}H_{27}O_{2} was determined by HRFABMS. 1H NMR spectra of 14, including decoupling experiments, with HREI data of a fragment ion at m/z 89 023896 for the formula of C_{4}H_{8}O_{2} (Scheme II) showed that the structure of 14 was 3-hydroxydecanic acid. This was confirmed by comparison of 1H NMR data of 14 and its methyl ester 14a with those of synthetic (R,S)-3-hydroxydecanic acid (15) and its methyl ester.

The absolute stereochemistry of 14a was determined by direct comparison of the 1H NMR data of the (+)-10-camphorsulfonlfy derivative 14b with those of the synthetic methyl-3-(R)- and methyl-3-(S)-[(+)-10-camphorsultyl] decanoates, 16a and 16b, respectively.

The preparation of optically pure synthetic methyl esters of 3-(R)- and 3-(S)-hydroxydecanic acids 15a and 15b was carried out by separating an epimeric mixture of the (R)-methylbenzyl carbamates (Pirkle, W.H., J.R. Hauske [1977] J. Org. Chem. 42:2781) (17a and 17b) by HPLC using a phenyl bonded silica gel column. The isolated, optically pure carbamates were then cleaved with trichlorosilane to give methyl-3-(R)-hydroxydecanate (15a) [(M)_{P} = 37.3°] and methyl-3-(S)-hydroxydecanate (15b) [(M)_{P} = 37.2°]. The esters were converted to (+)-10-camphorsulfonlates (16a and 16b) (Scheme III). 1H NMR spectra of the derivatives showed a very distinctive AB quartet for the C-10 position of the camphor moiety. The 1H NMR spectrum of 14b was superimposable on that of 16a (Figure 5).

Therefore, the structure of 1 was determined as (R)-3-hydroxydecanoyl-L-Gln-L-Gln-L-Gln-didemmin B.

Didemnin Y (2), isolated as a minor component, showed a molecular ion mass spectrum at m/z 1579.0119 (M + H) and HRFABMS mass spectra gave the molecular formula C_{45}H_{38}N_{3}O_{23}. 1 and 2 differ in molecular formula by C_{2}H_{7}N_{2}O_{2}, corresponding to a glutamyl unit. These data, along with 1H NMR spectra very similar to those of 1, suggest that the structure of 2 is 3-hydroxydecanoyl-L-Gln-L-Gln-L-Gln-L-Gln-didemmin B. This was confirmed by MS/MS, LR- and HRFABMS for the molecular ion of 2 and partially hydrolyzed compound 9 (Scheme IV). The stereochemistry at C-3 of the 3-hydroxydecanoyl moiety was determined analytically. Compound 2 (6 mg) was hydrolyzed to give side chain fragment 9 (2.3 mg). FABMS and MS/MS data of 9 secured the sequence (Scheme IV). Acid hydrolysis of 9 (1 mg) followed by treatment with (R)-methylbenzylisocyanate gave the diastereomeric carbamate 10, whose retention time on HPLC (normal phase cyano-column) compared with those of synthetic carbamates indicated that the configuration at C-3 of 3-hydroxydecanoyl subunit was also R (Scheme V).

A three-day L1210 cell growth inhibition assay showed ID_{50} 0.004 and 0.0064 μg/ml for 1 and 2, respectively, which are of the same magnitude as that of 4. The results are summarized in Table 1, supra.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

**Example 1 - Extraction and Initial Separation**

Extraction and initial separation to obtain fraction A were done as follows: Tunicate sample AHCE #614 was collected on the southwest side of Long Cay, Lighthouse Reef, Belize, 17° 11’ B’ N by 87° 36’ 5’ W at a depth of 50 to 100 feet. The sample was placed in 2-propanol and stored at -10°C until it was extracted by the procedure shown in Scheme I. A part of fraction A (18 g) was used to recover didemmins X and Y.

