amine, 51-45-6; *N*-(trifluoroacetyl)-3-(2-naphthyl)-DL-alanine, 103733-06-8; trifluoroacetic anhydride, 407-25-0; *N*-(trifluoroacetyl)-3-(2-naphthyl)-D-alanine, 103774-97-6; Boc-3-(2-naphthyl)-D-alanine, 76985-10-9; Boc-6-nitro-D-tryptophan, 103733-07-9; diethyl acetamidomalonate, 1068-90-2; 3-picolyl-chloride hydrochloride, 39901-94-5; diethyl acetamido(3-pyridylmethyl)malonate, 103733-08-0; 3-(3-pyridyl)-D-alanine, 70702-47-5; Boc-3-(3-pyridyl)-D-alanine, 98266-33-2; *N*-acetyl-3-(3-pyridyl)-DL-alanine ethyl ester, 103733-09-1; *N*-acetyl-3-(3-pyridyl)-D-alanine ethyl ester, 103774-98-7.

Mechanism of Action of the Marine Natural Product Stypoldione: Evidence for Reaction with Sulfhydryl Groups

E. Timothy O'Brien,[†,‡] David J. Asai,[†,§] Amiram Groweiss,[∥] Bruce H. Lipshutz,[⊥] William Fenical,[∥] Robert S. Jacobs,[†,#] and Leslie Wilson*[†]

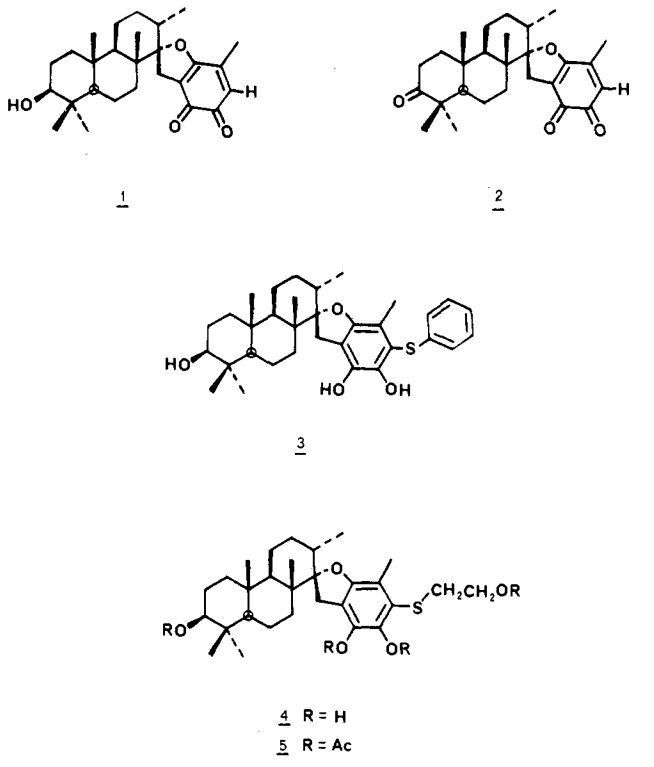
Departments of Biological Sciences and Chemistry and The Marine Science Institute, University of California, Santa Barbara, California 93106, and The Institute of Marine Resources, Scripps Institution of Oceanography, La Jolla, California 92093.
Received July 22, 1985

Stypoldione, a marine natural product that possesses an *o*-quinone functional group, has been shown to inhibit a variety of biological processes including cell division. We found that stypoldione binds covalently to sulfhydryl groups of thiol-containing compounds via addition of sulfur to the C-4′ position of the quinone ring. We examined the ability of stypoldione to add to sulfhydryl groups of a number of thiol-containing substances, including glutathione, thiophenol, β-mercaptoethanol, and the protein tubulin. We suggest that the biological actions of stypoldione may be caused by the addition of this compound to thiol groups of biological molecules.

Stypoldione (1), an *o*-quinone derived from the tropical marine alga *Stypopodium zonale*, inhibits a large number of biological and biochemical processes.[1-9] Initially identified as one of the major products of the alga responsible for fish toxicity,[1,2] stypoldione subsequently was found to inhibit division of cultured mammalian cells, Ehrlich ascites tumor cells and P388 lymphocytic leukemia cells in mice, and fertilized sea urchin eggs.[3,4,6] More detailed analysis revealed that low concentrations of stypoldione could inhibit cell division in fertilized sea urchin eggs without affecting spindle organization or chromosome movement,[7] while higher concentrations inhibited spindle formation, amino acid uptake and incorporation into protein, and DNA synthesis.[4] Stypoldione was also found to inhibit polymerization of bovine brain tubulin into microtubules in vitro[5] and to inhibit flagellar movement of intact or demembranated sea urchin sperm.[8,9] Importantly, the presence of dithiothreitol in the demembranated sperm reactivation buffer prevented the inhibition of flagellar movement by stypoldione.[9]

