Granulatimide and Isogranulatimide, Aromatic Alkaloids with G2 Checkpoint Inhibition Activity Isolated from the Brazilian Ascidian *Didemnum granulatum*: Structure Elucidation and Synthesis†

Roberto G. S. Berlinck,*,‡,§ Robert Britton,§ Edward Piers,*,|| Lynette Lim,⊥ Michel Roberge,⊥ Rosana Moreira da Rocha,⊗ and Raymond J. Andersen*,§

Departments of Chemistry and Earth and Ocean Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z1, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3, and Departamento de Zoologia, Setor de Ciencias Biologicas, Universidade Federal do Parana, Curitiba, Parana, Brazil

Received August 6, 1998

Crude methanol extracts of the ascidian *Didemnum granulatum* collected in Brazil showed activity in a new screen for G2 cell cycle checkpoint inhibitors. Bioassay-guided fractionation of the extract yielded the known alkaloids didemnimides A (**1**) and D (**2**), the new alkaloid didemnimide E (**3**), and a new G2 checkpoint inhibitor. Two candidate structures for the inhibitor, named granulatimide (**4**) and isogranulatimide (**5**), have been prepared via a short and efficient biomimetic synthesis involving the photolysis of didemnimide A (**1**). The synthesis revealed that the correct structure for the naturally occurring G2 checkpoint inhibitor is isogranulatimide (**5**). Granulatimide (**4**), the other candidate structure, was also found to be a G2 checkpoint inhibitor, and it was subsequently detected in chromatographic fractions associated with purification of *D. granulatum* alkaloids. Granulatimide (**4**) and isogranulatimide (**5**) represent the first examples of a new class of G2 specific cell cycle checkpoint inhibitors and the first ones identified through a rational screening program.

Introduction

The ultimate goal in the chemotherapeutic treatment of cancer is to develop drugs that are highly effective against tumor cells and have little or no effect on healthy cells. Recent discoveries of clear genetic differences between healthy and cancer cells have presented a number of interesting opportunities for developing strategies to selectively target cancer cells by taking advantage of their genetic abnormalities. One such approach would be to use a DNA damaging agent in combination with a G2 specific cell cycle checkpoint inhibitor.[1]

Normal cells respond to DNA damage by activating checkpoints that temporarily halt growth and division to allow time for DNA repair.[2] The G1 checkpoint allows DNA repair before it is replicated in S phase, and the G2 checkpoint permits DNA repair before chromosomes are segregated in mitosis, thus preventing the propagation of genetic abnormalities. Cells with mutated p53 tumor suppressor genes (≈50% of all solid tumor cancer cells) are unable to activate the G1 checkpoint in response to DNA damage. However, the G2 checkpoint, although usually weaker than in normal cells, still provides them with an opportunity to repair their DNA after damage and to continue successfully dividing. Inhibitors of the G2 checkpoint when used alone should have no effect on normal or cancer cells. When used in combination with a DNA-damaging agent, they should have little effect on the ability of normal cells to survive, but should dramatically increase the killing of p53− cancer cells. This is because normal cells should have time to repair DNA damage as they can still activate their G1 checkpoint, and their G2 checkpoint is typically strong and would require a higher dose of checkpoint inhibitor to be blocked. However, p53− cancer cells, which lack a G1 checkpoint and have a weak G2 checkpoint, should be forced into a premature and lethal mitosis.

Very few G2 checkpoint inhibitors are known. Two groups have been found serendipitously: the purine analogues (caffeine, pentoxifylline, etc.), and staurosporine and UCN-01 (7-hydroxystaurosporine).[3] In a variety of paired cell lines differing only in their p53 status, G2 checkpoint inhibitors induced greater sensitivity to DNA damage in p53− cells than in p53+ cells.[4] For example,

* Address correspondence to Raymond Andersen, Department of Chemistry, University of British Columbia, Vancouver, B.C., V6T 1Z1. FAX 604 822 6091. Email: randersn@unixg.ubc.ca.

† Dedicated to Professor Sergio de Almeida Rodrigues on the occasion of his 60th birthday.

‡ On leave from the Instituto de Quimica de Sao Carlos, Universidade de Sao Paulo, CP. 780, CEP 13560−970, Sao Carlos, SP, Brazil. Tel. 00-55-16-2739954. Fax: 00-55-16-2739987. Email: rberlink@iqsc.sc.usp.br.

§ Departments of Chemistry and Earth and Ocean Science, University of British Columbia.

|| Department of Chemistry, University of British Columbia.

⊥ Department of Biochemistry and Molecular Biology, University of British Columbia.

⊗ Universidade Federal do Parana.

(1) (a) Holloway, S. L.; Glotzer, M.; King, R. W.; Murray, A. W. *Cell* **1993**, *73*, 1393. (b) Weinert, T.; Lydall, D. *Seminars Cancer Biol.* **1993**, *4*, 129. (c) Nurse, P. *Cell* **1997**, *91*, 865. (d) Kao, G. D.; McKenna, W. G.; Maity, A.; Blank, K.; Muschel, R. J. *Cancer Res.* **1997**, *57*, 753.

(2) (a) Hartwell, L.; Weinert, T.; Kadyk, L.; Garvick, B. *Cold Spring Harbor Symp. Quantum Biol.* **1994**, *59*, 259. (b) Kaufmann, W. K.; Paules, R. S. *FASEB J.* **1996**, *10*, 238.

(3) (a) Busse, P. M.; Bose, S. K.; Jones, R. W.; Tomlach, L. J. *Radiat. Res.* **1978**, *76*, 292. (b) Schlegel, R.; Pardee, A. B. *Science* **1986**, *232*, 1264. (c) Downes, C. S.; Musk, S. R. R.; Watson, J. V.; Johnston, R. T. *J. Cell Biol.* **1990**, *110*, 1855. (d) Steinmann, K. E.; Belinski, G. S.; Lee, D.; Schlegel, R. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 6843. (e) Andreassen, P. R.; Margolis, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2272. (f) Tam, S. W.; Schlegel, R. *Cell Growth Differ.* **1992**, *3*, 811. (g) Wang, Q.; Fan, S.; Eastman, A.; Worland, P. J.; Sausville, E. A.; O'Connor, P. M. *J. Natl. Cancer Inst.* **1996**, *88*, 956.