Isolation of didemmins. A part of fraction A (9 g) was partitioned between the lower and the upper phases of a mixture of ethyl acetate/heptane/methanol/water (7:4:4:3). Both lower and upper phases were concentrated to give solids (4.5 g each). A portion of the solid from the lower layer (1 g) was separated by CCC using toluene/ethyl acetate/methanol/water (6:7:7:4) as the solvent system with the lower phase being used as the mobile phase, at a flow rate of 2 mL/min at 600 rpm. A total of 40 fractions (24 mL each) were collected. The stationary phase was recovered from the first 10 fractions. Fraction 11 was concentrated in vacuo to give crude 8 (169 mg). Fractions 12 and 13 were combined, and the solvent was removed to give semi-pure 9 (416 mg). A portion of crude 8 was purified by successive...
C-18 reversed-phase gravity column chromatography and HPLC using a C-18 column with methanol/water (8:2) to give pure peptide 8 as a faint green solid (Finehart, Gier, Hughes et al. [1981], supra): mp 154-164°C (lit. Finehart, Gier, Hughes et al., supra mp 159-161°C); [alpha]D = -81.5° (c 0.4, CHCl3) (lit. Finehart, Gier, Hughes et al., supra [alpha]D = -89.4°). 1H NMR (CDCl3) delta 7.04 (2 H, d, J = 8.4 Hz), 6.80 (2 H, d, J = 8.4 Hz), 3.75 (3 H, s), 3.02 (3 H, s), 2.50 (3 H, s); HRFABMS calcld. for C17H18N4O12, 360.1182. Found: 360.1181.

[0026] A semi-pure sample of diidemnin E was purified by the procedure employed for 8 to give pure peptide 9 as a colorless solid: mp 156-166°C (lit. Finehart, Gier, Hughes et al., supra mp 164-166°C); [alpha]D = -86.6° (c 1.98, CHCl3) (lit. Finehart, Gier, Hughes et al., supra [alpha]D = -90.6°). 1H NMR (CDCl3) delta 7.05 (2 H, d, J = 8.1 Hz), 6.80 (2 H, d, J = 8.1 Hz), 3.76 (3 H, s), 3.10 (3 H, s), 2.51 (3 H, s); HRFABMS calcld. for C27H19N12O21, 1479.7967 (M + H). Found: 1479.7993.

[0027] Fractions 14-18 were combined to give 100 mg of a solid. A methanol soluble part of the solid, after filtration, was chromatographed on a gravity column packed with a NS gel with methanol to give 49.5 mg of a peptide fraction. This was purified on HPLC using an amino column with methanol followed by a silica gel column with chloroform/methanol (3:1) to give pure peptide 2 (11.3 mg), amorphous, [alpha]D = -65° (c 0.93, CHCl3-MeOH); IR (Neat) 3310, 2950, 1720, 1650 cm⁻¹, 1H NMR (CDCl3-methanol-d4) delta 7.30 (2 H, d, J = 8.1 Hz), 6.70 (2 H, d, J = 8.1 Hz), 3.74 (3 H, s), 2.98 (3 H, s), 2.49 (3 H, s); HRFABMS calcld. for C29H39N2O25, 1795.0145 (M + H). Found: 1795.0119.

[0028] Fractions 19-29 from the CCC separation were also combined to afford a light green solid (248 mg). This solid was separated by NS gel column chromatography using methanol to afford a peptide solid. This material was passed through a Sep-Pak silica gel column which had been treated with ammonia gas with chloroform/methanol (4:1) to remove green pigments. The peptide was purified on HPLC using a silica gel column with chloroform/methanol (4:1) to give 1 (107 mg): solid: mp 156-156°C; [alpha]D = -86.6° (c 6.35, CHCl3); IR (Neat) 3450, 3300, 2950, 1720, 1650 cm⁻¹, 1H NMR (CDCl3-methanol-d4) delta 7.02 (2 H, d, J = 8.4 Hz), 6.76 (2 H, d, J = 8.4 Hz), 3.72 (3 H, s), 3.00 (3 H, s), 2.47 (3 H, s); HRFABMS calcld. for C42H39N3O23, 1666.9559 (M + H). Found: 1666.9533.