The wide range of inhibitory activities indicated that the compound might be affecting a number of different receptor classes, while the ability of dithiothreitol to prevent the action of stypoldione on movement of demembranated sperm suggested that sulfhydryl groups might be involved in the action. Further, reactivity of *o*-quinones with sulfhydryl groups had been described previously.[10] In the present study, we determined that stypoldione can react with sulfhydryl groups from a number of different types of thiol-containing compounds including β-mercaptoethanol, cysteine, glutathione, and a number of proteins including tubulin. Our data suggest that the wide spectrum of biological activity of stypoldione is due to a common mechanism: adding to sulfhydryl groups at or near sensitive reactive sites on cellular proteins, and/or by adding to the sulfhydryl group of glutathione, thereby affecting the function of sensitive sulfhydryl-dependent proteins indirectly.

Scheme I



[†] Department of Biological Sciences, University of California.
[‡] Present address: Department of Anatomy, Duke University Medical Center, Durham, NC 27710.
[§] Present address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.
[∥] Scripps Institution of Oceanography.
[⊥] Department of Chemistry, University of California.
[#] Marine Science Institute, University of California.

(1) Gerwick, W. H.; Fenical, W.; Fritsch, N.; Clardy, J. *Tetrahedron Lett.* **1979**, 145.
(2) Gerwick, W. H.; Fenical, W. *J. Org. Chem.* **1981**, *46*, 22.
(3) Jacobs, R. S.; White, S.; Wilson, L. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1981**, *40*, 26.
(4) White, S. J.; Jacobs, R. S. *Mol. Pharmacol.* **1983**, *24*, 500.
(5) O'Brien, E. T.; Jacobs, R. S.; Wilson, L. *Mol. Pharmacol.* **1983**, *24*, 493.
(6) O'Brien, E. T.; White, S. J.; Jacobs, R. S.; Boder, G. B.; Wilson, L. In *Developments in Hydrobiology, 11th International Seaweed Symposium*; Bird, C. J., Ragan, P., Eds.; Dr. Junk: Dordrecht, The Netherlands, 1984; Vol. 22, p 141.
(7) O'Brien, E. T.; Asai, D. J.; Jacobs, R. S.; Wilson, L., unpublished data.

0022-2623/86/1829-1851$01.50/0

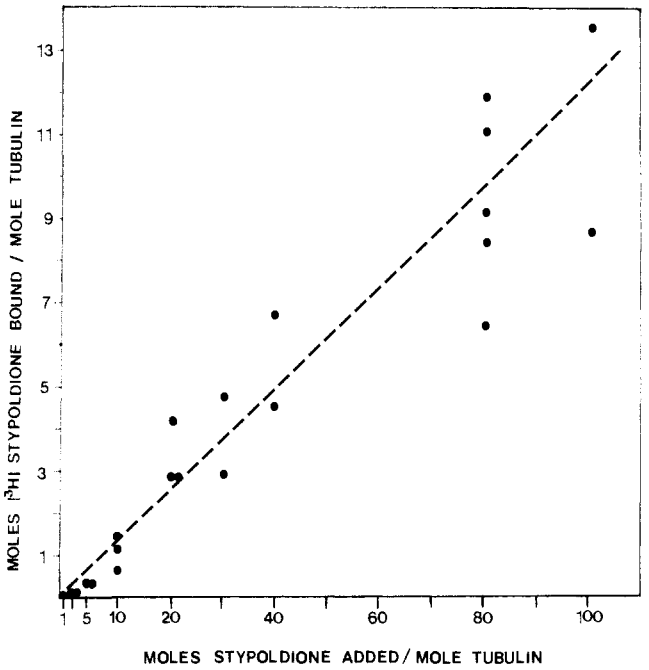


Figure 1. Binding of [^{3}H]stypoldione to tubulin. [^{3}H]Stypoldione was incubated with tubulin (1 μM) for 30 min at 20–22 °C at the indicated ratios of stypoldione to tubulin. Bound stypoldione was analyzed after separation from unbound stypoldione by gel filtration (see Experimental Section).

Results

Reaction of Stypoldione with Tubulin. Stypoldione inhibits polymerization of bovine brain microtubule protein in vitro, apparently by inactivating the tubulin so that it cannot polymerize.[5] To begin an examination of the mechanism of inhibition of microtubule polymerization by the compound, we prepared [^{3}H]stypoldione and examined the binding of the labeled compound to tubulin. We found that stypoldione bound to tubulin in a concentration-dependent manner, with the binding stoichiometry increasing approximately linearly with increasing compound concentration to the limit of solubility of the compound (Figure 1). Though the affinity and the maximal stoichiometry could not be determined, the results indicated that stypoldione was binding to multiple sites on the tubulin dimer. For example, at least 10 mol of stypoldione was bound per mol of tubulin at 100 μM stypoldione (a 100:1 ratio of stypoldione to tubulin).

