A New and Simply Available Class of Hydrosoluble Bioconjugates by Coupling Paclitaxel to Hyaluronic Acid through a 4-Hydroxybutanoic Acid Derived Linker

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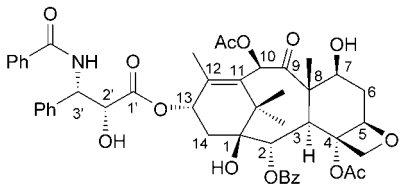
A simple preparation of a new hydrosoluble paclitaxel bioconjugate **8**, representing a new class of paclitaxel derivatives, is described. Bioconjugate **8** was obtained by coupling hyaluronic acid (**2**) to paclitaxel (**1**) by means of a 4-hydroxybutanoic acid derived linker (*Scheme 2*).

Introduction. – Since the 1980s, paclitaxel (**1**) [1], a well-known antitumor diterpenoid [2], has been adopted in the therapy of some cancer types [3]. Owing to its low solubility, it is administered as a castor oil (*Cremophor*®)/EtOH solution. This type of administration requires hospitalization, since side effects, such as hypersensitivity, may occur [4]. Because of this, for many years, extensive research has been carried out to obtain hydrosoluble paclitaxel derivatives.

The search for paclitaxel derivatives with such properties is also stimulated by clinical studies that hypothesize a direct role of concentrated *Cremophor*® in reducing the free paclitaxel fraction because of entrapment in *Cremophor*® micelles [5].

Thus, a number of hydrosoluble derivatives in which paclitaxel is conjugated, *inter alia*, to hydrophilic polymers including hyaluronic acid (HA; **2**), a well-known biopolymer, have been described [6][7].



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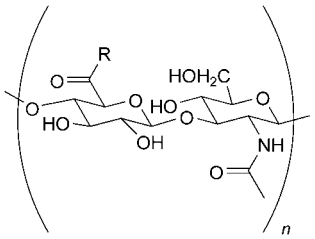
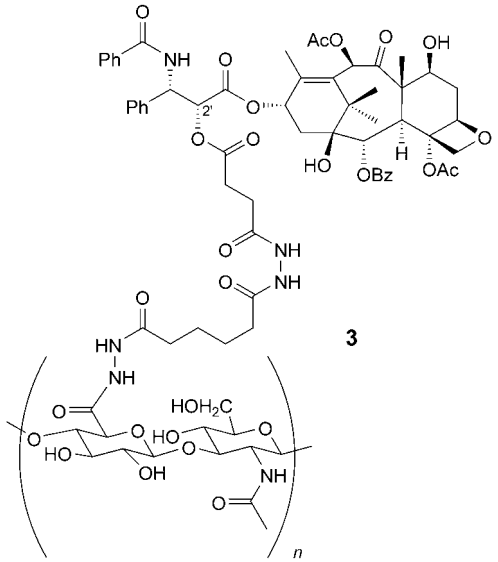
Linking a bioactive compound to a biocompatible polymer offers, in general, several advantages, like drug solubilization, stabilization, localization, and controlled



2 (= HA) R = OH
5 (= HA-ADH) R = ADH = $H_2NNHC(=O)(CH_2)_4C(=O)NHNH$
7 (= HA-TBA) R = $(Bu_4N)O$

release, as pointed out by *Maeda* and co-workers [8]. Furthermore, in the case of some tumors, such as the ovarian and mammalian, whose cells overexpress HA CD44 receptors, linking the antitumor drug to hyaluronic acid (**2**) might allow selective drug targeting.

Bioconjugate **3** (and its fluorescent derivative [7u]), described by *Prestwich* and co-workers some years ago, are the only paclitaxel–HA bioconjugates known to date; releasing of free paclitaxel (**1**) from **3** proceeds by hydrolytic cleavage of the labile 2′-ester linkage [7t][1]).



