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Thiocolchicine−Podophyllotoxin Conjugates: Dynamic Libraries Based on Disulfide Exchange Reaction

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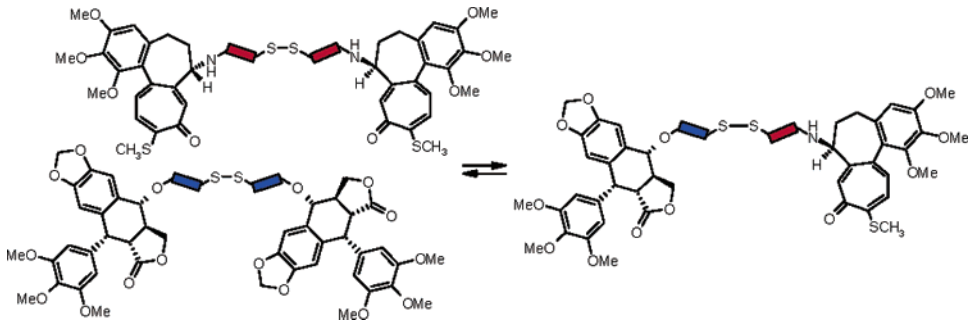
Received December 31, 2005



A dynamic combinatorial library of thiocolchicine−podophyllotoxin derivatives based on the disulfide bond exchange reaction is described. The influence of a biological target on the composition of the reaction mixture has been demonstrated. Use of high-resolution ESI mass spectrometry to evaluate the composition of the mixture shows promise for the design of new large libraries. The biological evaluation demonstrates that formation of a divalent compound affords a new chemical entity whose biological activity is not merely the sum of the single ligands activities, thus reflecting a different interaction with the biological target.

Introduction

A major challenge in cancer therapy is the discovery of molecules that could be selective for tumor cells and that could be characterized by reduced undesirable effects. In fact, tumor cells are a rapidly changing target leading to selection and overgrowth of drug-resistant tumor cells. Various strategies are being developed to increase the therapeutic index of different drugs. In particular, the multivalence strategy has proven to be useful to enhance the activity and selectivity of some monomeric leads by forming bivalent heterodimers.[1,2] The concept of a multivalent molecule is now accepted as an effective strategy for designing ligands, inhibitors, and drugs that influence biological systems.[3] In our recent research on the synthesis of new potential antitumor compounds, we prepared several bivalent hybrid compounds obtained by combination of podophyllotoxin, thiocolchicine, Taxol, and vinblastine derivatives.[4] In this context, we reported the synthesis of taxoid−thiocolchicine hybrids,[5] and in addition to obtaining interesting information regarding cytotoxicity of some compounds, we demonstrated that the biological activity is affected not only by the anchor position of thiocolchicine to the taxoid moiety but also by the dimension of the spacer. Therefore, the search for new

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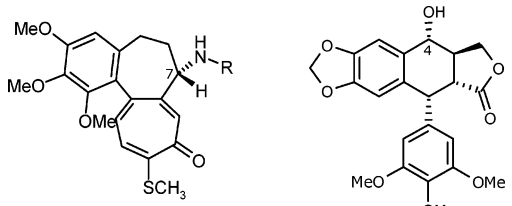
10.1021/jo052677g CCC: $33.50
Published on Web 03/10/2006

CHART 1. Chemical Structure of Thiocolchicine, Deacetylthiocolchicine, and Podophyllotoxin



1 R = $COCH_3$
2 R = H
3

multivalent molecules is a three-variable problem: two scaffolds and one linker. A possible way to solve it is the synthesis of a large number of compounds deriving from different combinations of these three variables. Combinatorial chemistry could offer a shortcut for the preparation of large collections of compounds, but evaluation of activity and purification of single components still remain arduous problems to overcome. In a series of recent developments, the dynamic variant of combinatorial chemistry (DCC)[6] has proven its capacity to select potent enzyme inhibitors.[7] This kind of approach is based on the use of known high-affinity scaffolds that could reversibly self-assemble with several complementary building blocks to form a DCL (dynamic combinatorial library). Reversibility ensures that the members of a DCL are in thermodynamic equilibrium and responsive to external influences. When the DCL is exposed to a molecular target, a selection process can alter the composition of the mixture. Those library members that bind to the target are stabilized, the equilibrium shifts and strong binders are amplified, while the poor binders are suppressed in the library.

In our project to create a DCL, we selected two well-known tubulin binders such as thiocolchicine (**1**)[8] and podophyllotoxin (**3**)[9] (Chart 1) as scaffolds, and we planned to condense them in a new molecule entity using a dithiodiacyl chain as linker.