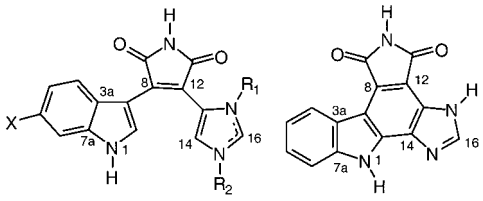
10.1021/jo981607p CCC: $15.00
Published on Web 11/25/1998

pentoxifylline enhanced cisplatin-induced killing of p53− MCF-7 cells 30-fold. The known G2 checkpoint inhibitors are of limited clinical use because they also interact with a broad range of other cellular processes. However, the demonstration that even imperfect G2 checkpoint inhibitors can potentiate the cytotoxicity of DNA-damaging agents in a manner specific for p53− cells suggests that a rational search for novel inhibitors could yield agents with greater specificity and potency. It is therefore of considerable importance to find new and specific checkpoint inhibitors that may form the basis of a different approach to cancer treatment.

The first high-throughput bioassay for G2 cell cycle checkpoint inhibitors has recently been developed in one of our laboratories.[5] This ELISA-based screen has been used to evaluate ca. 1300 extracts of marine invertebrates and marine bacterial cultures for G2 checkpoint inhibitors. Crude methanol extracts of the ascidian *Didemnum granulatum* collected in Brazil showed promising activity in the assay. Fractionation of *D. granulatum* extracts led to the isolation of the known alkaloids didemnimides A (**1**) and D (**2**)[6] and a new alkaloid didemnimide E (**3**), which are all inactive in the checkpoint assay. A small amount of a novel G2-specific cell cycle checkpoint inhibitor was also isolated. Two candidate structures for the inhibitor, named granulatimide (**4**) and isogranulatimide (**5**), have been prepared via a short and efficient biomimetic synthesis involving the photolysis of didemnimide A (**1**). The synthesis revealed that the correct structure for the naturally occurring G2 checkpoint inhibitor was isogranulatimide (**5**). Granulatimide (**4**),[7] the other candidate structure, was also found to be an in vitro G2 checkpoint inhibitor, and it was subsequently detected in chromatographic fractions associated with purification of *D. granulatum* alkaloids, possibly as an artifact. Herein we report the structures of the novel alkaloids didemnimide E (**3**), granulatimide (**4**), and isogranulatimide (**5**) and the total synthesis of didemnimide A (**1**), granulatimide (**4**), and isogranulatimide (**5**).

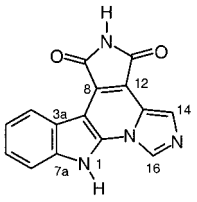
Results and Discussion

The ascidian *D. granulatum* occurs in rocky, shallow water marine habitats along the coastline of southern Brazil. Specimens of *D. granulatum* were collected by hand using SCUBA at three different sites, and the freshly collected ascidians were immediately immersed in ethanol and stored at −20 °C. Further workup involved decanting the ethanol followed by blending the ascidian tissue with methanol and extracting the solid residue multiple times with fresh methanol. The combined crude ethanol/methanol extract showed promising activity in a novel assay designed to detect G2 checkpoint inhibitors.[5] Initial fractionation of the crude methanol extract from the Araca Beach, Sao Sebastiao collection of *D. granulatum* via Sephadex LH20 (MeOH) chromatography gave a number of deep red and orange colored fractions. One of these fractions showed G2 cell cycle checkpoint inhibition activity, and further purification of that fraction by C18 RPHPLC yielded the pure G2 checkpoint inhibitor isogranulatimide (**5**) as a red amorphous solid. RPHPLC fractionation of colored but inactive fractions gave pure didemnimides A (**1**) and D (**2**). Fractionation of the crude methanol extracts of a second collection of *D. granulatum* made at Arquipelago do Arvoredo and in the Sao Sebastiao Channel using the same chromatography procedures gave the new alkaloid didemnimide E (**3**) as the major constituent, in addition to **1**, **2**, **4**, and **5**. The known alkaloids didemnimides A (**1**) and D (**2**) were identified by comparison of their spectroscopic data with the literature values.[6]

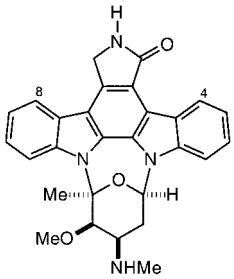


1 R_1 = H, X = H
2 R_1 = Me, X = Br
3 R_2 = Me, X = H
6 R_1 = Me, X = H

4 Granulatimide



5 Isogranulatimide



7 Staurosporine

Didemnimide E (**3**) was obtained as an orange amorphous solid that gave a $[M + H]^+$ ion at *m/z* 293.1048 in the HRFABMS appropriate for the molecular formula $C_{16}H_{12}N_4O_2$, identical with the molecular formula of didemnimide C (**6**). Comparison of the ^1H and ^{13}C NMR data obtained for didemnimide E (**3**) with the literature data for didemnimide C (**6**)[6] indicated that the molecules were closely related (Tables 1 and 2). The ^1H and ^{13}C NMR spectra of didemnimide E (**3**), like the NMR spectra of didemnimide C (**6**), showed well-resolved resonances for all the protons and carbons in the molecule, indicating that the tautomerism which caused signal broadening in the NMR spectra of didemnimide A (**1**) was no long present. Significant differences in the ^1H NMR data for the two compounds was only observed in the chemical shifts of the *N*-methyl and imidazole proton resonances (Table 1), indicating that didemnimide E (**3**) and didemnimide C (**6**) simply differed in the position of imidazole *N*-methylation. The HMBC data obtained for didemnimide E (**3**) showed correlations between the *N*-methyl proton resonance at δ 3.45 (Me-18) and two proton-bearing imidazole carbon resonances at δ 124.3 (C-14) and 137.2 (C-16), confirming the structure of didemnimide E (**3**). All of the additional COSY, HMQC, and HMBC