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Bioconjugate **3** was obtained by converting in two steps paclitaxel (**1**) into paclitaxel 2′-ester **4** containing an *N*-hydroxysuccinimide moiety, and by reacting the latter with adipic dihydrazide modified HA **5** (HA-ADH), prepared in turn from HA (**2**) and adipic dihydrazide (= hexanedioic acid dihydrazide) [7t,u] (*Scheme 1*).

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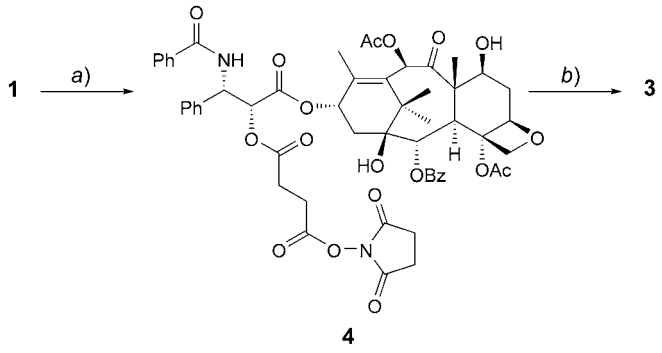
[1]) For a recent review of hydrolysis in drug and prodrug metabolism, see [9].

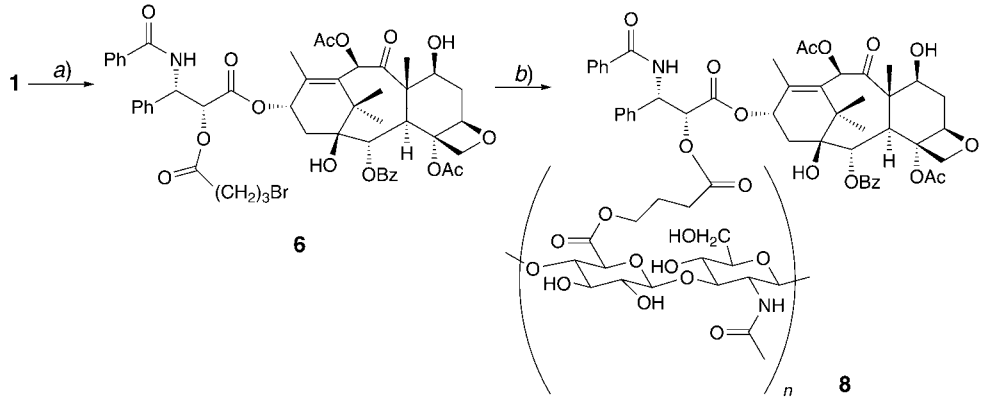
Scheme 1. Luo*'s and* Prestwich*'s Preparation of Bioconjugate* **3** *Starting from* **1**



a) 1) Succinic anhydride, CH_2Cl_2, pyridine, r.t., 3 d. 2) *N*-Hydroxysuccinimido diphenyl phosphate, MeCN, Et_3N, r.t., 6 h. *b*) HA-ADH (**5**), DMF/H_2O 2 : 1, r.t., 24 h.

In the frame of our studies in the field of biopolymers [10] and bioactive natural products [11], wishing to contribute to the access to a hydrosoluble paclitaxel – HA bioconjugate suitable for large-scale production, we assumed that a simple way of achieving this goal would be esterification of HO – C(2′) of paclitaxel (**1**) with 4-bromobutanoic acid to give **6** (*Scheme 2*). Paclitaxel derivative **6**, on reacting with the tetrabutylammonium derivative HA-TBA (**7**)[2]), would then reasonably produce the desired bioconjugate **8**. The type of linkage of paclitaxel (**1**) to HA (**2**) in **8** would be novel, simple, of general access, and not yet adopted to join **2** to **1** or other bioactives.

Scheme 2. *Preparation of Bioconjugate* **8** *Starting from* **1**



a) 4-Bromobutanoic acid, EDC, DMAP, CH_2Cl_2, r.t. *b*) HA-TBA (**7**), NMP, r.t.