By exploiting the reversible connecting system of the disulfide bond,[10] a DCL (Figure 1) of potential tubulin binders could be designed.[11,12] Disulfide bond exchange has the peculiarity to function under mild conditions. It can be anticipated that no interaction takes place with the protein disulfide bridges that are often in the interior of the macromolecule and are unlikely to be accessible to solution-phase disulfides at low concentration.[7]

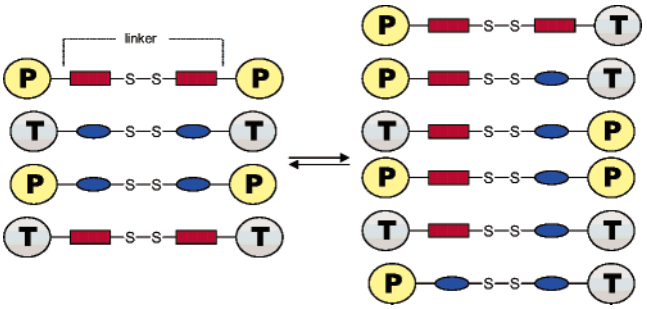
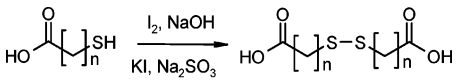


FIGURE 1. Schematic representation of a library based on disulfide exchange reaction. **P** = podophyllotoxin, **T** = deacetylthiocolchicine. The total number of the possible components of the library is $(n^2 + n)/2$ (n = number of homodimers).

SCHEME 1. Preparation of Dicarboxylic Acids



4 n = 1
5 n = 2
6 n = 1
7 n = 2

Results and Discussion

Two types of dithiodicarboxylic (**6**, **7**) acids have been prepared by a conventional procedure[13] using mercaptoacetic and 3-mercaptopropionic acids, respectively, as starting material (Scheme 1). The subsequent condensation with *N*-deacetylthiocolchicine (**2**)[14] and podophyllotoxin (**3**) in the presence of DCC and DMAP in CH_2Cl_2 at room temperature gave the desired homodimers **8**, **9** and **10**, **11**, respectively (Chart 2).

The structure of the compounds **8**−**11** has been confirmed on the basis of their NMR spectra that present all the characteristic signals of thiocolchicine (**8**, **9**) and podophyllotoxin (**10**, **11**). In particular, the signals of the H-7 of thiocolchicine moiety in the range of δ 4.66−4.59 (m) and of H-4 of podophyllotoxin moiety in the range of δ 5.95−5.80 (d) demonstrate the presence of an acyl group on the NH_2 of *N*-deacetylthiocolchicine (where H-7 resonates at δ 3.80, m) and on the C4-OH of podophyllotoxin (where H-4 resonates at δ 4.77). The definitive confirmation of the structures has been given by ESI spectra registered with a ICR-FT-MS instrument that permits the isotopic mass measurement with few ppm error (see the Experimental Section) and a resolution that moves in the range 15.000−25.000.

We first investigated if the disulfide exchange reaction between the two homodimers **9** and **11** could take place in a solvent in the presence of Et_3N as base and if the nature of the solvent can influence the mixture composition. The use of HPLC to monitor the reaction showed acetone and CH_2Cl_2 as convenient solvents because in these the exchange reaction takes place cleanly and only the peaks of starting materials **9** and **11** and the mixed hybrid **13** could be detected. The reactions were considered at the equilibrium when the mixtures composition did not change in a period of 24 h, and it generally happened after 20 days (Table 1).

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CHART 2. Chemical Structure of Homo- and Heterodimers