(4) (a) Russell, K. J.; Wiens, L. W.; Demers, G. W.; Galloway, D. A.; Plon, S. E.; Groudine, M. *Cancer Res.* **1995**, *55*, 1639. (b) Powell, S. N.; DeFrank, J. S.; Connell, P.; Eogan, M.; Preffer, F.; Dombkowski, D.; Tang, W.; Friend, S. *Cancer Res.* **1995**, *55*, 1643. (c) Russell, K. J.; Wiens, L. W.; Demers, G. W.; Galloway, D. A.; Le, T.; Rice, G. C.; Bianco, J. A.; Singer, J. W.; Groudine, M. *Int. J. Radiat. Oncol. Biol. Phys.* **1996**, *36*, 1099. (d) Yao, S. L.; Akhtar, A. J.; McKenna, K. A.; Bedi, G. C.; Sirdransky, D.; Mabry, M.; Ravi, S. J.; Collector, M. I.; Jones, R. J.; Sharkis, S. J.; Fuchs, E. J.; Bedi, A. *Nature Med.* **1996**, *2*, 1140. (e) Bracey, T. S.; Williams, A. C.; Paraskeva, C. *Clin. Cancer Res.* **1997**, *3*, 1371.

(5) Roberge, M.; Berlinck, R. G. S.; Xu, L.; Anderson, H.; Lim, L.; Curman, D.; Stringer, C. M.; Friend, S. H.; Davies, P.; Vincent, I.; Haggarty, S.; Kelly, M. T.; Britton, R.; Piers, E. Andersen, R. J. *Cancer Res.* **1998**, in press.

(6) Vervoort, H. C.; Richards-Gross, S. E.; Fenical, W.; Lee, A. Y.; Clardy, J. *J. Org. Chem.* **1997**, *62*, 1486−1490.

(7) The structure and synthesis of granulatimide was reported at the 9th International Symposium on Marine Natural Products, Townsville, Australia, July 6−10, 1998.

Table 1. ^{1}H NMR Data for Didemnimides and Granulatimides. Recorded in DMSO-d_6

atom no.	didemnimide A (**1**)[6]	didemnimide C (**6**)[6]	didemnimide E (**3**)	granualtimide (**4**)	isogranulatimide (**5**)
1	12.45, bs	12.02, bs	12.31, bs	12.58, bs	13.48, bs
2	8.05, bs	8.07, bs	8.20, bs	–	–
4	7.07, br m	6.38, d, 8 Hz	6.43, d, J = 7 Hz	8.89, d, J = 7 Hz	8.51, d, J = 7 Hz
5	6.87, br m	6.77, dd, 8, 8 Hz	6.90, dd, J = 7, 7 Hz	7.30, dd, J = 7, 7 Hz	7.35, dd, J = 7, 7 Hz
6	7.07, br m	7.10, dd, 8, 8 Hz	7.16, dd, J = 7, 7 Hz	7.48, dd, J = 7, 7 Hz	7.43, dd, J = 7, 7 Hz
7	7.39, d, J = 7.8 Hz	7.44, d, 8, Hz	7.50, dd, J = 7, 7 Hz	7.61, d, J = 7 Hz	7.67, d, J = 7 Hz
10	11.66, bs	11.13, bs	11.37, bs	10.96, s	11.11, bs
14	7.71, bs	7.07, s	7.75, bs	–	8.10, b
16	7.68, bs	7.70, s	9.07, bs	8.50, s	9.12, b
17	10.87, bs			13.57, s	–
18	–	3.15, s	3.45, s	–	–

Table 2. ^{13}C NMR Data for Didemnimides and Granulatimides. Recorded in DMSO-d_6

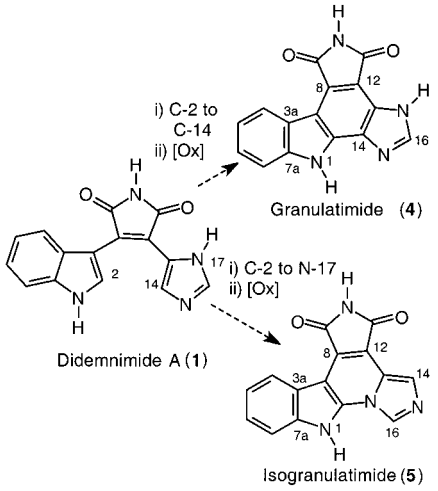
C no.	didemnimide A (**1**)[6]	didemnimide C (**3**)[6]	didemnimide E[d] (**3**)	granulatimide[d] (**4**)	isogranulatimide[d] (**5**)
2	130.7	131.8	133.4	135.4[c]	n.o.
3	104.7	105.2	104.4	113.0	97.9
3a	125.6	125.0	124.3	121.4	121.0
4	121.7	119.9	119.1	123.8	122.4
5	119.6	120.8	121.3	120.0	121.9
6	121.7	122.6	122.9	126.1	124.8
7	112.0	112.5	112.9	111.6	112.4
7a	136.5	136.6	136.7	140.4	135.5
8	n.o.	134.1	138.2[a]	122.7[a]	126.4[a]
9	172.9[a]	172.0[a]	170.9[b]	169.8[b]	169.0[b]
11	172.8[a]	171.8[a]	171.2[b]	171.0[b]	170.0[b]
12	n.o.	134.1	113.5[a]	109.5[a]	114.8[a]
13	125.7	122.6	122.3	125.7[c]	n.o.[e]
14	119.6	131.8	124.3	133.4[c]	n.o.
16	136.5	140.4	137.2	144.5	n.o.
NMe		32.3	34.3		

[a–c] May be interchanged. [d] Assignments are based on HMQC and HMBC data. [e] Not observed.

data obtained for didemnimide E (**3**) were consistent with the proposed structure.