Example 2 - Methanolyis of Didemnin X (1)

[0029] To a solution of 1 (122 mg) in 2 mL of methanol was added excess sodium carbonate (25 mg) with stirring at room temperature until TLC indicated the starting material had been consumed (0.5 hr). The reaction mixture was filtered, and concentrated to give a mixture of methanol-soluble products. The solid was dissolved in DMSO and filtered to remove the residual salts. Removal of the DMSO by a stream of nitrogen gave a colorless solid 13 (35 mg): [alpha]D = 19° (c 0.14, DMSO); LR and HRFABMS see Scheme II. The methanol-soluble portion was filtered to remove residual solid, then further separated on reversed-phase HPLC using a C-18 column with methanol/water (4:1) to give a major component along with five minor components. The major product was re-chromatographed to give a pure solid 12: mp 152-156°C; 1H NMR (CDCl3) see Figure 3, HRFABMS calcld. for C57H39N7O15, 1112.6495 (M + H). Found: 1112.6502.

Example 3 - GC Analysis of Hydrolyzed 1, 3, 4, and 9

[0030] A sample of 1 (3 mg) was heated with 0.5 mL of 6 N HCl at 110°C for 21 hours. Dichloromethane was added to the mixture and the aqueous phase was evaporated to dryness. The residual material was treated with a mixture of methanol/acetic chloride (10:1) at 110°C for 0.5 hr. The solvent was removed with a stream of nitrogen gas, and the resulting oil was then treated with trifluoroacetic anhydride/trifluoroacetic acid (0.2 mL, each) at 110°C for 4 minutes. Excess acid was removed with a stream of nitrogen gas, and the residue was dissolved in 1 mL of dichloromethane for GC analysis. Samples of hydrolyzed 3, 4, and 9 were prepared by the same procedure.

Example 4 - Hydrolysis of 13 with 3 N HCl

[0031] Compound 13 (12.3 mg) was dissolved in 3 N HCl (1 mL) and heated at 120°C in a sealed sample vial for 8 hours. The mixture was extracted with dichloromethane (2 X 1 mL), the organic layer was dried over sodium sulfate, and the solvent was removed to give 14 as a white solid: 1H NMR (CDCl3) delta 4.03 (1 H, br s), 2.55 (1 H, br d, J = 17.7 Hz), 2.45 (1 H, dd, J = 17.1 Hz), 1.63-1.36 (2 H, m), 1.25 (br s), 0.68 (br t, J = 5.7 Hz); HRFABMS calcld. for C10H12O3, 189.1491 (M + H). Found: 189.1486. HREIMS calcld. for C10H12O3, 89.02386. Found: 89.02386. This compound was then treated with a mixture of methanol/acetic anhydride (9:1) for 30 minutes at 120°C in a sealed vial. The solvent was removed with a stream of nitrogen. The residual material was separated on a silica Sep-Pak column with dichloromethane/ethyl acetate (5:1) to give 0.89 mg of 14a as an oil: 1H NMR (CDCl3) delta 4.00 (1 H, m), 3.71 (3 H, s), 2.46 (1 H, dd, J = 3.3, 16.5 Hz), 2.40 (1 H, dd, J = 9.0, 16.8 Hz), 1.54-1.34 m, 1.28 br s, 0.88 (3 H, t, J = 6.3 Hz).
Example 5 - Synthesis of (R,S)-3-Hydroxydecanoic Acid and its Methyl Ester