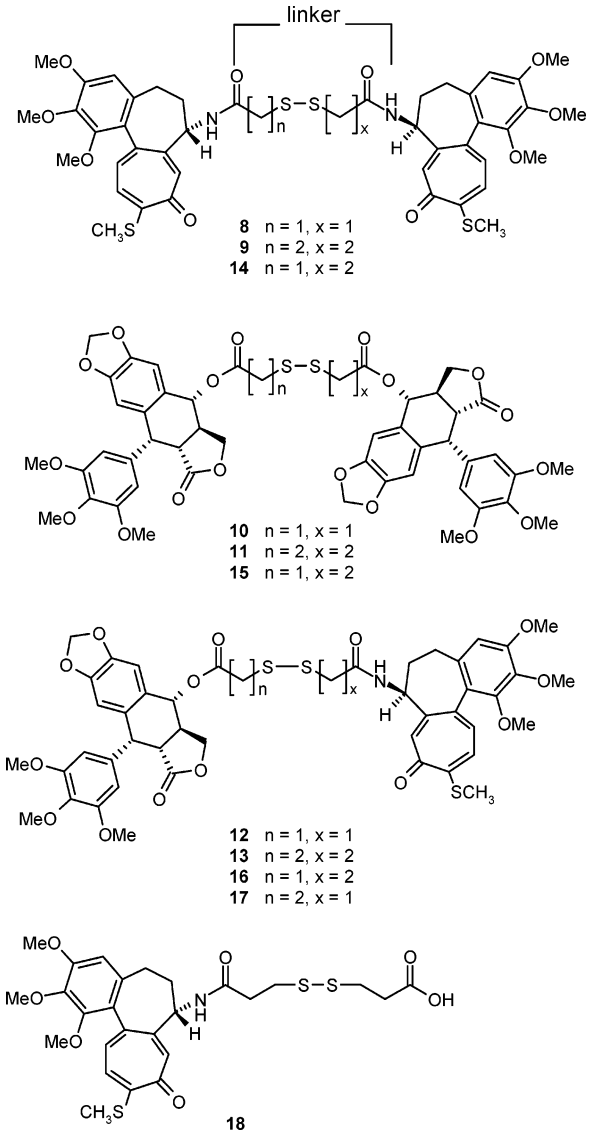

TABLE 1. Equilibrium Composition Determined by HPLC of the Disulfide Exchange Reaction (9 and 11 as Starting Materials) in the Presence of Et_3N and Different Solvents (Reaction Time: 20 days)

solvent	**9**[a] (%)	**11**[a] (%)	**13**[a] (%)
CH_3COCH_3	42	42	15
CH_2Cl_2	42	41	17
CH_3CN[b]	40	37	22
THF[b]	61	38	0.3

[a] The values indicate the relative percentage of the peak areas of compounds **9**, **11**, and **13**. [b] Extensive decomposition has been observed.

Mixed hybrid **13** was isolated from the reaction mixture and identified by spectroscopic analysis of the product. The structure was definitely confirmed by comparison with an authentic sample synthesized by condensing *N*-deacetylthiocolchicine (**2**) with dithiodipropionic acid to give **18** followed by coupling with podophyllotoxin (**3**). The use of other solvents such as CH_3CN and THF revealed an extensive degradation of starting materials, and the figures reported in Table 1 for these two solvents represent only the ratio of the areas of the peaks of compounds **9**, **11**, and **13**. It is to be noted that, whereas for CH_3CN the ratio of the areas of compounds **9**, **11**, and **13** was

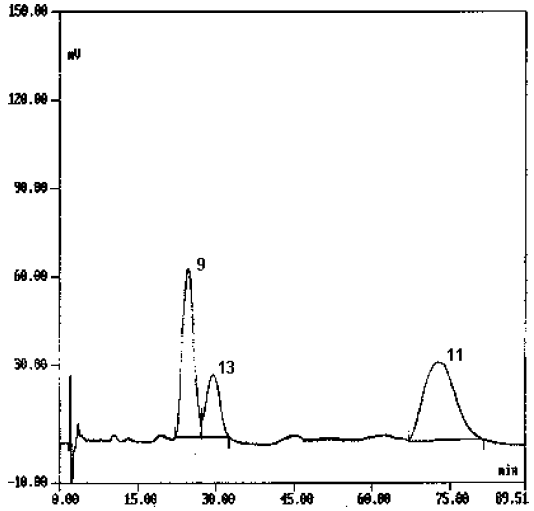

FIGURE 2. HPLC analysis of the reaction mixture obtained by the disulfide exchange reaction using **9** and **11** as starting materials.

similar to that of acetone and CH_2Cl_2, in THF a suppression of the exchange seems to occur. As a consequence, acetone was considered the best choice for the next studies. The exchange reaction could be triggered by the addition of catalytic amount of benzylmercaptan (1%) or an excess of dithiothreitol[15] with substantial reduction of the mixing time (~120 hs) without altering the product ratio. The composition of the disulfide exchange reaction mixture was also checked by ESI-FT-ICR-MS,[16] which showed three peaks at *m*/*z* 943,2350, 984.2355, and 1025.2314 for the sodiated pseudomolecular ion of **9**, **13**, and **11**, respectively. By this way we realized that the relative intensity of the peaks nicely reflects the products distribution determined by HPLC analysis (Figures 2 and 3 and Table 2).

This result was important for us because it opened the possibility to analyze larger dynamic libraries without facing the problems of the HPLC differentiation of products with small structural variations insufficient to guarantee significant differences in the retention times.

Analogously, compounds **8** and **10** reacted to give the formation of the heterodimer **12**, which presented spectroscopic characteristics very similar to the ones of compound **13**. The next step to enlarge the dimension of the dynamic library was the mixing of the **8**−**11** homodimers. If equilibration occurs, a mixture of 10 disulfides could be generated, containing in principle the four starting materials, the two nonsymmetrical homodimers **14** and **15**, and the four heterodimers **12**, **13**, **16**, and **17**, all differing in the number of methylene groups in the spacer. The four compounds were mixed in acetone in the presence of a catalytic amount of benzylmercaptan. After 120 h, we submitted the mixture directly to ESI-MS analysis. We did not try the HPLC separation of the product mixture because

(15) The formation of the heterodimers (**12**, **13**) could be improved (60−65% yield) by a microwave-assisted reaction. A solution of **8** (or **9**) (0.012 mmol), **10** (or **11**) (0.012 mmol), TEA (600 μL), and benzylmercaptan (1%) in CH_2Cl_2/MeOH (9:1, 5 mL) was warmed in a microwave oven at 45 °C for 2 h. Evaporation and purification by chromatography gave **12** (or **13**) in 65% (60%) yield.