The G2 checkpoint inhibitor gave a $[M + H]^+$ ion in the HRFABMS at m/z 277.0730 appropriate for a molecular formula of $C_{15}H_8N_4O_2$. Its UV spectrum (MeOH) showed bands at 210, 231, 280, and 470 nm consistent with a polycyclic aromatic chromophore, and its IR spectrum contained bands at 3294 and 1678 cm^{-1} indicating NH and amide carbonyl groups. The molecular formula of the inhibitor differed from that of didemnimide A (**1**) only by the loss of two hydrogen atoms, and a comparison of their NMR data (Tables 1 and 2) showed that the two compounds were closely related. A NH proton resonance at δ 11.11 gave HMBC correlations to carbonyl resonances at δ 169.0 and 170.0 and sp^2 carbon resonances at δ 126.4 and 112.2, confirming the presence of a maleimide substructure in the inhibitor. The COSY spectrum identified a four-proton spin system (δ 8.51, d, J = 7 Hz (H-4)); 7.43, dd, J = 7, 7 Hz (H-6); 7.35, dd, J = 7, 7 Hz (H-5); 7.67, d, J = 7 Hz (H-7)) that could be assigned to the H-4 to H-7 protons on an indole residue. Irradiation of a broad proton resonance at δ 13.48 induced a NOE only in the aromatic doublet at δ 7.68, which assigned the doublet to H-7 and the broad proton resonance to the indole NH. The absence of resonances in the ^{1}H NMR spectrum of the inhibitor that could be assigned to the indole H-2 or H-3 protons indicated the indole nucleus had substituents at both C-2 and C-3. Subtraction of the atoms present in the maleimide and indole fragments ($C_{12}H_6N_2O_2$) from the molecular formula of the inhibitor left $C_3H_2N_2$. These atoms could be accounted for by a disubstituted imidazole moiety, leading to the two polycyclic aromatic candidate structures **4**[7] and **5** that represent alternative modes of forming a bond between the C-2 carbon of the indole fragment and either a carbon (C-14) or nitrogen atom (N-17) of the imidazole fragment in the putative biosynthetic precursor didemnimide A (**1**) (Scheme 1).

Scheme 1



Evidence in support of a planar polycyclic aromatic structure for the checkpoint inhibitor could be found in the ^{1}H NMR data. The large chemical shift observed for the H-4 resonance (δ 8.51) in the inhibitor relative to the chemical shift observed for the H-4 resonance in didemnimide A (**1**) (δ 7.07) can be attributed to a neighboring group effect from the C-9 maleimide carbonyl in either candidate structure **4** or **5**. A similar diamagnetic anisotropic effect deshields the H-4 resonance (DMSO-d_6 δ 9.30) relative to the H-8 resonance (δ 7.96) in staurosporine (**7**).[8] The presence of the neighboring group deshield-

(8) Takahashi, I.; Saitoh, Y.; Yoshida, M.; Sano, H.; Nakano, H.; Morimoto, M.; Tamaoki, T. *J. Antibiot.* **1989**, *42*, 571–576.

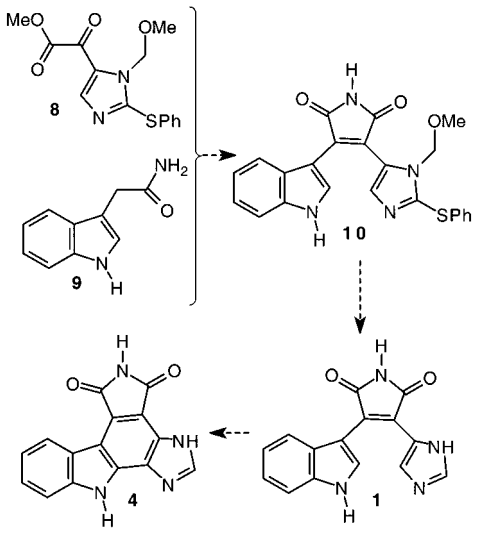
ing effect of the maleimide carbonyl in the checkpoint inhibitor and not in didemnimide A (**1**) is consistent with the presence of a rigid planar heterocycle in the inhibitor, in contrast to the uncyclized didemnimide A (**1**), where the X-ray data shows that the indole and maleimide rings are twisted relative to each other along the C-3 to C-8 bond.[6]

In principle, a variety of different NMR experiments should readily distinguish between the candidate structures **4** and **5**. However, the remaining two resonances at δ 8.10 and 9.12 in the ^{1}H NMR spectrum, that could be assigned to the imidazole fragment of the inhibitor, gave very broad signals that failed to show any HMBC/HMQC correlations or NOEs precluding a clear spectroscopic resolution to the structural problem. The broadness of the imidazole resonances in the NMR spectra of the checkpoint inhibitor was attributed to a tautomeric equilibrium involving the NH proton. Similar broadening of the imidazole CH and NH proton resonances was observed in the ^{1}H NMR spectrum of didemnimide A (**1**) and related analogues which do not have a methyl substituent on one of the imidazole nitrogens.[6] Didemnimide C (**6**) and E (**3**), which do have methyl substitution on one of the imidazole ring nitrogens, give sharp, well-resolved NMR spectra. By analogy with the didemnimides, only one of the candidate structures for the checkpoint inhibitor, i.e., **4**, would be expected to undergo tautomeric equilibrium involving an imidazole NH. Therefore, the observed broadening of the ^{1}H NMR signals assigned to the imidazole fragment of the inhibitor appeared to be most consistent with candidate structure **4**. From a biogenetic perspective, it seemed reasonable that the co-occurring alkaloid didemnimide A (**1**) represented a logical precursor to either candidate structure **4** or **5** (Scheme 1). Structure **4** is related to staurosporine (**7**),[9] arcyriaflavins,[10] and other polycyclic naturally occurring bisindole maleimides, whereas structure **5** is apparently without precedent among natural products. Therefore, from a biogenetic perspective, **4** was also deemed to be the most probable structure.

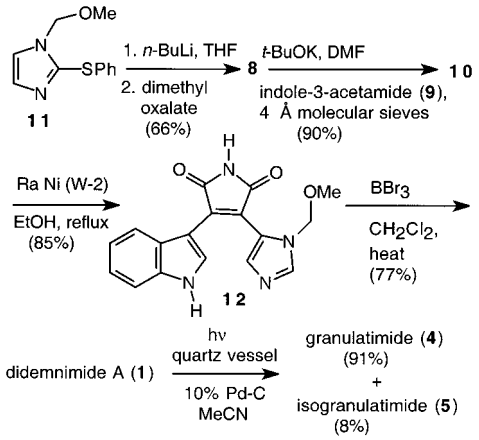
Since it was not possible to distinguish between candidate structures **4** and **5** using the available spectroscopic data, it was decided to resolve the issue by total synthesis. The synthesis was also required to generate sufficient material for complete biological evaluation. Based on the combination of chemical and biogenetic arguments presented above, candidate structure **4**, which we named granulatimide, was felt to be the most probable structure for the checkpoint inhibitor, and it was chosen as the initial synthetic target.