One possible explanation for the high binding stoichiometry was that stypoldione was interacting with a specific amino acid residue in tubulin. We chose to investigate the interaction of stypoldione with the sulfhydryl groups of cysteine, because mercaptoethanol was found to prevent the ability of stypoldione to inhibit motility of reactivated sperm flagella.[9]

The sulfhydryl groups of tubulin can be carboxymethylated specifically with iodoacetate in the presence of mercaptoethanol,[11,12] and we investigated whether stypoldione could prevent carboxymethylation of tubulin by iodo[^{3}H]acetic acid. In the absence of stypoldione, approximately 20 mol of acetate was bound per mol of tubulin, consistent with the number of cysteine residues in tubulin[11-14] (Table I). Incubation of tubulin with stypoldione prior to addition of iodo[^{3}H]acetic acid decreased the number of reactive sulfhydryl groups in a concentration-dependent manner. At the highest stypoldione concentrations testable (ratios of added stypoldione to tubulin of 69 and 86), stypoldione eliminated the reactivity of all but four or five sulfhydryl groups.

Table I. Inhibition of S-Carboxymethylation of Tubulin by Stypoldione[a]

mol of stypoldione added per mol of tubulin	mol of bound [^{3}H]acetate per mol of tubulin
0	20
8.6	22
17	16
34	12
69	4.8
86	4.3

[a] See Experimental Section for description of the method.

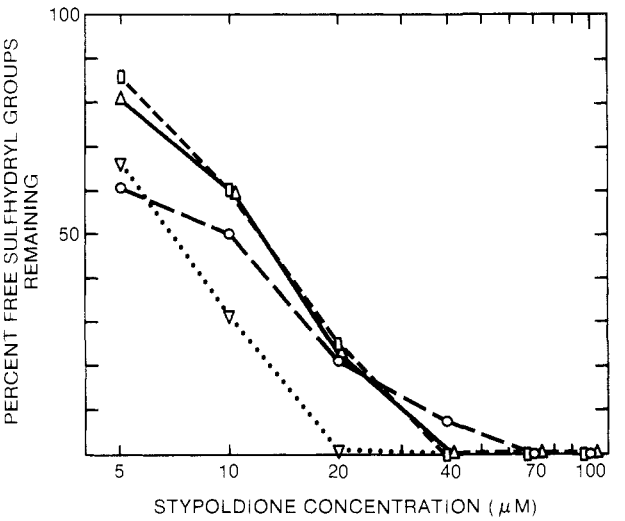


Figure 2. Concentration dependence for sulfhydryl blocking by stypoldione of a number of different thiol compounds. Concentrations of β-mercaptoethanol (○), cysteine (△), glutathione (▽), and purified tubulin (□), equivalent in DTNB reactivity to 20 μM β-mercaptoethanol, were reacted with DTNB in the absence or presence of the indicated concentrations of stypoldione as described in the Experimental Section.

General Reactivity of Stypoldione with Sulfhydryl Groups. A colorimetric assay using Ellman's reagent [5,5′-dithiobis(2-nitrobenzenoic acid)][15] was employed to quantitate the reactivity of stypoldione with sulfhydryl groups of a number of compounds and proteins. Sulfhydryl-containing compounds were first reacted with stypoldione under a standardized set of conditions (see Experimental Section), and then the remaining-free-sulfhydryl concentration was determined colorimetrically. We found that stypoldione reacted with all sulfhydryl-containing compounds tested. For example, stypoldione produced a concentration-dependent block of the sulfhydryl groups of β-mercaptoethanol, cysteine, glutathione, and tubulin (Figure 2). In addition to the compounds examined in Figure 2, we also examined the reactivity of the sulfhydryl groups of a number of proteins other than tubulin, including hexokinase, deoxyribonuclease I, alkaline phosphatase, and bovine serum albumin, exactly as de-

(8) White, S. J., unpublished data.
(9) Asai, D. J.; Brokaw, C., unpublished data.
(10) Prota, G.; D'Agostino, M.; Misuia, M. *Experientia* **1971**, *27*, 15.
(11) Bryan, J.; Wilson, L. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1762.
(12) Luduena, R. F.; Shooter, E. M.; Wilson, L. *J. Biol. Chem.* **1977**, *252*, 7006.
(13) Ponstingl, H.; Krauhs, E.; Little, M.; Kempf, T. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2757.
(14) Krauhs, E.; Little, M.; Kempf, T.; Hofer-Warbinek, R.; Ade, W.; Ponstingl, H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4156.
(15) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.

scribed for the compounds shown in Figure 2. Similar to the situation with tubulin, stypoldione completely blocked the sulfhydryl groups of all proteins tested at a 2- to 3-fold excess of stypoldione relative to the sulfhydryl content of the protein (data not shown). The major difference detected in the reactivity of stypoldione with the various sulfhydryl compounds tested was the 1.5-fold greater reactivity of the sulfhydryl group of glutathione relative to that of the other sulfhydryl compounds tested. The sulfhydryl groups of most compounds tested were completely blocked by stypoldione at a stypoldione:sulfhydryl ratio of 2–3 to 1 at stypoldione concentrations between 40 and 70 μM under the conditions used.