[0032] Octanly chloride (16.2 g, 0.068 mol) was added to a solution of 2.2-dimethyl-1,3-dioxane-4,6-dione (11.5 g, 0.090 mol) in dichloromethane (100 mL) and pyridine (12.8 mL) at 0°C over 10 minutes. The reaction mixture was stirred for 1 hour at room temperature. The reaction product was partitioned successively between dichloromethane, and aqueous HCl (10%), and then water. The organic portion was concentrated to give a deep red oil and then refluxed with methanol (250 mL) for 12 hours. The product was purified by silica gel gravity column chromatography (dichloromethane) to give a light yellow oil (14.6 g). A part of the oil (650 mg) was dissolved in THF (10 mL). To the solution was added a suspension of sodium borohydride (120 mg, 3.2 mmol) and water (1 mL) at 0°C with stirring for 1 hour. The reaction was quenched by adding acetone (5 mL), the solvent was removed in vacuo, and the residual material was triturated with dichloromethane. The organic solutes were purified by silica gel gravity column chromatography with dichloromethane/ethanol acetate (3:1) to give (R,S)-methyl-3-hydroxydecanoate as an oil. 1H NMR, identical with that of 14a, 13C NMR (CDCl3) δ 173.79, 67.92, 51.50, 41.17, 36.52, 31.74, 29.42, 29.17, 25.44, 22.56, 14.00. A mixture of the methyl ester (166 mg, 0.92 mmol) with 6 N sodium hydroxide was heated at 110°C for 1 minute. The saponified material was dissolved in water (1 mL) and the pH of the solution was adjusted to 1 by adding 6 N HCl. Dichloromethane was added to the solution, and the organic layer was dried over sodium sulfate. The solvent was removed in vacuo to give 127 mg (83%) of 15 as fine crystals: mp 74°C. 1H NMR (CDCl3), identical with that of 14.

Example 6 - Conversion of (R,S)-methyl-3-hydroxydecanoate to its (+)-10-camphorsulfonate 16a and 16b

[0033] A mixture of (R,S)-methyl-3-hydroxydecanoate (87 mg, 0.43 mmol) and (+)-10-camphorsulfonyl chloride (125 mg, 0.499 mmol) dissolved in pyridine (0.5 mL) was allowed to stand at room temperature for 12 hours. Pyridine was removed in vacuo, and the residual oil was subjected to gravity silica gel column chromatography with chloroform/ethanol acetate (9:1) to give 132 mg of an epimeric mixture (73%) of 16a and 16b as an oil: 1H NMR (CDCl3), see Figure 4.

Example 7 - Conversion of 14a to (+)-10- Camphorsulfonate, 14b

[0034] A mixture of ester 14a (0.89 mg), (+)-10-camphorsulfonyl chloride (4.6 mg), and pyridine (0.2 mL) was allowed to stand at room temperature for 12 hours, then pyridine was removed with a stream of nitrogen. The resulting oil was passed through a gel Sep-Pak column with dichloromethane/ethanol acetate (5:1) then purified by HPLC using a cyanophenyl column with hexane/2-propanol (20:1) to give 0.95 mg of an oil. 1H NMR (CDCl3), identical with that of 16a; see Figure 5.

Example 8 - Reaction of (R,S)-3-methyl-3-hydroxydecanoic acid with (R)-alpha-methylbenzyl isocyanate

[0035] A mixture of (R)-alpha-methylbenzyl isocyanate (668 mg, 4.5 mmol) and (R,S)-methyl-3-hydroxydecanoate (650 mg, 4.2 mmol) was dissolved in dichloromethane (2 mL) and pyridine (0.5 mL), and refluxed for 42 hours. The solvent was removed in vacuo, and the resulting oil was purified by gravity column chromatography using silica gel with hexane/2-propanol (20:1) to give 1.19 g of a mixture of carbamates 17a and 17b (78%) as an oil: CIMS (M + H, methane) m/z (rel. intensity) 352.0 (100), 334.2 (12), 318.2 (8), 272.2 (2), 246 (43), 233.1 (37), 203.2 (43), 185.2 (95), 164.1 (60), 153.1 (33), 120.1 (40), 105.1 (60), 85.0 (35), 71.1 (30), 59.1 (38). A mixture of 17a and 17b (70 mg) was separated on HPLC, using a phenyl column with hexane/2-propanol (60:1), to afford 28 mg of optically pure carbamate 17a as the less polar isomer: 1H NMR (CDCl3) δ 7.30 (5 H.m), 5.07 (1 H, m), 4.95 (1 H, br d, J = 6 Hz), 4.92 (1 H, br m), 3.68 (3 H, s); [alpha]D = 33.3° (c 2.80, CHCl3). The more polar fraction gave 29 mg of the other optically pure carbamate 17b: [alpha]D = 36.6° (c 2.86, CHCl3); 1H NMR (CDCl3) δ 7.30 (5 H, m), 5.06 (1 H, m), 4.95 (1 H, br d), 4.88 (1 H, br m), 3.56 (3 H, s).