The highly convergent total synthesis that was envisaged, as outlined in general terms in Scheme 2, was to include two key steps. The first of these was the condensation of the α-keto ester **8** with indole-3-acetamide (**9**), which was expected[11] to furnish the maleimide **10**. The second key transformation was envisioned to be the photochemically induced bond formation[12] between the indole C-2 carbon and the imidazole C-4 carbon (C-14) of the natural product didemnimide A (**1**)[6,13] to afford granulatimide (**4**). Presumably, **1** would be readily derived by removal of the two protecting groups from the imidazole moiety of **10**.

Scheme 2



Scheme 3



The total syntheses of didemnimide A (**1**) and granulatimide (**4**) are summarized in Scheme 3. Treatment of the substituted imidazole **11**[14] with *n*-BuLi in THF at −78 °C, followed by reaction of the resultant 5-lithio imidazole intermediate with dimethyl oxalate, provided the required α-keto ester **8** in 66% yield. The condensation of **8** with indole-3-acetamide (**9**) was effected efficiently by treatment of a solution of the two reactants with *t*-BuOK in warm DMF *in the presence of molecular sieves*. The use of molecular sieves was crucial to the success of this reaction, since, in the absence of this material, a second major product resulting from displacement of the phenylthio group by a methoxyl function (i.e., **10**, OMe in place of SPh) was formed.

(9) Tamaoki, T.; Nomoto, H.; Takahashi, I.; Kato, Y.; Morimoto, M.; Tomita, F. *Biochem. Biophys. Res. Commun.* **1986**, *135*, 397.

(10) Steglich, W.; Steffan, B.; Kopanski, L.; Eckhardt, G. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 459.

(11) Related condensations of indole-3-acetamide and other monosubstituted acetamides with dialkyl oxalates have been reported. See, for example, (a) Rooney, C. S.; Randall, W. C.; Streeter, K. B.; Zeigler, C.; Cragoe, E. J.; Schwam, H.; Michelson, S. R.; Williams, H. W. R.; Eichler, E.; Duggan, D. E.; Ulm, E. H.; Noll, R. M. *J. Med. Chem.* **1983**, *26*, 6, 700. (b) Neel, D. A.; Jirousek, M. R.; McDonald, J. H. Bioorg. *Med. Chem. Lett.* **1998**, *8*, 47. After the work reported herein had been completed and accepted for publication, a paper describing condensations similar to **8** + **9** → **10** was published: Faul, M. M.; Winneroski, L. L.; Krumrich, C. A. *J. Org. Chem.* **1998**, *63*, 6053.

(12) For related cyclizations, see (a) Rawal, V. H.; Jones, R. J.; Cava, M. P. *Tetrahedron Lett.* **1985**, *26*, 2423. (b) Rawal, V. H.; Jones, R. J.; Cava, M. P. *J. Org. Chem.* **1987**, *52*, 2, 19. (c) Terpin, A.; Winklhofer, C.; Schumann, S.; Steglich, W. *Tetrahedron* **1998**, *54*, 1745.

(13) For the first total synthesis of a didemnimide (didemnimide C) via a route less convergent than that reported herein for didemnimide A, see ref 12c.

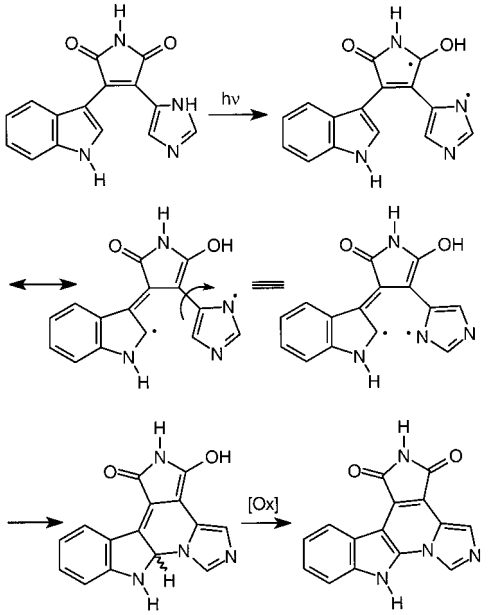
(14) Achab, S.; Guyot, M.; Potier, P. *J. Am. Chem. Soc.* **1978**, *100*, 3918, and references cited therein.

Treatment of the tetracycle **10** with W-2 Raney nickel in refluxing EtOH[15] furnished an excellent yield of the desulfurized product **12**. Removal of the methoxymethyl group was accomplished by reaction of the latter substance with 10 equiv of boron tribromide in refluxing CH_2Cl_2. Reaction at room temperature and (or) use of lesser amounts of the reagent resulted in the recovery of considerable amounts of starting material. The product of this transformation, didemnimide A (**1**), exhibited spectral data identical with those reported[6] for the natural product.

Photolysis of a solution of didemnimide A (**1**) in acetonitrile containing a small amount of palladium on carbon[12a] produced, in 91% yield, granulatimide (**4**), and in 8% yield, isogranulatimide (**5**). The spectroscopic data obtained for granulatimide (**4**) were in full accord with the assigned structure. A series of difference NOE experiments were particularly instrumental in assigning the ^{1}H NMR spectra and confirming the structure. Irradiation of a broad singlet at δ 12.58 induced a NOE in a doublet at δ 7.61, thereby assigning the broad singlet to the indole N*H* and the doublet to H-7. Similarly, irradiation of the broad singlet at δ 13.57 induced a NOE in a sharp singlet at δ 8.5, which led to the assignment of the downfield singlet to the imidazole N*H* and the upfield singlet to H-16. It is interesting to note, that contrary to our expectations, granulatimide (**4**) gives sharp, well-resolved ^{1}H NMR resonances for all of the hydrogen atoms in the molecule. The imidazole N*H* in granulatimide (**4**) is strongly deshielded (i.e., δ 13.57), and it does not show a NOE to the indole N*H*, suggesting that it participates in a strong intramolecular hydrogen bond to the maleimide C-11 carbonyl. Semiempirical calculations carried out on the *H*N-17 and *H*N-15 tautomers of granulatimide (**4**) showed that the *H*N-17 tautomer is approximately 7 kcal lower in energy.[16] Calculations also show that the *H*N-15 tautomer has a severe steric interaction between the indole N*H* (N-1) and imidazole N*H* (N-15) that leads to a distortion of the planar aromatic heterocycle. The combination of an intramolecular hydrogen bond stabilizing the *H*N-17 tautomer and a severe steric interaction destabilizing the *H*N-15 tautomer may be responsible for suppressing the imidazole NH tautomerization in granulatimide (**4**).