Mechanism of Stypoldione Reactivity with Sulfhydryl Groups. The reactivity of stypoldione with β-mercaptoethanol and thiophenol was initially monitored by TLC analysis of the crude reaction mixtures. Incubation of stypoldione with these thiol-containing reagents generated simple mixtures that contained only one major component by TLC analysis. Small quantities of the thiol addition products were isolated from TLC plates and analyzed by high-resolution mass spectrometry. The adduct of thiophenol (3) showed a molecular ion at m/z 536.2982 (calcd 536.2982 for $C_{33}H_{44}O_4S$), and the β-mercaptoethanol adduct (4) showed a molecular ion at m/z 504.2923 (calcd 504.2916 for $C_{29}H_{44}O_5S$). These analyses not only proved that covalent thiol addition had occurred but also indicated that addition to stypoldione was accompanied by overall reduction of the *o*-quinone to the *o*-catechol. This was later proven by a comprehensive evaluation of the reaction product (see below).

The addition of β-mercaptoethanol to stypoldione was performed on a preparative scale such that the site of thiol addition could be determined. The mercaptoethanol adduct (4), although unstable and sensitive toward air oxidation, was isolated and successfully purified in ca. 60% overall yield. The proton NMR spectrum of 4 showed the presence of new bands characteristic of the mercaptoethanol methylene protons. The aromatic proton at C-4′, which is observed at 6.13 ppm in stypoldione, was conspicuously absent in this adduct. In addition, the adjacent methyl group, which is observed at 2.18 ppm in stypoldione, was deshielded to 2.31 ppm in the adduct (4). All other proton NMR bands that characterize the carbon skeleton of stypoldione were present and readily assigned (see Experimental Section). Thus the location of thiol addition was concluded to be the unsubstituted 4′-position.

The UV absorption spectrum of 4 (absorption at 292 nm) was similar to that of stypotriol,[2] thus supporting the observed reduction of the aromatic ring in the thiol addition reaction. Further confirmation of the overall reduction was obtained by acetylation of 4 to yield the tetraacetate 5. The high-resolution mass spectrum and NMR features of ester 5 confirmed these latter assignments (see Experimental Section for complete details).

Discussion

Chemical Reactivity of Stypoldione toward Sulfhydryl Groups. The chemical analyses of the sulfhydryl adducts of stypoldione described in the present study are consistent with previous analyses of the reactions of quinones with nucleophilic compounds[16–18] and particularly with the reaction of simple *o*-quinones with cysteine.[10] Our results indicate that stypoldione covalently blocks sulfhydryl groups via addition of sulfur to the C-4′ position of the quinone ring. Stypoldione reacted to an approximately equivalent extent (at a given ratio of stypoldione to total sulfhydryl concentration) with almost all sulfhydryl groups of the compounds tested, including those in a number of different proteins. Slightly higher reactivity was observed toward the sulfhydryl group of one compound, glutathione.

Relationship between the Reaction of Stypoldione with the Sulfhydryl Groups of Tubulin and the Inhibition of Microtubule Polymerization. Stypoldione inhibits polymerization of bovine brain microtubule protein into microtubules in vitro,[5] and on the basis of the results reported here, it seems likely that binding of stypoldione to sulfhydryl groups in tubulin is responsible for the inhibition. Microtubule assembly is inhibited by a number of agents that block or oxidize sulfhydryl groups, including *N*-ethylmaleimide, *p*-benzoquinone, *p*-(hydroxymercuri)-benzoate, *N*,*N*′-ethylenebis(iodoacetamide), and diamide,[19–26] and there is general agreement that blockage of as few as two or three sulfhydryl groups per tubulin dimer is sufficient to prevent microtubule assembly.

The colchicine binding activity of tubulin is not as sensitive to sulfhydryl blockage as is the ability of tubulin to polymerize, as the blockage of between 8 and 14 sulfhydryl groups is required to inactivate colchicine binding activity.[20,24] It is likely that addition of stypoldione to the sulfhydryl groups of tubulin is responsible for the ability of the compound to inhibit the colchicine binding activity of the protein.[5] We found that stypoldione noncompetitively inhibited all colchicine binding activity at a stypoldione to tubulin ratio of ca. 40:1. At this ratio, approximately half of the sulfhydryl groups of tubulin would be complexed with the stypoldione (Table I), consistent with the stoichiometry of sulfhydryl blockage required to inactivate the colchicine binding site with other sulfhydryl blocking agents. It is possible that inhibition of colchicine binding is due to addition of stypoldione to a sulfhydryl group in the vicinity of the colchicine binding site. However, because it appears that large numbers of blocked sulfhydryl groups are required to inhibit colchicine binding, a more likely possibility is that the compound adds to multiple sulfhydryl groups is regions distant from the colchicine site and inactivates the site allosterically by cumulative modification of the site.