(16) For other examples of electrospray ionisation Fourier-transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR) of dynamic combinatorial libraries, see: (a) Poulsen, S.-A.; Gates, P. J.; Cousins, G. R. L.; Sanders, J. M. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 44. (b) Cooper, H. J.; Case, M. A.; McLendon, G. L.; Marshall, A. G. *J. Am. Chem. Soc.* **2003**, *125*, 5331. (c) Reference 11b.

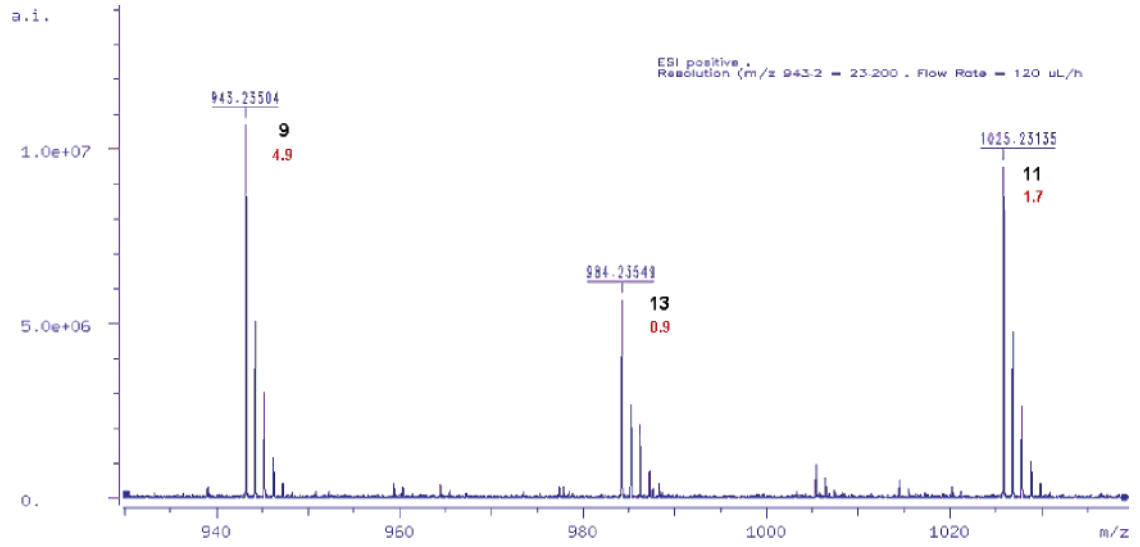

FIGURE 3. ESI-FT-ICR mass spectrum of the mixture obtained using **9** and **11** as starting materials. The peaks represent the corresponding MNa^+ ions. Errors (ppm) with respect to the calculated exact mass are shown in red.

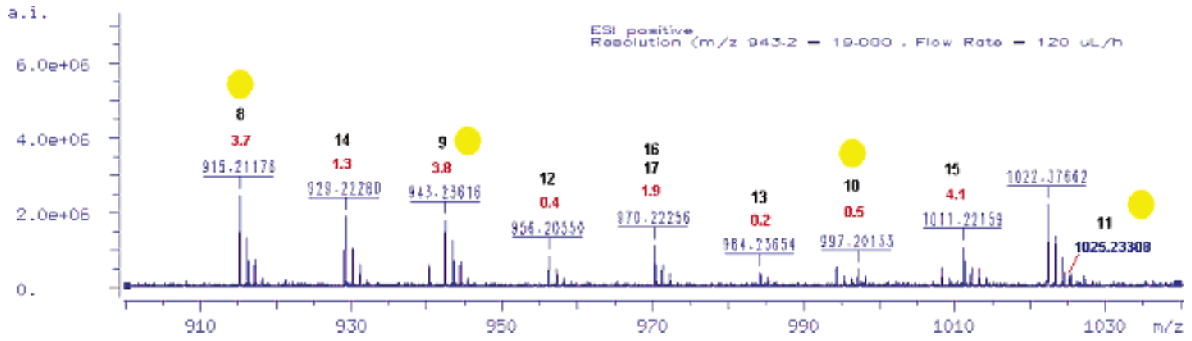

FIGURE 4. ESI-FT-ICR mass spectrum of the mixture obtained (after 120) using compounds **8**−**11** as starting materials. The peaks represent the corresponding MNa^+ ions. The peaks of the starting compounds are marked with a yellow point. Errors (ppm) with respect to the calculated exact mass are shown in red.