Example 9 - Cleavage of Carbamates 17a and 17b to Give Optically Pure Esters 15a and 15b

[0036] To a solution of 17b (22.5 mg) in 1 mL of dried benzene was added 20 μL of triethylamine and 25 μL of trichlorosilane. The mixture was stirred for 36 hours at room temperature, then saturated aqueous ammonium chloride (1 mL) was added. The organic layer was dried over sodium sulfate, and the solvent was removed with a stream of nitrogen. The resulting material was purified by HPLC using a cyanophenyl column with hexane/ethyl acetate (4:1) to give 4.4 mg (33%) of optically pure S ester 15b as an oil; [alpha]D = -37.2° (c 0.243, CHCl3). The other isomer (24.2 mg) 17a was cleaved by the same procedure to give 7.05 mg (54%) of pure R ester 15b as an oil; [alpha]D = -37.2° (c 0.565, CHCl3). (lit. [Parker, W.L. and M.L. Rathnum (1975) J. Antibiot. 28:379], [alpha]D = -37°).
Example 10 - Reaction of Optically Pure Esters 15a and 15b with (+)-10-Camphorsulfonyl Chloride

[0037] A mixture of (S)-ester 15b (1.89 mg) and (+)-10-camphorsulfonyl chloride (11.5 mg) was dissolved in pyridine (0.5 mL) and allowed to stand at room temperature for 5 hours. Pyridine was removed with a stream of nitrogen, and the residual material was separated by silica gel Sep-Pak column chromatography with dichloromethane/ethyl acetate (5:1). The resulting oil was purified on HPLC using a cyanopropyl column with dichloromethane/ethyl acetate (5:1) to give pure sulfonate 16b as an oil: $^1$H NMR (CDCl$_3$) delta 5.12 (1 H, ddt, $\delta$ = 6.1, 6.1, 6.1 Hz), 3.72 (3 H, s), 3.59 (1 H, $d$, $\delta$ = 15.0 Hz), 3.09 (1 H, $d$, $\delta$ = 15.0 Hz). The (R)-ester 15a was converted to the corresponding (+)-10-camphorsulfonate by using the same procedure to give 16a as an oil: $^1$H NMR (CDCl$_3$) delta 5.12 (1 H, ddt, $\delta$ = 6.1, 6.1, 6.1 Hz), 3.72 (3 H, s), 3.67 (1 H, $d$, 15.0 Hz), 3.01 (1 H, $d$, 15.0 Hz).

Example 11 - Salts of Didemmins

[0038] Since the didemmins are weakly basic, they form salts with mineral acids such as HCl, H$_2$SO$_4$, and H$_3$PO$_4$. Such salts can be prepared by suspending the didemmins in water, adding a dilute acid until the pH of the solution is about 3 to 4, and freeze-drying the solution to provide a dried residue of the didemmin salt.

Example 12 - Derivatives of Didemmins

[0039] The didemmins have free amino and hydroxyl groups available for derivatization. Thus, acyl amides and esters of the didemmins can be prepared by methods well known to those skilled in the art. Acyl derivatives of the didemmins can be used for the same biological purposes as the parent compounds.

[0040] Acids which can be used in the acylation of a didemmin are as disclosed in U.S. Patent No. 4,548,814, Columns 3 and 4. The administration of didemmins X and Y can be carried out as disclosed in U.S. Patent No. 4,548,814, Columns 9 through 15. This patent is incorporated herein by reference thereto for the above-noted disclosures.
Scheme I. Isolation of didemnins from the extract of *T. solidum*

*T. solidum*

1) Extraction (EtOH, toluene-MeOH)
2) Preparative LC (silica gel)