Effects of Stypoldione in Cells. Inhibition of cell division in developing sea urchin embryos by low concentrations of stypoldione does not appear to be due to disruption of microtubules.[7,27] Karyokinesis, which depends upon the proper functioning of mitotic spindle microtubules, proceeds normally. Inhibited cells exhibit multiple nuclei, indicating that inhibition of cell division may be due to inhibition of cytokinesis. High concentrations of

(16) Horner, L.; Klupfel, K.; Herz, H.; Spietschka, W. *Justus Liebigs Ann. Chem.* **1953**, *579*, 170.
(17) Horner, L.; Gowecke, S. *Chem. Ber.* **1961**, *94*, 1267.
(18) Horner, L.; Gowecke, S.; Duckheimer, W. *Chem. Ber.* **1961**, *94*, 1276.
(19) Mann, K.; Geisel, M.; Fasold, H.; Haase, W. *FEBS Lett.* **1974**, *92*, 45.
(20) Kuriyama, R.; Sakai, H. *J. Biochem. (Tokyo)* **1974**, *76*, 651.
(21) Mellon, M. B.; Rebhun, L. I. *J. Cell Biol.* **1976**, *70*, 226.
(22) Rebhun, L. I.; Nath J.; Remilard, S. P. In *Cold Spring Harbor Conferences on Cell Proliferation: Cell Motility*; Goldman, R., Pollard, T., Rosenbaum, J. L., Eds.; Cold Spring Harbor Laboratory: New York, 1976; p 1343.
(23) Nishida, E.; Kobayashi, T. *J. Biochem. (Tokyo)* **1977**, *81*, 343.
(24) Ikeda, Y.; Steiner, M. *Biochemistry* **1978**, *17*, 3454.
(25) Irons, R. D.; Neptun, D. A.; Pfeifer, R. W. *J. Reticuloendothel. Soc.* **1981**, *30*, 359.
(26) Pfeifer, R. W.; Irons, R. D. *J. Immunopharmacol.* **1983**, *5*, 463.
(27) O'Brien, E. T.; Asai, D. J.; Jacobs, R. S.; Wilson, L. *J. Cell Biol.* **1984**, *99*, 182a.

stypoldione do disrupt the microtubule organization of developing eggs, but such concentrations exceed the minimal concentration required for inhibition of cell growth.

A compound that blocks sulfhydryl groups would be expected to exhibit pleiotropic effects over a wide concentration range. Thus, it seems reasonable to suggest that inhibition of cell division and perhaps all of the actions that have been described for stypoldione can be attributed to its covalent bonding to sulfhydryl groups of sensitive cellular molecules.

Although the specific target responsible for the ability of stypoldione to inhibit cytokinesis is unknown, it is reasonable to suggest that the function of one or perhaps only a small number of sulfhydryl-containing cellular substances exhibits a greater sensitivity to stypoldione than most others and that the putative highly sensitive "target" molecule plays an important role in cytoplasmic division. One possibility is that stypoldione might inhibit cytokinesis in sea urchin embryos by reacting with the sulfhydryl group of intracellular glutathione, which is considered to be important in the maintenance of the reduced state of cysteine residues on cellular proteins.[28] Another possibility is that stypoldione might block a sulfhydryl group on a protein whose function is required for cytokinesis and whose function depends critically upon a specific stypoldione-accessible sulfhydryl group.[22] such a protein may be myosin, which might provide the motive force for cytokinesis[29,30] and which is known to be sensitive to sulfhydryl-substances such as *N*-ethylmaleimide.[31]

Experimental Section

Preparation of [^{3}H]Stypoldione. Stypoldione was oxidized to stypotrione (**2**) by use of Jones reagent (CrO_3 in H_2SO_4), and the product was purified by silica gel (5 μm, Merck Si 60) high-performance liquid chromatography (HPLC), eluting with EtOAc in isooctane (1:1). Stypotrione was then reduced with sodium [^{3}H]borohydride in MeOH (sp act. 5.5 Ci/mmol, New England Nuclear Corp.), and the [^{3}H]stypoldione produced was separated from residual stypotrione, dried ($MgSO_4$), and subsequently purified by HPLC under the conditions specified above. Under the conditions of HPLC, stypotrione had a retention time of 16.5 min, stypoldione a retention time of 18.5 min, and stypotriol a retention time of 20.2 min. The identity and purity of the stypoldione produced were confirmed by TLC (silica gel/EtOAc, R_f 0.5) and IR and UV spectroscopy (UV λ_{max} ($CHCl_3$) 475 nm (ϵ 1100)). The labeled stypoldione was stored in $CHCl_3$ at 0 °C under N_2 gas. The borohydride reaction clearly produced the β epimeric alcohol (stypoldione) rather than the alternative α isomer. This was concluded by interpretation of the specific proton NMR bands originating from the alcohol methine proton.