TABLE 2. Comparison of the Composition of the Mixture (in Acetone) by HPLC (Area of the Peaks) and ESI-MS (Intensities of the Monoisotopic Peaks)

	9 (%)	**11** (%)	**13** (%)
HPLC	42	42	15
ESI-MS (rel intensity)	41	36	21

TABLE 3. Composition (after 120 h, by ESI-MS) of the Mixture Obtained Using 8−11 as Starting Materials

MNa^+	%	MNa^+	%
8	20	**13**	2
14	15	**10**	4
9	14	**15**	8
12	6	**11**	1
16−**17**	9		

it could be anticipated that the small structural variation among the compounds would have repercussions on insignificant differences in the retention times. Figure 4 illustrates the ESI mass spectrum of the mixture and Table 3 describe the relative intensities of the peaks. The sodiated molecular ions of all the possible compounds are detectable, the two nonsymmetrical thiocolchicine and podophyllotoxin homodimers **14** and **15** at *m*/*z* 929.22890 and 1011.2215, respectively, the two thiocolchicine−podophyllotoxin heterodimers **12** and **13** at *m*/*z* 954.2055 and 984.23654. Of course, it is not possible to distinguish between compounds **16** from **17** because they present the same mass value at *m*/*z* 970.22256. The mass peak of compound **11** is very weak and, unfortunately, very close but definitely separated from the peak of an unidentified impurity (at *m*/*z* 1022.3766). A rapid glance at these data indicates that an extensive chemical equilibration occurs, although the low intensity of some peaks containing podophyllotoxin suggests that some decomposition occurs involving mainly this moiety.

Having verified that the disulfide exchange reaction among homodimers takes place, we then studied if the equilibration could be influenced by the presence of a biological target that in our case could be identified as the tubulins in order to define the compound with the best affinity. Unfortunately, the homodimers are not soluble in water, and conversely, tubulins are proteins that collapse when treated with organic solvents. As a consequence, before moving to the design of new spacers to improve the hydrophilicity of the hybrid compounds, we decided to study the influence on the equilibration reaction of albumin and of subtilisin that could be considered as models of a general receptorial system. These biological targets offer a good compatibility with organic solvents, and their use was to demonstrate the possibility to induce a detectable change in the equilibrium distribution. The homodimers **9** and **11** were dissolved in acetone and TEA in the presence of albumin or

TABLE 4. Equilibrium Composition in the Presence of Subtilisin and Albumin Receptorial Systems Using Acetone as Solvent (20 Days)

	9[a]	11[a]	13[a]
no target	42	42	15
subtilisin	48	49	3
albumin	49	47	4.1

[a] The values indicate the percentage of the peak areas.

TABLE 5. In Vitro Cytotoxicity in a Human Lung Carcinoma Cell Line NCI-H460

compd	IC_{50} (μg/mL)[a]	IC_{50} (μmol/ml)[a] $\times 10^{-6}$
3, podophyllotoxin	0.005	12.1
10 (P-C_2-S-S-C_2-P)	0.03	30.8
11(P-C_3-S-S-C_3-P)	0.3	299
1, thiocolchicine	0.002	4.82
8 (T-C_2-S-S-C_2-T)	0.034	38.1
9 (T-C_3-S-S-C_3-T)	0.03	32.6
12 (P-C_2-S-S-C_2-T)	0.3	322
13 (P-C_3-S-S-C_3-T)	0.16	166

[a] Refers to amount of drug in μg/mL (and μmol/mL) to inhibit the growth of H460 cells by 50% in 72 h. Each value is the average of three sets of experiments.

subtilisin. In both cases, we observed with pleasure that the equilibrium between **9** and **11** is strongly influenced by the presence of the biological polymers and results in the suppression of the formation of the heterodimer **13** (Table 4). Noteworthy, we could demonstrate that neither albumin nor subtilisin inhibited the disulfide exchange but that they truly influence the distribution of the products. In fact, by treating the heterodimer **13** in acetone with Et_3N in the presence of subtilisin and albumin, we got after 20 days a reaction mixture containing the homodimers **9** and **11** in almost equimolar ratio, accompanied by smaller amount of the heterodimeric starting material.

The antiproliferative activity of the novel homodimers and heterodimers was evaluated against the NCI-H460 human non small cell lung carcinoma cell line. The results of the studies are summarized in Table 5, reporting the IC_{50} values of each compound. The formation of disulfide dimers of podophyllotoxin resulted in a progressive reduction of the antiproliferative activity with increasing of the dimension of the spacer; i.e., compound **11** was less active than compound **10**, which in turn was less active than podophyllotoxin. In the case of thiocolchicine, the homodisulfide derivatives presented evident reduction of potency compared to the monomer, but the dimension of the spacer was negligible. Both the tested heterodimers presented very low antiproliferative activity.