2. Results and Discussion. – Thus, paclitaxel (**1**) was treated at room temperature with 4-bromobutanoic acid in the presence of 1-[3-(dimethylamino)propyl]-3-ethyl-

[2]) For the esterification of HA-TBA with alkyl halides, see [12].

carbodiimide hydrochloride (EDC) and *N*,*N*-dimethylpyridine-4-amine (DMAP) to cleanly give in 78% yield paclitaxel 2′-(4-bromobutanoate) (**6**)[3]) (*Scheme 2*). The formation of a 2′-*O*-substituted paclitaxel derivative was confirmed by the characteristic downfield shifts in the ^{1}H- and ^{13}C- NMR of the signals of H–C(2′) from δ(H) 4.77 [7a] to 5.50 and of C(2′) from δ(C) 73.2 [7a] to 74.2, respectively.

Compound **6** was then dissolved in 1-methylpyrrolidin-2-one (NMP) and treated for 7 days at room temperature with HA-TBA (**7**; M_w 185 kDa; **6**/**7** 1:4) to give **8**. In our opinion, the chosen ratio of reactants should ease the substitution-degree evaluation of the bioconjugate and ensure its hydrosolubility due to the presence of a still large number of free COONa groups. Bioconjugate **8**[4]) was isolated from the reaction mixture after EtOH/NaCl precipitation; this treatment converted residual COO-(Bu_4N) groups at HA into COONa groups. The collected crude material was then purified by extensive dialysis (cut off *ca.* 2 kDa) against distilled H_2O and finally freeze-dried [15]. A *ca.* 25% substitution degree was deduced for **8**[5]) by comparing its UV absorbance in EtOH/H_2O 7:3 with a UV-absorption calibration plot of paclitaxel (**1**) and HA-Na (*Fig.*).

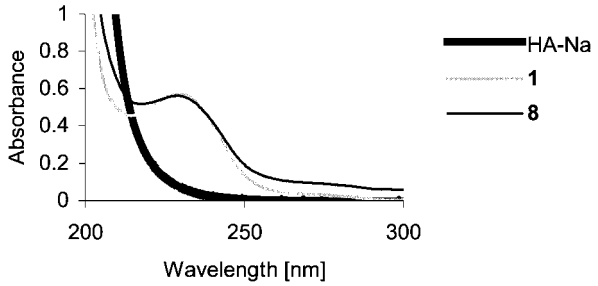


Figure. *UV Spectra* (EtOH/H_2O = 7:3) *of HA-Na, paclitaxel* (**1**; $c = 2.00 \cdot 10^{-5}$ M), *and the paclitaxel–HA bioconjugate* **8**

Compound **8** constitutes the first member of a new class of paclitaxel–HA bioconjugates. The simplicity of its preparation appears suitable for large-scale production. If *in vitro* and *in vivo* anticancer activities prove comparable to those of paclitaxel (**1**), a less severe *i.v.* patient administration as well as the treatment of tumors developed on sensitive tissues should be possible. Finally, selective drug targeting in the case of tumors overexpressing HA CD44 receptors makes paclitaxel–HA bioconjugates of type **8** even more interesting.

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[3]) For previous examples of the paclitaxel esterification at HO–C(2′), see [7][13].

[4]) Related preliminary experiments were presented at 'Hyaluronan 2003', Oct. 11–16, 2003, Cleveland, Ohio [14].

[5]) Assuming for **8** the ε_{max} of **1**.