Binding of [^{3}H]Stypoldione to Tubulin. Bovine brain microtubule protein consisting of ca. 70% tubulin and 30% microtubule-associated proteins was prepared by three cycles of assembly and diassembly as described previously.[3] Tubulin was purified from the microtubule protein mixture by phosphocellulose column chromatography (Whatman P11) in 20 mM sodium phosphate, 100 mM sodium glutamate, 1 mM $MgSO_4$, and 1 mM EGTA, pH 6.75 (L-GNPEM buffer), and used in [^{3}H]stypoldione binding experiments at a concentration of 1 μM (0.1 mg/mL). For binding experiments, a trace quantity of [^{3}H]stypoldione was added to freshly dissolved unlabeled stypoldione (10 mM) in $CHCl_3$, and the drug was repurified by TLC. The stypoldione was eluted with $CHCl_3$, and the $CHCl_3$ solution was filtered through a sintered glass filter to remove remaining silica gel. The $CHCl_3$ solutions were then dried with a stream of N_2 gas, and the drug was redissolved in 100% Me_2SO and incubated with the tubulin solutions for 30 min at room temperature (20–22 °C) (final Me_2SO concentration, 1%, v/v). Bound [^{3}H]stypoldione was separated from free [^{3}H]stypoldione by passage through columns of Bio-Gel P-10 (1.0-mL bed volume) that had been equilibrated previously with the L-GNPEM buffer. Bound radioactivity was quantitated in a Beckman LS8000 liquid scintillation sepctrometer using Aquasol 2 (New England Nuclear Corp.) as the scintillation solvent.

S-Carboxymethylation of Tubulin. Carboxymethylation of the cysteine residues of tubulin was carried out by use of a modification of the procedure of Crestfield et al.[32] A 1.2 mg/mL solution of tubulin in L-GNPEM buffer (100 μL) was added to 1.5-mL polyethylene vials with 1- to 5-μL volumes of stypoldione (20 mM, in EtOH) and incubated at room temperature for 15 min. A reducing and denaturing buffer solution consisting of 8 M urea, 0.2% EDTA, 0.36 M Tris (Sigma Chemical Co.), and 0.83% β-mercaptoethanol (v/v), pH 8.6, that had been bubbled with N_2 gas was then added to the tubulin and stypoldione mixtures (total volumes of 1 mL). The vials were sealed and placed in large dark bottles, which were flushed with N_2 gas, sealed, and incubated at room temperature for 4 h. Fifty microcuries of iodo[^{3}H]acetic acid (New England Nuclear Corp., 232.5 mCi/mmol, 5 mCi/mL) was added to 1 mL of 1.3 M unlabeled iodoacetic acid that had been freshly dissolved in 1.0 N NaOH, and 83.3 μL of this solution was added to each vial to carboxymethylate the remaining free sulfhydryl groups with tritium-labeled acetic acid. After 15 min of further incubation at room temperature, tubulin-bound tritium was separated from unreacted iodo[^{3}H]acetic acid by gel filtration of 1-mL volumes of each reaction mixture on 1 × 18 cm columns of Bio-Gel P-10 (L-GNPEM buffer) and quantitated.

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) Sulfhydryl Assay. Solutions of sulfhydryl compounds in L-GNPEM buffer in the presence or absence of stypoldione (final volume, 490 μL; 485 μL of L-GNPEM buffer and 5 μL of 100% EtOH) were incubated for 15 min at room temperature. DTNB (10 μL, 100% EtOH, final concentration, 200 μM) was added, and after an additional 30 min of incubation, absorbancy at 412 nm was quantitated by use of a Gilford model 2400 spectrophotometer. Concentrations of sulfhydryl-containing compounds were chosen for analysis such that the absorbance produced was equivalent to that produced by 20 μM β-mercaptoethanol. Determination of the sulfhydryl reactivity of tubulin was complicated because gradual denaturation of the protein produced a gradual increase in the DTNB reactivity. Thus, the DTNB reactivity of tubulin reported represents the sulfhydryl reactivity of the protein at 30 min of incubation under the conditions used. Absorbance due to reaction of stypoldione with the various sulfhydryl-containing compounds tested, due mostly to a shift of the 475-nm peak of stypoldione to shorter wavelengths, was subtracted for each thiol compound tested by measuring the absorbance of parallel reaction mixtures that were not incubated with DTNB.