Conclusions

In conclusion, this study documents the potentiality of disulfide-exchange reaction to generate a dynamic library of thiocolchicine−podophyllotoxin adducts and supports that this type of reaction could be useful to generate large libraries of compounds. Amplification or suppression of any component of the library could be induced by the presence of a selected biological target. The introduction of new scaffolds will offer new dimension to the libraries. High-resolution ESI mass spectrometry resulted as a useful analytical method for monitoring the compound distribution of large libraries. Finally, the results of the in vitro biological tests provide evidence that the formation of divalent compound gives a new chemical entity whose activity is not predicted by the sum of the single ligands activities, thus reflecting a different interaction with the putative biological target. The results here described strengthen our challenging project devoted to the synthesis of divalent compounds with the aim to disclose a successful approach to the design of new antitumor compounds. The investigation is going on in the search of new spacers that could enhance the hydrophilicity of the hybrid molecules to make possible the generation of a dynamic library based on the disulfide-exchange reaction in water in the presence of tubulin or other biological targets, which may be relevant to the behavior and proliferation of tumor cells.

Experimental Section

Preparation of Dithiodiethanoic Acid (6) and 4,4′-Dithiodipropanoic Acid (7). General Procedure. To a solution of mercaptoacetic acid (or 3-mercaptopropionic acid) (2 mmol) in MeOH (10 mL) were added NaOH (2 mmol) and KI (0.6 mmol). Iodine (1 mmol) was added portionwise until the yellow color persisted, and then Na_2SO_3 was added until complete decoloring of the solution occurred. The solvent was evaporated, HCl (1 N) was added to reach pH 2, and the aqueous phase was extracted with EtOAc. The crude was treated with Et_2O to give the dithiodiacid as a white solid.

6: yield 76%; ^{1}H NMR (200 MHz, CD_3OD) δ 3.61 (s, 2H); mp 132 °C. CAS Registry No.: 505-73-7.

7: yield 74%; ^{1}H NMR (200 MHz, CD_3OD) δ 2.87−3.08 (m, 2H), 2.61−2.81 (m, 2H); mp 156 °C. CAS Registry No.: 1119-62-6.

General Procedure for the Preparation of Homodimers. A solution of dithiodiacid (0.18 mmol), DMAP (0.18 mmol), DCC (0.54 mmol), and *N*-deacetylthiocolchicine (or podophyllotoxin) (0.52 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature for 3 days. Filtration through a Celite layer and evaporation of the solvent afforded a crude that was purified by column chromatography.

8: purified by chromatography (CH_2Cl_2/EtOH 27:1); yield 67%; R_f 0.25 (CH_2Cl_2/MeOH 20:1); $[\alpha]_D = +0.6$ ($CHCl_3$, $c = 1$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.56 (d, $J = 5.6$ Hz, 1H), 7.73 (1H, s), 7.37 (d, $J = 10.5$ Hz, 1H), 7.14 (d, $J = 10.6$ Hz, 1H), 6.53 (s, 1H), 4.69−4.59 (m, 1H), 3.94 (s, 3H), 3.90 (s, 3H), 3.67 (s, 3H), 2.54 (m, 2H), 2.45 (s, 3H), 2.37 (m, 2H), 2.08−1.93 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 182.2, 169.0, 158.3, 153.7, 152.0, 151.1, 141.6, 138,8, 135.1, 134.5, 128.2, 127.1, 125.6, 107.4, 61.7, 61.4, 56.1, 53.5, 43.6, 35.7, 29.9, 15.1; ESI positive MS calcd for $C_{44}H_{48}O_{10}S_4N_2 + Na^+$ 915.20840, found 915.20892.

9: purified by chromatography (CH_2Cl_2/MeOH 20:1); yield = 74%; R_f 0.21 (CH_2Cl_2/MeOH 20:1); $[\alpha]_D = +1.8$ ($CHCl_3$, $c = 1$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.08 (d, $J = 7$ Hz, 1H), 7.62 (s, 1H), 7.33 (d, $J = 10$ Hz, 1H), 7.13 (d, $J = 10$ Hz, 1H), 6.54 (s, 1H), 4.66 (m, 1H), 3.97 (s, 3H), 3.88 (s, 3H), 3.68 (s, 3H), 2.98−2.78 (m, 4H), 2.75−2.61 (m, 2H), 2.34 (s, 3H), 1.98−1.86 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 182.1, 170.9, 158.2, 153.6, 152.5, 151.1, 141.6, 138.9, 135.0, 134.5, 128.5, 125.6, 125.6, 107.3, 61.7, 61.3, 56.1, 52.5, 36.9, 35.4, 35.6, 30.0, 15.0; ESI positive MS calcd for $C_{46}H_{52}O_{10}S_4N_2 + Na^+$ 943.23970, found 943.23877.