Comparison of the NMR data for synthetic granulatimide (**4**) with the data for the checkpoint inhibitor isolated from the extract of the first *D. granulatum* collection (Tables 1 and 2) clearly revealed that the two molecules were different. Therefore, we concluded that the G2 checkpoint inhibitor must be isogranulatimide (**5**), the only alternate candidate structure consistent with the spectroscopic data and a biogenesis from didemnimide A (**1**) (Scheme 1). A minor product from the photochemical conversion of didemnimide A (**1**) to granulatimide (**4**) had TLC properties identical with the natural isogranulatimide (**5**). Their ^{1}H NMR spectra were also nearly identical in all respects, except for the two resonances that could be assigned to the imidazole H-14 and H-16 protons. The synthetic product gave sharp, well-resolved singlets at δ 7.88 and 8.93, while the natural sample gave broad resonances at δ 8.10 and 9.12.

Scheme 4



These NMR discrepancies were attributed to the use of different chromatographic procedures to purify the two samples resulting in different protonation states for the natural and synthetic isogranulatimides. Therefore, both the natural and synthetic samples were repurified using identical reversed phase HPLC conditions (1:1 CH_3CN−0.05% TFA) resulting in them having identical HPLC retention times and ^{1}H NMR spectra. The formation of isogranulatimide (**5**) via the photolysis of didemnimide A (**1**), while unanticipated, confirms the structure of the natural product. Scheme 4 proposes a possible radical mechanism for this interesting transformation.[17]

During the investigation of the photochemical conversion of didemnimide A (**1**) to granulatimide (**4**), it was discovered that the reaction proceeded rapidly and in good yield in DMSO upon irradiation with natural sunlight and oxidation with air. *D. granulatum* grows in very shallow water where it is exposed to intense sunlight and didemnimide A (**1**) is a major constituent of *D. granulatum* extracts, so it seemed reasonable to expect that granulatimide (**4**) would be a co-occurring metabolite. Granulatimide (**4**) is extremely insoluble in most common organic solvents including methanol and ethanol, and it can only be effectively solubilized with DMF or DMSO. Therefore, in hindsight it was apparent that our standard extraction procedures using ethanol/methanol would be expected to yield only small amounts of **4**. Nevertheless, with synthetic granulatimide (**4**) as a TLC reference it was possible to identify **4** in chromatography fractions generated during purification of *D. granulatum* alkaloids. Unfortunately, the extracted ascidian tissue had been thrown away by the time the solubility and chromatographic properties of synthetic granulatimide (**4**) were discovered. Therefore, the true concentration of granulatimide in *D. granulatum* and the issue of whether the granulatimide found in the chromatography fractions is an artifact formed by photochemical transformation of didemnimide A (**1**) during fractionation or a bona fide natural product remain to be resolved by a future collection of *D. granulatum*.

(15) Ohta, S.; Yamamoto, T.; Kawasaki, I.; Yamashita, M.; Katsuma, H.; Nasako, R.; Kobayashi, K.; Ogawa, K. *Chem. Pharm. Bull.* **1992**, *40*, 2681.

(16) Calculations were performed using HyperChem release 4 for Windows, Polack-Ribiere Alogrithm, in vacuo.

(17) See: *CRC Handbook of Organic Photochemistry and Photobiology*; Harspool, W. M., Ed., CRC Press: New York, 1995; p 513.

Granulatimide (**4**) and isogranulatimide (**5**) are novel alkaloids that both possess heterocyclic aromatic skeletons without precedent in natural products. Biogenetically they represent alternative modes of cyclization of the putative precursor didemnimde A (**1**) (Scheme 1). A short and highly efficient biomimetic synthesis that gives granulatimide (**4**) in high yield and isogranulatimide (**5**) in relatively low yield has been completed (Scheme 3). The facile photochemical transformation of didemnimide A (**1**) into both granulatimide (**4**) and isogranulatimide (**5**) in the laboratory suggests that a similar photochemical reaction may be responsible for their formation in *D. granulatum*. Granulatimide (**4**) and isogranulatimide (**5**) are both G2 specific cell cycle checkpoint inhibitors with in vitro IC_{50} values in the range of 1 to 1.8 μM.[5] These novel alkaloids represent a new structural class of G2 checkpoint inhibitors and the first one discovered as part of a rational screening program. The availability of synthetic granulatimide (**4**) and isogranulatimide (**5**) should facilitate their use as tools to test, for the first time in vivo, the hypothesis that a G2 checkpoint inhibitor combined with a DNA damaging agent will lead to selective killing of p53− cancer cells.

Experimental Section

General Experimental Procedures. TLC was carried out by using commercial aluminum-backed silica gel 60 plates. Flash chromatography[18] was carried out with 230−400 mesh silica gel (E. Merck). THF was distilled from sodium/benzophenone, and CH_2Cl_2 was distilled from CaH_2. Commercial EtOH (reagent grade) and MeCN (HPLC grade) were used without further purification. Molecular sieves were dried under vacuum with heating for 5 h prior to use. All reactions were carried out under an atmosphere of dry argon using glassware that had been thoroughly flame dried.