Reaction of Stypoldione with β-Mercaptoethanol. β-Mercaptoethanol was added dropwise to a stirred solution of stypoldione (33 mg) in CH_3OH at room temperature until the red solution became colorless. Then the excess solvent and reagent were removed in vacuo. Chromatography of the resulting material (high-performance LC, 5-μm silica gel, elution with 7:3 ethyl acetate–isooctane) yielded adduct **4** as pale yellow gum, which turned dark yellow if allowed to stand in air. Adduct **4**: UV λ_{max} (MeOH) 397 nm (ϵ 3200), 292 (5300), 241 (8700), 218 (8900); IR (CH_2Cl_2) 3620, 3550, 3050, 2940, 1450, 1430, 1290, 1245 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) 3.68 (t, J = 5.8 Hz, 2 H), 3.25 (dd, J = 11.2, 5.0 Hz), 3.21 (d, J = 16.8 Hz), 2.81 (t, J = 5.8 Hz, 2 H), 2.80 (d, J = 16.8 Hz), 2.31 (s, 3 H), 0.95 (s, 3 H), 0.94 (s, 3 H), 0.85 (s, 3 H), 0.77 (s, 3 H), 0.66 (d, J = 6.4 Hz, 3 H).

Adduct **4** was also characterized as its more stable tetraacetate **5**, obtained after treatment with acetic anhydride–pyridine. Tetraacetate **5**: ^{1}H NMR (360 MHz, $CDCl_3$) 4.51 (dd, J = 10.8, 5.5 Hz), 4.15 (t, J = 6.6 Hz, 2 H), 3.13 (d, J = 16.7 Hz), 2.92 (t, J = 6.6 Hz, 2 H), 2.69 (d, J = 16.7 Hz), 2.39 (s, 3 H), 2.32 (s, 3 H), 2.26 (s, 3 H), 2.05 (s, 3 H), 2.01 (s, 3 H), 0.93 (s, 3 H), 0.87 (s, 3 H), 0.84 (s, 3 H), 0.83 (s, 3 H), 0.70 (d, J = 6.5 Hz, 3 H); high-resolution mass spectrum, m/z 672.3308 (M^+, calcd for $C_{37}H_{52}O_9S$ 672.3349, 4.4%), 630.4 ($M^+ - CH_2CO$, 100%).

(28) Nath, J.; Rebhun, L. I. *J. Cell Biol.* **1976**, *68*, 440.
(29) Kiehart, D. P.; Mabuchi, I.; Inoue, S. *J. Cell Biol.* **1982**, *94*, 165.
(30) Mabuchi, I.; Okuno, M. *J. Cell Biol.* **1977**, *74*, 251.
(31) Meeusen, R. L.; Cande, W. Z. *J. Cell Biol.* **1979**, *82*, 57.
(32) Crestfield, A. A.; Moore, S.; Stein, W. H. *J. Biol. Chem.* **1973**, *238*, 622.

Miscellaneous Procedures. Mass spectrometric analyses of the stypoldione reaction products were carried out on a VG ZAB-2F spectrometer at high resolution, using positive chemical ionization. ^{1}H NMR spectroscopy was carried out by using either a Nicolet NT-300 (300 MHz) or a 360-MHz Oxford Magnetics–Nicolet 1180E spectrometer. IR and UV spectra were obtained with Perkin-Elmer Model 137 and Beckman Acta IV spectrophotometers, respectively. DTNB, iodoacetate, amino acids, glutathione, and the proteins used for this study, except for tubulin, were obtained from Sigma Chemical Co.

Acknowledgment. This work was supported by USPHS Grant NS13560 from the Institute of Neurological, Communicative Disorders, and Stroke (L.W.), by USPHS Grant 16956 from the Division of Child Health and Human Development (D.J.A.), by NOAA National Sea Grant College Program, Department of Commerce Grant NA808A-D00102 through the California Sea Grant College Program (R.S.J.), and by NOAA, Department of Commerce, Office of Sea Grant, No. 04-7-158-44121 (W.F.). We thank Herbert Miller for preparing bovine brain microtubule protein.

Registry No. 1, 71103-05-4; [^{3}H]-1, 103533-00-2; 2, 103532-99-6; 4, 103532-97-4; 5, 103532-98-5; $HS(CH_2)_2OH$, 60-24-2; L-Cys, 52-90-4; glutathione, 70-18-8; hexokinase, 9001-51-8; deoxyribonuclease I, 9003-98-9; alkaline phosphatase, 9001-78-9.