Experimental Part

General. All solvents were anal. grade. HA-TBA (**7**; $M_w \approx 185$ kDa, batch n. 110) was obtained from *Fidia* (Abano Terme, PD, Italy). TLC: *Merck* silica gel *60 F254*. M.p.: *Mettler FP-61* apparatus (uncorrected). IR Spectra: *Shimadzu 470* scanning infrared spectrophotometer; in cm^{-1}. UV/Vis Spectra: *Perkin-Elmer Lambda-18* spectrometer equipped with a thermostated cell holder. ^{1}H- and ^{13}C-NMR: *Bruker AC-300-P* at 300.13 and 75.48 MHz, resp.; δ in ppm rel. to internal Me_4Si (= 0 ppm), J in Hz. Molecular-mass determination was performed by means of a *LabFlow 4000* HPLC pump (*LabService Analytica*, Bologna, Italy) equipped with a *Varian RI-4* refractive-index detector (*Varian Associates*, Palo Alto, CA, USA) and two *TSK Gel-GMPW* columns (*TosoHaas*, Montgomeryville, Pa). For protection, a *TSK Gel-PWH* guard column was placed before the two columns. All columns were maintained at 25°; the refractive-index detector was thermostatted at 30°. Eluent was 0.5M NaCl at 1 ml/min. M_w and M_n were determined by the universal calibration method, based on polyethylene oxide standards, M_w 885000–26000 Da, (*Tosoh Corporation*, Akasama, Tokyo) by using the *Mark–Houwink* parameters. For $M_w > 40000$, k and α are 0.01192 ml/g and 0.76, resp.; for $M_w < 40000$, k and α are 0.156 ml/g and 0.50, resp. For HA, k and α are 0.0318 ml/g and 0.777, resp. HA and bioconjugate **8** concentrations were 0.6–0.7 mg/ml. High-resolution (HR) MS: *Micromass Q-Tof-micro* mass spectrometer (*Waters*).

Paclitaxel 2′-(4-Bromobutanoate) (= *(αR,βS)-β-(Benzoylamino)-α-(4-bromo-1-oxobutoxy)benzenepropanoic Acid (2aR,4S,4aS,6R,9S,11S,12S,12aR,12bS)-6,12b-bis(acetyloxy)-12-(benzoyloxy)-2a,3,4,4a,5,6,9,10,11,12,12a,12b-dodecahydro-4,11-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-7,11-methano-1*H*-cyclodeca[3,4]benz[1,2-*b*]oxet-9-yl Ester*; **6**). To a soln. of paclitaxel (**1**; 500 mg, 0.59 mmol) in CH_2Cl_2 (25 ml), EDC (380 mg, 2 mmol), DMAP (36 mg, 0.3 mmol), and 4-bromobutanoic acid (300 mg, 1.8 mmol) were added. The mixture was stirred at r.t. until TLC ($CHCl_3$/MeOH 97:3) showed the disappearing of the starting material. The mixture was quenched with H_2O and the aq. phase extracted with CH_2Cl_2 (3 × 10 ml). The combined org. phase was washed with brine, dried (Na_2SO_4), and evaporated and the crude mixture (80 mg) purified by CC (SiO_2, $CHCl_3$/CH_3OH 99:1): **6** (49 mg, 70%). White solid. M.p. 175.0–177.0° (EtOH/hexane 1:1). IR ($CHCl_3$): 1736. ^{1}H-NMR ($CDCl_3$): 1.12 (*s*, 3 H); 1.22 (*s*, 3 H); 1.67 (*s*, 3 H); 1.80–2.85 (10 H); 1.93 (*s*, 3 H); 2.21 (*s*, 3 H); 2.45 (*s*, 3 H); 3.30–3.60 (*m*, 2 H); 3.80 (*d*, J = 7.0, 1 H); 4.19 (*A* of *AB*, J = 8.4); 4.30 (*B* of *AB*, J = 8.4); 4.43 (*m*, 1 H); 4.96 (*d*, J = 7.8, 1 H); 5.50 (*d*, J = 3.1, 1 H); 5.67 (*d*, J = 7.0, 1 H); 5.97 (*dd*, J = 2.8, 9.0, 1 H); 6.25 (*t*, J = 9.3, 1 H); 6.28 (*s*, 1 H); 6.90 (*d*, J = 9.4, 1 H); 7.30–8.20 (15 H). ^{13}C-NMR ($CDCl_3$): 9.6; 14.8; 20.8; 22.1; 22.7; 26.8; 27.5; 31.9; 32.3; 35.5; 43.2; 45.6; 52.7; 58.5; 71.9; 72.1; 74.2; 75.1; 75.6; 76.4; 79.1; 81.1; 84.4; 126.5; 127.1; 128.5; 128.7; 129.1; 130.2; 132.1; 132.8; 133.5; 133.7; 136.8; 142.7; 167.0; 167.1; 168.0; 169.8; 171.2; 171.6; 203.8. HR-MS: 1024.2734 ($C_{51}H_{56}BrNO_{15}^+$, $[M + Na]^+$; calc. 1024.2731).