Less polar fraction
didemnin A
didemnin B

**Fraction A (9g)**

Partition:
7:4:4:3
EtOAc-heptane-MeOH-H₂O

**Lower Phase (4.5g)**

1g

Centrifugal
Countercurrent
Chromatography

6:7:4:4
toluene-EtOAc-MeOH-H₂O

Crude Didemnins

D (159 mg)  E (416 mg)

**Upper Phase (4.5g)**
didemnin A and B pigments and fats

Y (120 mg)  X (248 mg)

Purification:
gravity column
HPLC

Didemnin Y (11.3 mg)  Didemnin X (107.0 mg)
Scheme II. FABMS fragmentation of the methanolation product (4) of didemnin X (1)

\[
\text{Didemnin X (1) } \xrightarrow{\text{MeOH, Na}_2\text{CO}_3} \text{Didemnin B (3) + six other acyclic peptides}
\]

\[
\text{C}_{82}\text{H}_{131}\text{N}_{13}\text{O}_{23} \quad (122 \text{ mg})
\]

\[
\text{C}_{57}\text{H}_{80}\text{N}_{15}\text{O}_{15}
\]

\[
\text{H NMR Identical with that of natural compound}
\]

\[
4 \quad (35 \text{ mg, 83%})
\]

\[
\text{C}_{26}\text{H}_{46}\text{N}_{6}\text{O}_{9}
\]

L-Glu by Chiral GC

\[
(C_{48}\text{H}_{19}\text{O}_{2}) \quad \text{N} \quad \text{H} \quad \text{O} \quad \text{C} \quad \text{N} \quad \text{H} \quad \text{O} \quad \text{C} \quad \text{OCH}_3
\]

\[
4: R = \text{CH}_2\text{CH}_2\text{CONH}_2
\]

D: m/z 555.3142, \( \Delta 0 \text{ mmu}, \text{C}_{20}\text{H}_{43}\text{N}_{6}\text{O}_{8} \)

C: m/z 427.2567, \( \Delta 1.0 \text{ mmu}, \text{C}_{20}\text{H}_{35}\text{N}_{4}\text{O}_{6} \)

B: m/z 299.1971, \( \Delta 0 \text{ mmu}, \text{C}_{15}\text{H}_{27}\text{N}_{2}\text{O}_{4} \)

A: m/z 188.1636, \( \Delta 0.5 \text{ mmu}, \text{C}_{10}\text{H}_{22}\text{NO}_{2} \)
Scheme III.

\[
\begin{align*}
\text{C}_7\text{H}_{15}\text{Cl} & \quad + \quad \text{O} & \quad \text{4 steps} & \quad \text{and HPLC} \\
\text{SiHCl}_3 & \quad \text{TEA/benzene} \\
\text{OH} & \quad \text{C}_7\text{H}_{15}\text{O} & \quad \text{CHCl}_3 & \quad [\text{M}]_D = +37.2^\circ, \text{CHCl}_3 \\
\text{S} & \quad \text{C}_7\text{H}_{15}\text{OH} & \quad \text{HCl} & \quad [\text{M}]_D = -37.2^\circ, \text{CHCl}_3 \\
\text{(+)-10-camphorsulfonyl chloride} & \quad \text{pyridine} \\
\text{O} & \quad \text{O} & \quad \text{OCH}_3 & \quad \text{OCH}_3
\end{align*}
\]

Scheme IV. (a) FABMS fragmentation pattern and MS/MS data of didemnin Y (2)