Collection and Extraction of *D. granulatum*. The ascidian *D. granulatum* (85 g wet wt.) was collected during the summer of 1995 at depths of 1 m at the rocky shore of Araca beach, Sao Sebastiao (Sao Paulo state, southeastern Brazil). A second collection was made at the Arquipelago do Arvoredo (Santa Catarina state, southern Brazil. 150 g wet wt.) and in the Sao Sebastiao Channel (Sao Paulo state. 185 g wet wt.) during November 1997. Freshly collected animals were stored in ethanol at −20 °C. Animals obtained from the different collections were processed separately as follows: after decantation of ethanol, the animal was blended and extracted three times with methanol. The ethanol and methanol extracts were combined, filtered, and evaporated in vacuo. The organic material was dissolved in 8:2 MeOH−CH_2Cl_2 and filtered for elimination of inorganic salts.

Isolation and Structure Determination. The organic extract (1.7 g) obtained from the first collection of *D. granulatum* was subjected to chromatography on Sephadex LH20 (MeOH), affording eight fractions with clearly distinctive constituents by TLC (CH_2Cl_2−MeOH 9:1, UV at 254 nm). The seventh fraction (0.0131 g) which exhibited G2 cell cycle checkpoint inhibition activity contained almost pure isogranulatimide (**5**). RPHPLC purification of this fraction (1:1 CH_3CN−0.05% TFA) yielded pure isogranulatimide (**5**) (0.006% wet wt.). RPHPLC purification (30:70 CH_3CN−0.05% TFA) of individual inactive fractions yielded didemnimides A (**1**) (0.004% wet wt.)[6] and D (**2**) (0.004% wet wt.).[6]

The organic extracts obtained (total weight: 5.8 g) from the second collection were separately subjected to chromatography on Sephadex LH20 (MeOH), giving fractions with compositions similar to those from the first collection as determined by TLC and RP HPLC analyses. These fractions were pooled according to TLC properties and further separated by RP HPLC to give pure didemnimide A (**1**), didemnimide D (**2**), didemnimide E (**3**) (0.012% wet wt.), and isogranulatimide (**5**). Once synthetic granulatimide (**4**) was in hand as an authentic TLC reference (see below), small amounts of **4** were also identified in the pooled chromatography fractions.

Didemnimide E (3). Obtained as an orange amorphous solid; for ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HRFABMS *m/z* 293.1048 $[M + H]^+$, calcd for $C_{16}H_{13}N_4O_2$, 293.1039.

Isogranulatimide (5). Obtained as a red amorphous solid; UV (MeOH) λ_{max} (ϵ) 210 (10234), 231 (10648), 280 (6552), 470 (1876); IR (film) 3294, 1678, 1586, 1568, 1421, 1367, 1205, 1135, 802, 745, 724 cm^{-1}; for ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HRFABMS *m/z* 277.0730 $[M + H]^+$, calcd for $C_{15}H_9N_4O_2$, 277.0714.

Synthesis. Methyl 2-(1-(methoxymethyl)-2-(phenylthio)imidazol-5-yl)glyoxylate (8). To a cold (−78 °C), stirred solution of 1-(methoxymethyl)-2-(phenylthio)imidazole[15] (**11**) (340 mg, 1.55 mmol) in dry THF (8.0 mL) was added a solution of *n*-BuLi (1.47 M in hexanes, 1.26 mL, 1.85 mmol) and the mixture was stirred for 1 h. A solution of dimethyl oxalate (540 mg, 4.58 mmol) in dry THF (2.0 mL) was added, and the mixture was stirred at −78 °C for an additional 1.25 h. The reaction mixture was treated with saturated aqueous NH_4Cl (5.0 mL) and Et_2O (20 mL). The phases were separated, and the aqueous layer was extracted with Et_2O (25 mL). The combined organic extracts were washed (brine, 10 mL), dried ($MgSO_4$), and concentrated. Flash chromatography (35 g of silica gel, 1:1 petroleum ether−Et_2O) of the crude material afforded 305 mg (66%) of **8** as a beige solid that exhibited mp 59−60 °C; IR (KBr) 1729, 1657, 1305, 1273, 1167, 1114, 748 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.23 (s,1 H), 7.52−7.54 (m, 2 H), 7.37−7.38 (m, 3 H), 5.81 (s, 2 H), 3.91 (s, 3 H), 3.36 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.2, 161.6, 154.1, 145.4, 145.3, 133.2, 129.5, 129.2, 128.7, 75.8, 56.5, 53.0; HREIMS calcd for $C_{14}H_{14}N_2O_4S$ 306.0674, found 306.0674. Anal. Calcd C, 54.89; H, 4.61; N, 9.15. Found: C, 55.00; H, 4.66; N, 8.94.

3-[4-(1-(Methoxymethyl)-1*H*-2-(phenylthio)imidazol-5-yl)-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl]indole (10). To a stirred solution of *t*-BuOK (150 mg, 1.34 mmol) in DMF (3.0 mL) at room temperature were added sequentially 4 Å molecular sieves (∼500 mg) and a solution of **8** (167 mg, 0.54 mmol) and indole-3-acetamide **9** (114 mg, 0.66 mmol) in DMF (4.0 mL). The reaction mixture was heated to 45 °C and stirred for 12 h. The dark purple solution was treated with hydrochloric acid (1 N, 3.0 mL) and EtOAc (30 mL). The phases were separated, and the aqueous layer was extracted with EtOAc (2 × 10 mL). The combined organic extracts were washed (brine, 4 × 15 mL), dried ($MgSO_4$), and concentrated. Flash chromatography (25 g of silica gel, 30:1 CH_2Cl_2−MeOH) of the crude material afforded 208 mg (90%) of **10** as an orange solid that exhibited mp 243 °C; IR (KBr) 3400−2600, 1765, 1708, 1537, 1341, 741 cm^{-1}; ^{1}H NMR (400 MHz) δ 12.11 (br s, 1 H), 11.25 (br s, 1 H), 8.14 (s, 1 H), 7.46 (d, 1 H, J = 7.9 Hz), 7.18−7.33 (m, 6 H), 7.14 (dd, 1 H, J = 8, 8 Hz), 6.77 (dd, 1 H, J = 8, 8 Hz), 6.51 (d, 1 H, J = 8.0), 5.00 (s, 2 H), 2.97 (s, 3 H); ^{13}C NMR (100 MHz) δ 171.7, 171.6, 140.3, 136.7, 135.3, 133.8, 133.5, 132.5, 129.5, 128.4, 127.2, 125.7, 124.3, 122.7, 120.8, 120.2, 117.4, 112.6, 104.6, 75.8, 55.9; HREIMS calcd for $C_{23}H_{18}N_4O_3S$ 430.1099, found 430.1099.