10: purified by chromatography (CH_2Cl_2/MeOH 60:1); yield 50%; R_f 0.70 (CH_2Cl_2/MeOH 20:1); $[\alpha]_D = -0.5$ ($CHCl_3$, $c = 1$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.85 (s, 1H), 6.53 (s, 1H), 6.38 (s, 2H), 5.96 (d, $J = 8.5$ Hz, 2H), 5.92 (d, $J = 8.3$ Hz), 4.61 (d, $J = 7.5$ Hz, 1H), 4.41 (dd, $J = 8.1$ Hz, 1H), 4.18 (t, $J = 10.7$ Hz, 1H), 3.79 (s, 3H), 3.74 (s, 6H), 3.00−2.83 (m, 2H), 2,03−1,87 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 174.8, 170.0, 153.0 (2C), 148.0 (2C), 138.5, 134.9, 133.0, 127.9, 110.0, 108.1 (2C), 107.5, 101.7, 75.0, 71.5, 60.8, 56.2 (2C), 46.0, 44.0, 39.3, 34.0; ESI positive MS calcd for $C_{48}H_{46}O_{18}S_2 + Na^+$ 997.20178, found 997.20410.

11: purified by chromatography (CH_2Cl_2/MeOH 50:1); yield 74%; R_f 0.69 (CH_2Cl_2/MeOH 20:1); $[\alpha]_D = -0.1$ ($CHCl_3$, $c = 1$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.67 (s, 1H), 6.56 (s, 1H), 6.04−5.85 (m, 3H), 4.61 (d, $J = 2.7$ Hz, 1H), 4.38 (t, $J = 10.8$ Hz, 1H), 4.18 (t, $J = 10.7$ Hz, 1H), 3.81 (s, 3H), 3.77 (s, 6H), 3.07−2.71 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.5, 172.0, 152.6 (2C), 147.5 (2C), 136.7, 134.7, 132.3, 127.9, 109.7, 108.1 (2C), 106.9, 101.6, 74.1, 71.2, 60.7, 56.2 (2C), 45.5, 43.7, 38.6, 34.0, 32.9; ESI positive MS calcd for $C_{50}H_{50}O_{18}S_2 + Na^+$ 1025.23308, found 1025.23886.

Formation of Heterodimer: Base-Catalyzed Equilibration. A solution of **8** (or **9**) (0.012 mmol) and **10** (or **11**) (0.012 mmol) and TEA (50 μL) in CH_2Cl_2 (or acetone, CH_3CN, THF) (10 mL) was stirred at 37 °C for 20 days. The solvent was removed and the mixture submitted to flash-chromatography column. The equilibrium's state was monitored qualitatively by TLC (eluent CH_2Cl_2/MeOH 20:1) and quantitatively by HPLC (RP18, CH_3CN/H_2O 6:4, 0,8 mL/min). In acetone: **8** (40%), **10** (39%), **12** (17%); For **9**, **11**, and **13,** see the text and Table 2. **Base-Catalyzed Equilibration with the Addition of benzylmercaptan.** A solution of **8** (or **9**) (0.012 mmol) and **10** (or **11**) (0.012 mmol), TEA (50 μL), and benzylmercaptan (0.07×10^{-4} mmol, 1%) in CH_2Cl_2 (10 mL) was stirred at 37 °C for 120 h. The solvent was removed and the mixture submitted to HPLC analysis. **Dithiothreitol-Induced Equilibration.** A solution of **8** (or **9**) (0.060 mmol) and dithiothreitol (0.18 mmol) in CH_2Cl_2 (15 mL) was stirred at room temperature for 2 days. Then, **10** (or **11**) (0.049 mmol) and TEA (400 μL) were added, and the mixture was stirred for 100−120 h.

12. The pooled equilibration reactions of **8** and **10** have been submitted to chromatography (CH_2Cl_2/MeOH 35:1) to give a pure sample of heterodimer: R_f 0.18 (CH_2Cl_2/MeOH 30:1); $[\alpha]_D = -1.1$ ($CHCl_3$, $c = 1$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00−7.05 (m, 4H), 6.80 (s, 1H), 6.53 (m, 2H), 6.38 (m, 2H), 6.05−5.85 (m, 3H), 4.80−4.15 (m, 4H), 3.94 (s, 3H), 3.90 (s, 3H), 3.82 (s, 3H), 3.75 (s, 6H), 3.61 (s, 3H), 3.00−1.80 (m, 10H), 2.48 (s, 3H); ESI positive MS calcd for $C_{46}H_{47}O_{14}S_3N + Na^+$ 956.20509, found 956.20598.