Paclitaxel 2′-(4-Hyaluronoylbutanoate) (= *(αR,βS)-β-(Benzoylamino)-α-(4-hyaluronoyl-1-oxobutoxy)-benzenepropanoic Acid (2aR,4S,4aS,6R,9S,11S,12S,12aR,12bS)-6,12b-bis(acetyloxy)-12-(benzoyloxy)-2a,3,4,4a,5,6,9,10,11,12,12a,12b-dodecahydro-4,11-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-7,11-methano-1*H*-cyclodeca[3,4]benz[1,2-*b*]oxet-9-yl Ester*; **8**). To a soln. of HA-TBA (**7**; 0.71 mmol, 0.46 g) dissolved in NMP (10 ml), **6** (0.18 mmol, 185 mg) was added, and the stirred soln. was kept at r.t. for 7 days. After that time, H_2O (5 ml) and brine (0.7 ml) were added, and the mixture was stirred for an additional 3 h. Abs. EtOH (79 ml) was then added dropwise, and the resulting white and filamentous precipitate was separated from its mother liquor by centrifugation. The precipitate was purified by extensive dialysis (cut off *ca.* 12 kDa) against distilled H_2O and finally freeze-dried: pure **8** (0.37 g). White cotton-like material. A *ca.* 25% substitution degree for **8** was deduced by using a UV-absorption calibration curve of paclitaxel (**1**) in EtOH/H_2O 7:3 (see *Fig.*).

REFERENCES

[1] M. C. Wani, H. L. Taylor, M. E. Wall, P. Coggon, A. T. McPhail, *J. Am. Chem. Soc.* **1971**, *93*, 2325.
[2] R. W. J. Miller, *J. Nat. Prod*. **1980**, *43*, 425.
[3] J. Lesley, P. W. Kinkade, R. Hyman, *Eur. J. Immunol.* **1993**, *23*, 1902.
[4] J. Szebeni, F. M. Muggia, C. R. Alving, *J. Natl. Cancer Inst*. **1998**, *90*, 300.
[5] I. Knemeyer, M. G. Wientjes, J. L. Au, *Cancer. Chemother. Pharmacol.* **1999**, *44*, 241; H. Gelderblom, J. Verweij, D. M. van Zomeren, D. Buijs, L. Ouwens, K. Nooter, G. Stoter, A. Sparrenboom, *Clin. Cancer Res.* **2002**, *8*, 1237.
[6] D. M. Vyas, H. Wong, A. R. Crosswell, A. M. Casazza, J. O. Knipe, S. W. Mamber, T. W. Doyle, *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1357; K. C. Nicolaou, R. K. Guy, E. N. Pitsinos, W. Wrasidlo, *Angew. Chem., Int. Ed.* **1994**, *33*, 1583; Y. Ueda, J. D. Matiskella, A. B. Mikkilineni, V. Farina, J. O. Knipe, W. C. Rose, A. M.