C_{18}H_{19}O_{2} · Gln — Gln — Gln — Gln — O — Lac — Pro — MeLeu — Thr

Didemnin Y (2), m/z 1795.0199 (M + H), C_{28}H_{46}N_{15}O_{23}

<table>
<thead>
<tr>
<th>fragment #</th>
<th>mass observed (Da)</th>
<th>formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>816.7 (H_2)</td>
<td></td>
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<tr>
<td>B</td>
<td>943.5765, Δ -4.5 mmu,</td>
<td>C_{28}H_{47}N_{14}O_{20} (H_2)</td>
</tr>
<tr>
<td>C</td>
<td>1040.6247, Δ -3.7 mmu,</td>
<td>C_{28}H_{46}N_{14}O_{19} (H_2)</td>
</tr>
<tr>
<td>D</td>
<td>1112.6483, Δ -1.2 mmu,</td>
<td>C_{28}H_{46}N_{15}O_{18} (H_2)</td>
</tr>
<tr>
<td>E</td>
<td>1240.7121, Δ 0.4 mmu,</td>
<td>C_{28}H_{46}N_{15}O_{17} (H_2)</td>
</tr>
<tr>
<td>F</td>
<td>1358.7 (H_2)</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1496.5 (H_2)</td>
<td></td>
</tr>
<tr>
<td>A'</td>
<td>979.7</td>
<td></td>
</tr>
<tr>
<td>C'</td>
<td>755.4</td>
<td></td>
</tr>
<tr>
<td>D'</td>
<td>701.3811, Δ -2.3 mmu,</td>
<td>C_{28}H_{46}N_{15}O_{16} (H_2)</td>
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<tr>
<td>E'</td>
<td>555.3147, Δ 0.5 mmu,</td>
<td>C_{28}H_{46}N_{15}O_{15}</td>
</tr>
<tr>
<td>F'</td>
<td>427.2</td>
<td></td>
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<tr>
<td>G'</td>
<td>299.2</td>
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</tr>
</tbody>
</table>

* Low resolution data were obtained from MS/MS (FAB) experiment.

(b) Fab MS/MS data of side chain fragment (9) of didemnin Y (2)

C_{18}H_{19}O_{2} · Gln — Gln — Gln — Gln — OH

Compound 9 (M + H) = 701.1

<table>
<thead>
<tr>
<th>fragment #</th>
<th>mass</th>
<th>fragment #</th>
<th>mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>684</td>
<td>B'</td>
<td>147 (H_2)</td>
</tr>
<tr>
<td>B</td>
<td>555</td>
<td>C'</td>
<td>275 (H_2)</td>
</tr>
<tr>
<td>C</td>
<td>427</td>
<td>D'</td>
<td>403 (H_2)</td>
</tr>
<tr>
<td>D</td>
<td>299</td>
<td>E'</td>
<td>531 (H_2)</td>
</tr>
<tr>
<td>E</td>
<td>188 (H_2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Scheme V Absolute stereochemistry of 3-hydroxydecanoyl unit of didemnin Y (2)

\[
\begin{align*}
K_2CO_3 & \\
MeOH & \\
\text{Didemnin Y (6.0 mg)} & \rightarrow \\
\text{Fragment B (2.3 mg, 98%)} & \\
\text{1 mg} & \\
1. 6 N HCl, 12 h, 100 ^\circ C & \\
2. partition & \\
\text{Organic} & \\
\text{Aqueous} & \\
\text{benzene/TEA} & \\
\text{Chiral GC Analysis} & \\
\text{L-Glutamic acid} & \\
\end{align*}
\]

HPLC (Cyano Anal.)
Retention
Synthetic R,S: 10.4 min
Synthetic R,R: 12.6 min
Natural: 12.6 min

Naturally derived Carbamate
HRCIMS (M + H) 350.2330703
C_{20}H_{32}NO_4, m / z 0.1 mmu
CIMS 350.2, 246.1, 203.1, 185.1 etc.
(identical with that of synthetic sample)
Claims

1. A didemnin of the formula

\[
\text{CH}_3\text{O}
\]

wherein \( R = \)

2. Didemnin X, according to claim 1, wherein \( R = \)
or the salts, acyl amides, and esters thereof.

3. Didemnin Y, according to claim 1, wherein \( R = \)

or the salts, acyl amides, and esters thereof.

4. Use of a didemnin according to any preceding claim, for the manufacture of a medicament for use in the treatment of a neoplastic disease.

5. A process for the preparation of a didemnin according to any of claims 1 to 3, which comprises extraction from the marine tunicate *Trididemnum solidum*.

6. A process for the manufacture of a medicament for use in the treatment of a neoplastic disease, wherein a didemnin prepared according to the process of claim 5 is employed as active agent.