Opioid Agonist and Antagonist Bivalent Ligands. The Relationship between Spacer Length and Selectivity at Multiple Opioid Receptors

P. S. Portoghese,*† D. L. Larson,† L. M. Sayre,† C. B. Yim,† G. Ronsisvalle,† S. W. Tam,‡ and A. E. Takemori§

Departments of Medicinal Chemistry and Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455, and Biomedical Products Department, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898.
Received January 14, 1986

Bivalent ligands containing the oxymorphamine or naltrexamine pharmacophores connected to spacers of varying length were synthesized and evaluated for their selectivity at μ, κ, and δ opioid receptors. The oxymorphamine bivalent ligands (**1–8**) behaved as μ agonists on the electrically stimulated guinea pig ileum longitudinal muscle preparation (GPI). The spacer that conferred peak agonist activity in these series contains a total of four glycyl units (n = 2). Binding studies with guinea pig brain membranes showed a qualitatively similar profile at μ receptors as a function of spacer length. Also, δ receptor selectivity increased as the spacer was lengthened. The naltrexamine bivalent ligands (**9–13**) effectively antagonized the μ receptor agonist morphine in the GPI at the same optimal spacer length (n = 2) as in the agonist series. However, the peak antagonism of ethylketazocine, a κ receptor agonist, occurred with the bivalent ligand **9** containing the shortest spacer (n = 0), and it was found that **9** is the most selective κ antagonist in the series. While receptor binding roughly parallels that of κ antagonist activity in the GPI, no correlation between binding and antagonist activity was observed at μ opioid receptors. The possible significance of these results is discussed.

Compounds that contain two pharmacophores joined through a connecting unit (spacer) have been termed "bivalent ligands".[1-3] A classic example of bivalent ligands is the bis onium cholinergic blocking agents.[4] Bivalent ligands that contain opioid pharmacophores have attracted interest as molecular probes for opioid receptors.[1-3,5-11] In this connection, they have been employed as an approach to obtaining ligands with high selectivity for a single receptor type in the presence of multiple opioid receptors.[3]

The expectation for such high selectivity was based on three assumptions:[1,2] (1) simultaneous interaction (bridging) of both pharmacophores with vicinal receptors occurs with greatest frequency when the spacer chain of the bivalent ligand is an optimal length, (2) bridging under such conditions is preferred over a univalent binding mode, and (3) different opioid receptor types may be characterized by different vicinal interreceptor relationships in the clustered state.

According to this model, the relationship between spacer length and the activity of the bivalent ligand should be parabolic if very short or very long spacers decrease the frequency of binding to vicinal receptors. Moreover, the structure–activity profile for different receptor types should differ if they possess different interreceptor distances.

The present paper describes the results of studies conducted to investigate the interaction of bivalent ligands **1–13** at opioid receptors. Opioid pharmacophores with agonist and antagonist activities were employed in order to compare the structure–activity–relationship profiles. The results of these studies are consistent with the bridging principle.

Design Considerations and Chemistry

The pharmacophores that we selected for this study are

†University of Minnesota, Department of Medicinal Chemistry.
‡E. I. du Pont de Nemours and Co.
§University of Minnesota, Department of Pharmacology.

(1) Erez, M.; Takemori, A. E.; Portoghese, P. S. *J. Med. Chem.* **1982**, *25*, 847.
(2) Portoghese, P. S.; Ronsisvalle, G.; Larson, D. L.; Yim, C. B.; Sayre, L. M.; Takemori, A. E. *Life Sci.* **1982**, *31*, 1283.
(3) Portoghese, P. S.; Takemori, A. E. *Life Sci.* **1985**, *36*, 802.
(4) *Wilson and Gisvold's Textbook of Organic Medicinal Chemistry*, 8th ed.; Doerge, R. F., Ed.; J. B. Lippincott: Philadelphia, 1982; pp 490–496.
(5) Hazum, E.; Chang, K-J.; Leighton, H. J.; Lever, O. W.; Cuatrecases, P. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 347.
(6) Shimohigashi, T.; Costa, T.; Chen, H-C.; Rodbard, D. *Nature (London)* **1982**, *297*, 333.
(7) Krumins, S. A.; Costa, T.; Rodbard, D. *Life Sci.* **1982**, *32*, 511.
(8) Costa, T.; Shimohigashi, Y.; Krumins, S. A.; Munson, P. J.; Rodbard, D. *Life Sci.* **1982**, *31*, 1625.
(9) Shimohigashi, Y.; Costa, T.; Matsuura, S.; Chen, H.-C.; Rodbard, D. *Mol. Pharmacol.* **1982**, *21*, 558.
(10) Lipkowski, A. W.; Konecka, A. M.; Sroczynska, I. *Peptides* **1982**, *3*, 697.
(11) Lipkowski, A. W.; Konecka, A. M.; Sadowski, B. *Pol. J. Pharmacol. Pharm.* **1982**, *34*, 69.

0022-2623/86/1829-1855$01.50/0