13. The pooled equilibration reactions of **9** and **11** have been submitted to chromatography (CH_2Cl_2/MeOH 35:1) to give a pure sample of heterodimer: R_f 0.18 (CH_2Cl_2/MeOH 30:1); $[\alpha]_D = -1.6$ ($CHCl_3$, $c = 1$); ^{1}H NMR (400 MHz,$CDCl_3$) δ 7.1 (d, $J = 10$ Hz, 1H), 7.49 (d, $J = 8$ Hz, 1H), 7.42 (s, 1H), 7.34 (d, $J = 10$ Hz, 1H), 6.79 (s, 1H), 6.56 (s, 1H), 6.53 (s, 1H), 6.41 (s, 2H), 5.98 (m, 2H), 5.95 (d, $J = 8$ Hz, 1H), 4.75−4.63 (m, 1H), 4.62 (d, $J = 3.5$ Hz, 1H), 4.43 (dd, $J = 10.0$, 7.0 Hz, 1H), 4.22 (t, $J = 10.0$ Hz, 1H), 2.91 (m, 1H), 2.78 (m, 1H), 2.62−2.51 (m, 1H), 2.48 (s, 3H), 2.47 − 2.38 (m, 1H), 2.34−2.18 (m, 1H), 1.98−1.68 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 182.4, 173.7, 158,3, 153.5, 151.2 (2C), 141.7, 138.3, 137.2, 135.3, 134.9, 128.6, 126.6, 109.7, 108.2 (2C), 1075.5, 107.1, 101.6, 74.0, 71.4, 61.6, 61.4, 60.7, 56.2, 56.1, 52.1, 45.5, 44.4, 38.7, 36.8, 34.4, 34.1, 33.6, 30.0, 15.1; ESI positive MS calcd for $C_{48}H_{51}O_{14}S_3N + Na^+$ 984.23639, found 984.23254.

Preparation of 18. A solution of dithiodipropionic acid (1.01 mmol), DMAP (0.34 mmol), DCC (1.01 mmol), and deacetylthiocolchicine (0.67 mmol) in CH_2Cl_2 (30 mL) was stirred at room temperature for 2 h and then filtered through a Celite layer. The removal of the solvent gave a crude mixture that was dissolved in CH_2Cl_2 and extracted with NH_4OH (sol 3.5%). The aqueous phase was treated with HCl (1 N) and extracted with CH_2Cl_2 to give a crude compound that was directly used for the next step. **18:** yield 71%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.92 (s, 1H), 7.41 (d, $J = 9$ Hz, 1H), 7,23 (d, $J = 9$ Hz, 1H), 6,61 (s, 1H), 4,97−4,81 (m, 1H), 3.97 (s, 3H), 3.87 (s, 3H), 3.65 (s, 3H), 2.75−2.61 (m, 2H), 2.43 (s, 3H), 2.40−2.25 (m, 2H), 1.98−1.86 (m, 4H), 1.31−1.19 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 182.7, 174.7, 170.9, 158.9, 153.9, 152.4, 151.1, 141.7, 139.4, 135.6, 134.3, 128.6, 128.0, 125.4, 107.5, 61.6, 61.3, 56.1, 51.9, 37.3, 36.3, 34.9, 34.0, 33.8, 30.0, 15.0.

Preparation of 13 by Direct Condensation of 18 with Podophyllotoxin 3. A solution of **18** (0.18 mmol), DMAP (0.18 mmol), DCC (0.26 mmol), and podophyllotoxin (0.18 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature for 3 days. Filtration through a Celite layer and evaporation of the solvent afforded a crude that was purified by column chromatography.

Target-Assisted Equilibration. A solution of **9** (0.004 mmol), **11** (0.004 mmol), TEA (50 μL), and target (subtilisin A from *Bacillus* sp. or bovine albumin) (2 mg) in acetone (10 mL) was stirred for a period longer than 20 days. The equilibrium's state was quantitatively monitored by HPLC (RP18, CH_3CN/H_2O 6:4, 0.8 mL/min).

Equilibration of Homodimers 8−11. Samples (150 μL) of the four solutions obtained by dissolving 0.0012 mmol of each the compounds **8**, **9**, **10**, and **11** in acetone (1 mL) were mixed in the presence of 1 μL of a solution (1 mg/100 mL of acetone) of benzylmercaptan. The mixture was stirred at room temperature for 120 h. The mixture was directly submitted to ESI MS (see Figure 4 and Table 3).

14: ESI positive MS calcd for $C_{45}H_{50}O_{10}S_4N_2 + Na^+$ 929.22405, found 929.22199.

15: ESI positive MS calcd for $C_{49}H_{48}O_{18}S_2 + Na^+$ 1011.21743, found 1011.22305.

16 and 17: ESI positive MS calcd for $C_{47}H_{49}O_{14}S_3N + Na^+$ 970.22074, found 970.22226.

In Vitro Studies. The human tumor cell lines used in this study included NCI-H460, a human lung large cell carcinoma cell line (ATCC HTB 177). All of the cell lines were cultured in RPMI-1640 containing 10% fetal calf serum. Cytotoxicity was assessed by growth inhibition assay after 1 h of drug exposure. Briefly, cells in the logarithmic phase of growth were harvested and seeded in duplicate into six-well plates. Twenty-four hours after seeding, cells were exposed to the drug and harvested 72 h after exposure and counted with a Coulter counter. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control. All compounds are insoluble in water and were dissolved in DMSO prior to dilution into the biological assay.

Acknowledgment. We are grateful for the financial support provided by Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) COFIN 2001−2003 Program "Sostanze Naturali ed Analoghi Sintetici con Attività Antitumorale". G.A. expresses her gratitude to INDENA S.p.A. (Milano) for the Ph.D. fellowship.

Supporting Information Available: NMR spectra for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JO052677G