**Patentansprüche**

1. Didemnin der Formel
2. Didemnin X gemäß Anspruch 1, wobei R =

oder die Salze, Acylamide und Ester davon.

3. Didemnin Y gemäß Anspruch 1, wobei R =

oder die Salze, Acylamide und Ester davon.

4. Verwendung eines Didemmins gemäß einem der vorhergehenden Ansprüche für die Herstellung eines Medikaments zur Verwendung bei der Behandlung eines Tumorleidens.
5. Verfahren für die Darstellung eines Didemmins gemäß einem der Ansprüche 1 bis 3, das die Extraktion aus dem marinen Manteltierchen *Trididemnum solidum* umfaßt.


**Revidcations**

1. Didemnine de la formule:

2. Didemnine X, suivant la revendication 1, dans laquelle R vaut
3. Didemnine Y, suivant la revendication 1, dans laquelle \( R \) vaut

ou des sels, des acylamides et des esters correspondants.

4. Utilisation d'une didemnine suivant l'une quelconque des revendications précédentes, pour la fabrication d'un médicament en vue d'une utilisation dans le traitement d'une maladie néoplasique.

5. Procédé de préparation d'une didemnine suivant l'une quelconque des revendication 1 à 3, qui comprend une extraction à partir du tunicate marin *Trididemnum solidum*.

6. Procédé de fabrication d'un médicament en vue d'une utilisation dans le traitement d'une maladie néoplasique, dans lequel une didemnine préparée suivant le procédé de la revendication 5 est employée en tant qu'agent actif.
Figure 1
<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
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<tbody>
<tr>
<td>5</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃CH₉=CH⁻</td>
</tr>
<tr>
<td>6</td>
<td>CH₃,CH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>7</td>
<td>CH₃,CH₃</td>
<td>H</td>
<td>CH₅CHOH⁻N⁻</td>
</tr>
<tr>
<td>8</td>
<td>CH₄</td>
<td>H</td>
<td>O</td>
</tr>
<tr>
<td>9</td>
<td>CH₃</td>
<td>H</td>
<td>O</td>
</tr>
<tr>
<td>11</td>
<td>CH₃</td>
<td>R₂ = CH₂</td>
<td>O</td>
</tr>
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</table>

Figure 1A
12 \equiv 4

\[
\text{natural} \quad \text{synthetic}
\]

<table>
<thead>
<tr>
<th></th>
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<th>R₂</th>
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<tbody>
<tr>
<td>14</td>
<td>H</td>
<td>H</td>
<td>15a</td>
<td>H</td>
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<td>16a</td>
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<td>Rₓ</td>
<td>CH₃</td>
<td>17a</td>
<td>Rᵧ</td>
<td>CH₃</td>
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\[\text{synthetic}\]

<table>
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<th>R₆</th>
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<tbody>
<tr>
<td>15b</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>16b</td>
<td>Rₓ</td>
<td>CH₃</td>
</tr>
<tr>
<td>17b</td>
<td>Rᵧ</td>
<td>CH₃</td>
</tr>
</tbody>
</table>

Figure 1B
Didemnin X (1): Solid, mp 156-158°C, $[\alpha]_D = -88.6^\circ$ (CHCl₃)

Didemnin Y (2): $[\alpha]_D = -65^\circ$ (CHCl₃)

Figure 1C
Figure 2. $^1$H NMR data of didemmins X (1) and E (9), Y (2), and D (8) in CDCl$_3$ + Methanol-d$_4$. 
Figure 3. $^1$H NMR of 12 and authentic didemnin B (4).
Figure 4. Chiral GC data for acid hydrolysate (bis-TFA-OMe) of Didemmins E, X, and Y. Peak assignments were based on coinjection data. Me₂Tyr is not identified in these data.
Figure 5. $^1$H NMR data of 3-((+)-10-camphorsulfonyl)decanoic acid methyl ester, (A) 3-(R) synthetic (16a), (B) 3-(R) natural (14b), and (c) 3-(S) synthetic (16b), derivatives.