3-[4-(1-(Methoxymethyl)-1*H*-imidazol-5-yl)-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl]indole (12). To a refluxing solution of **10** (52 mg, 0.12 mmol) in EtOH (5.0 mL) was added Raney Ni (W-2, 50% slurry in water, ∼150 mg), and the suspension was refluxed for 1 h. An additional amount of Raney Ni (W-2, 50% slurry in water, ∼100 mg) was added, and the mixture was refluxed for an additional 3 h. The reaction mixture was then cooled to room temperature and filtered through Celite. The Celite was washed with CH_2Cl_2−MeOH (1:1, 75 mL), and the combined organic washes were concentrated. Flash chromatography (14 g of silica gel, 20:1 CH_2Cl_2−MeOH) of the crude material afforded 33 mg (85%) of **12** as an orange solid: mp > 235 °C dec; IR (KBr) 3400−2600, 1765, 1703, 1537, 1440, 1342, 1113 cm^{-1}; ^{1}H NMR (400

(18) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

MHz) δ 12.03 (br s, 1 H), 11.18 (br s, 1 H), 8.07 (s, 1 H), 7.95 (s, 1 H), 7.44 (d, 1 H, $J = 8.5$ Hz), 7.09 (dd, 1 H, $J = 7, 7$ Hz), 7.01 (s, 1 H), 6.79 (dd, 1 H, $J = 7, 7$ Hz), 6.45 (d, 1 H, $J = 8.0$ Hz), 5.02 (s, 2 H), 3.07 (s, 3 H); ^{13}C NMR (100 MHz) δ 171.8, 171.7, 140.5, 136.5, 134.3, 132.9, 131.8, 124.3, 122.4, 121.7, 120.5, 120.3, 118.4, 112.3, 104.7, 76.4, 55.6; HREIMS cacld for $C_{17}H_{14}N_4O_3$ 322.1066, found 322.1066.

Didemnimide A (1). To a stirred suspension of **12** (67.4 mg, 0.209 mmol) in CH_2Cl_2 (15.0 mL) at room temperature was added a solution of BBr_3 in CH_2Cl_2 (1.0 M, 2.1 mL, 2.1 mmol), and the deep blue solution was heated under reflux for 5 h. The mixture was cooled to room temperature and treated with saturated aqueous $NaHCO_3$ (10 mL) and EtOAc (20 mL) and then was stirred for 0.25 h. The phases were separated, and the aqueous layer was extracted with EtOAc (2 × 15 mL). The combined organic extracts were washed (brine, 10 mL), dried ($MgSO_4$), and concentrated. Flash chromatography (20 g of silica gel, 15:1 CH_2Cl_2−MeOH) of the crude material afforded 15.8 mg of recovered **12** as well as 45.0 mg (77%, 100% based on recovered starting material) of **1** as an orange solid: IR (KBr) 3247, 1760, 1708, 1557, 1423, 1345, 1240, 747 cm^{-1}; ^{1}H NMR (400 MHz, major tautomer) δ 12.45 (br s, 1 H), 11.66 (br s, 1 H), 10.87 (br s, 1 H), 8.05 (s, 1 H), 7.71 (br s, 1 H), 7.68 (br s, 1 H), 7.39 (br d, 1 H, $J = 7.8$ Hz), 7.07 (br m, 2 H), 6.87 (br m, 1 H); ^{13}C NMR (100 MHz) δ 172.8, 172.6, 136.1, 135.9, 130.9, 130.5, 126.0, 126.0, 121.7, 121.3, 119.7, 119.2, 112.2, 111.5, 105.0; HREIMS calcd for $C_{15}H_{10}N_4O_2$ 278.0804, found 278.0804.

Granulatimide (4) and Isogranulatimide (5). To a solution of **1** (12.9 mg, 0.046 mmol) in MeCN (5.0 mL) was added a catalytic amount of palladium-on-carbon (10% Pd), and the resulting mixture was sparged with argon for 0.5 h. The mixture was irradiated (450 W Hanovia medium-pressure mercury vapor lamp, quartz reaction vessel) for 6.5 h. The reaction mixture was filtered through Celite, the Celite was washed with DMF (10 mL), and the combined filtrate was concentrated. The remaining material was taken up in EtOAc (30 mL), and the resultant solution was washed (brine, 4 × 20 mL), dried ($MgSO_4$), and concentrated. Flash chromatography (20 g of silica gel, 10:1 CH_2Cl_2−MeOH) of the crude material afforded 11.7 mg (91%) of **4** as a yellow solid and 1.0 mg (8%) of **5** as a red solid. Final purification was achieved on RPHPLC (1:1 CH_3CN−0.05% TFA). **Granulatimide (4):** IR (KBr) 3246, 2925, 1743, 1698, 1328, 1227, 743 cm^{-1}; for ^{1}H NMR (400 MHz), see Table 1; for ^{13}C NMR, see Table 2; HREIMS calcd for $C_{15}H_8N_4O_2$ 276.0647, found 276.0647. **Isogranulatimide (5):** see above and Tables 1 and 2.

Acknowledgment. The authors thank Professor Jose Carlos de Freitas, director of the Centro de Biologia Marinha of the Universidade de Sao Paulo, for providing many facilities during the ascidian collection, and M. Netherton and J. Scheffer for help with the photoreactions. R.G.S.B. thanks the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP) for a visiting professor fellowship (97/06799-6). Financial support was provided by grants from FAPESP (to R.G.S.B.), the National Cancer Institute of Canada (to R.J.A.), the Natural Sciences and Engineering Research Council of Canada (a Postgraduate Fellowship to R.B. and grants to R.J.A. and E.P.), and the Canadian Breast Cancer Research Initiative (to M.R.).

Supporting Information Available: NMR spectra for didemnimide A (**3**), synthetic granulatimide (**4**), natural and synthetic isogranulatimide (**5**), synthetic didemnimide A (**1**), and the synthetic intermediates **8**, **10**, and **12** (41 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO981607P