Casazza, D. M. Vyas, *Bioorg. Med. Chem. Lett.* **1995**, *5*, 247; J. Golik, H. S. L. Wong, S. H. Chen, T. W. Doyle, J. J. K. Wright, J. Knipe, W. C. Rose, A. M. Casazza, D. M. Vyas, *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1837; T. Takahashi, H. Tsukamoto, H. Yamada, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 113; A. Safavy, K. P. Raisch, M. B. Khazaeli, D. J. Buchsbaum, J. A. Bonner, *J. Med. Chem.* **1999**, *42,* 4919; F. M. H. de Groot, L. W. A. van Berkom, H. W. Scheeren, *J. Med. Chem.* **2000**, *43,* 3093.

[7] a) W. Mellado, N. F. Magri, D. G. I. Kingston, R. Garcia-Arenas, G. A. Orr, S. B. Horwitz, *Biochem. Biophys. Res. Commun.* **1984**, *124*, 329; b) N. F. Magri, D. G. I. Kingston, *J. Nat. Prod.* **1988**, *51*, 298; c) H. M. Deutsch, J. A. Glinski, M. Hernandez, R. D. Haugwitz, V. L. Narayanan, M. Suffness, L. H. Zalkow, *J. Med. Chem.* **1989**, *32*, 788; d) Z. Zhao, D. G. I. Kingston, A. R. Crosswell, *J. Nat. Prod.* **1991**, *54*, 1607; e) A. E. Mathew, M. R. Mejillano, J. P. Nath, R. H. Himes, V. J. Stella, *J. Med. Chem.* **1992**, *35*, 145; f) Y. Ueda, A. B. Mikkilineni, J. O. Knipe, W. C. Rose, A. M. Casazza, D. M. Vyas, *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1761; g) K. C. Nicolaou, C. Riemer, M. A. Kerr, D. Rideout, W. Wrasidlo, *Nature (Washington, D.C.)* **1993**, *364*, 464; h) R. B. Greenwald, A. Pendri, D. Bolikal, C. W. Gilbert, *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2465; i) R. B. Greenwald, A. Pendri, D. Bolikal, *J. Org. Chem.* **1995**, *60*, 331; j) A. A. Souto, A. U. Acuna, J. M. Andreu, I. Barasoain, M. Abal, F. Amat-Guerri, *Angew. Chem., Int. Ed.* **1995**, *34*, 2710; k) R. B. Greenwald, C. W. Gilbert, A. Pendri, C. D. Conover, J. Xia, A. Martinez, *J. Med. Chem.* **1996,** *39,* 424; l) C. Li, D. Yu, T. Inoue, D. J. Yang, L. Milas, N. R. Hunter, E. E. Kim, S. Wallace, *Anti-Cancer Drug* **1996**, *7*, 642; m) D. B. A. de Bont, R. G. G. Leenders, H. J. Haisma, I. van der Meulen-Muileman, H. W. Scheeren, *Bioorg. Med. Chem. Lett.* **1997**, *5*, 405; n) Y. L. Khmelnitsky, C. Budde, J. M. Arnold, A. Usyatinsky, D. S. Clark, J. S. Dordick, *J. Am. Chem. Soc.* **1997**, *119,* 11554; o) N. Harada, K. Ozaki, T. Yamaguchi, H. Arakawa, A. Ando, K. Oda, N. Nakanishi, M. Ohashi, T. Hasiyama, K. Tsujihara, *Heterocycles* **1997**, *46*, 241; p) C. Li, D.-F. Yu, R. A. Newman, F. Cabral, L. C. Stephens, N. Hunter, L. Milas, S. Wallace, *Cancer Res.* **1998**, *58*, 2404; q) A. Pendri, C. D. Conover, R. B. Greenwald, *Anti-Cancer Drug Design* **1998**, *13*, 387; r) R. Paradis, M. Page, *Anticancer Res.* **1998**, *18*(*4A*), 2711; s) R. Paradis, M. Page, *Int. J. Oncol.* **1998**, *12*, 391; t) Y. Luo, G. D. Prestwich, *Bioconjug. Chem.* **1999**, *10*, 755; u) Y. Luo, M. R. Ziebell, G. D. Prestwich, *Biomacromol.* **2000,** 1, 208; v) T. A. Kirschberg, C. L. VanDeusen, J. B. Rothbard, M. Yang, P. A. Wender, *Org. Lett.* **2003**, *5*, 3459.

[8] H. Maeda, L. W. Seymour, Y. Miyamoto, *Bioconjug. Chem.* **1992**, *3*, 351.

[9] B. Testa, J. M. Mayer, 'Hydrolysis in Drug and Prodrug Metabolism', Verlag Helvetica Chimica Acta, Zürich, 2003.

[10] A. E. J de Nooy, G. Masci, V. Crescenzi, *Macromolecules* **1999**, *32*, 1318; V. Crescenzi, A. Francescangeli, A. L. Segre, D. Capitani, L. Mannina, D. Renier, D. Bellini, *Macromol. Bioscience* **2002**, *2*, 272; V. Crescenzi, A. Francescangeli, D. Capitani, L. Mannina, D. Renier, D. Bellini, *Carbohydr. Polym.* **2003**, *53*, 311; V. Crescenzi, A. Francescangeli, A. Taglienti, D. Capitani, L. Mannina, *Biomacromol.* **2003**, *4*, 1045.

[11] R. Marini Bettolo, P. Tagliatesta, A. Lupi, D. Bravetti, *Helv. Chim. Acta* **1983**, *66*, 760; R. Marini Bettolo, P. Tagliatesta, A. Lupi, D. Bravetti, *Helv. Chim. Acta* **1983**, *66*, 1922; M. Berettoni, R. Marini Bettolo, V. Montanari, T. Prencipe, S. Romeo, *Helv. Chim. Acta* **1991**, *74*, 1671; M. Berettoni, G. De Chiara, T. Iacoangeli, P. Lo Surdo, R. Marini Bettolo, L. Montagnini di Mirabello, L. Nicolini, R. Scarpelli, *Helv. Chim. Acta* **1996**, *79*, 2035; S. Di Stefano, F. Leonelli, B. Garofalo, L. Mandolini, R. Marini Bettolo, L. M. Migneco, *Org. Lett.* **2002**, *4*, 2783; A. La Bella, F. Leonelli, I. De Salve, L. M. Migneco, R. Marini Bettolo, *Helv. Chim. Acta*, **2004**, *87,* 2120.

[12] F. Della Valle, A. Romeo, to *Fidia S.p.A.*, Eur. Pat. 0216453, 1987.

[13] H. Lataste, V. Senilh, M. Wright, D. Guenard, P. Potier, *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81,* 4090; J. M. Rimoldi, D. G. Kingston, A. G. Chaudhary, G. Samaranayake, S. Grover, E. Hamel, *J. Nat. Prod.* **1993**, *56*, 1313; M. L. Rodrigues, P. Carter, C. Wirth, S. Mullins, A. Lee, B. K. Blackburn, *Chem. Biol.* **1995**, *2*, 223; S. Ali, S. Minchey, A. Janoff, E. Mayhew, *Biophys. J.* **2000**, *78*, 246; S. Ali, I. Ahmad, A. Peters, G. Masters, S. Minchey, A. Janoff, E. Mayhew, *Anti-Cancer Drugs* **2001**, *12*, 117.

[14] A.-L. Capodilupo, V. Crescenzi, A. Francescangeli, R. Joudioux, F. Leonelli, R. Marini Bettolo, L. M. Migneco, M. Quagliariello, P. Bellato, G. De Luca, E. Galbiati, D. Renier, A. Banzato, A. Rosato, in 'HA 2003 Proceedings', Chapt. 5, Ed. Matrix Biology Institute, Edgewater, N.J., 2004.

[15] R. Marini Bettolo, L. M. Migneco, G. De Luca, PCT Int. Appl., Patent No. WO 2004035629, 2004.

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