Synthesis and Evaluation of Some Water-Soluble Prodrugs and Derivatives of Taxol with Antitumor Activity

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The synthesis and evaluation of some 2'- and 7-amino acid derivatives of taxol (1) are reported. Reaction of taxol with N-protected amino acids gave 2'-N-protected amino acid esters of taxol. However, deprotection of the amino group and subsequent isolation of products were complex and only successful when formic acid was used to deprotect a *t*-BOC protecting group. Esterification of taxol using N,N-dialkylated amino acids gave 2'-amino acid esters of taxol, 2'-(*N*,*N*-dimethylglycyl)taxol (4) and 2'-[3-(*N*,*N*-diethylamino)propionyl]taxol as its methanesulfonic acid salt (**5b**), in good yield. The 7-derivatives, 7-(*N*,*N*-dimethylglycyl)taxol (9) and 7-L-alanyltaxol (12), were prepared by two alternate methods. In the first approach, the 2'-hydroxyl group was protected using the [(2,2,2-trichloroethyl)oxy]carbonyl, or troc, protecting group followed by the esterification of the 7-hydroxyl and subsequent deprotection of the amino and troc groups. In the second approach, taxol was allowed to react with more than 2 molar equiv of the N-protected amino acids or N,N-dialkylated amino acids to give 2',7-diamino acid esters of taxol. For the protected amino acids, the deprotection of the amino group followed by removal of the 2'-substituent gave the 7-amino acid esters of taxol. The methanesulfonic acid salts of both 2'- and 7-amino acid esters showed improved solubility ranging from 2 to >10 mg/mL. The 7-derivatives were effective in promoting microtubule assembly in vitro while 2'-derivatives showed little in vitro activity. The derivatives 2'-(*N*,*N*-dimethylglycyl)taxol (4) and 2'-[3-(*N*,*N*-diethylamino)propionyl]taxol (5) inhibited proliferation of B16 melanoma cells to an extent similar to that of taxol, while the other derivatives were about 50% as cytotoxic. In a mammary tumor screen, 2'-[3-(*N*,*N*-diethylamino)propionyl]taxol showed the greatest antitumor activity compared to the other analogues. The lower activities of the 7-derivatives in inhibiting tumor growth and melamona cell proliferation (although they were almost as active as taxol in inducing microtubule assembly in vitro) may be due to differences in drug uptake by the cells. The similar cytotoxic and antitumor activities of the 2'-analogues and taxol can be explained by their conversion to taxol or an active taxol metabolite. Therefore, the 2'-analogues appear to behave as prodrugs and have the potential to be developed as chemotherapeutic agents.

Introduction

Taxol is a diterpenoid taxane derivative which was first isolated from *Taxus brevifolia* by Wani et al. in 1971.[1] Taxol has shown excellent antitumor activity in a wide variety of tumor models such as B16 melanoma, L1210 and P388 leukemias, MX-1 mammary tumor, and CX-1 colon tumor xenografts.[2-6] The antitumor property of taxol is known to be due to its ability to promote tubulin assembly into microtubules.[7-10] In the presence of taxol, microtubules resist depolymerization, thus interfering with the G2 and M phases of the cell cycle.[8] In spite of its excellent antitumor activity, there are considerable difficulties in developing taxol as a chemotherapeutic agent. One major difficulty with taxol is its low solubility in water which makes its formulation for injection difficult. Other problems are its low availability and structural complexity.

Due to poor water solubility, taxol is administered as a 6 mg/mL Cremophor EL/ethanol mixture diluted with normal saline or 5% dextrose in water to the desired final concentration. However, because of the relatively large doses of taxol needed, dilutions result in the patient receiving large doses of Cremophor EL. Exposure to this surfactant has resulted in a high incidence of idiosyncratic histamine release.[11] The solubility difficulties with taxol could be overcome by the development of a water soluble, chemically stable, but in vivo labile prodrug or analogue.[12-15] This paper describes the studies leading to the synthesis of several water-soluble derivatives of taxol, some of which appear to behave as prodrugs.

Although limited structure–activity studies are reported on taxol, the C-13 ester side chain and the 2'-hydroxyl group on the side chain appear essential for biological activity.[16,17] Mellado et al.[17] have reported the synthesis

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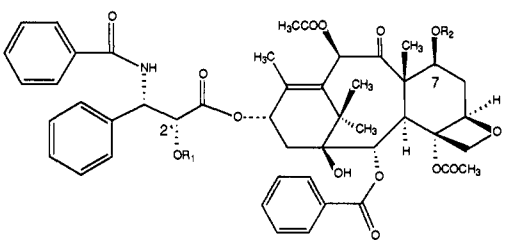
and biological activity of 2′-acetyl, 7-acetyl, and 2′,7-diacetyltaxol. Introduction of an acetyl moiety at the 2′-position resulted in the loss of ability to promote microtubule assembly, but not cytotoxicity. Taxol and 7-acetyltaxol were similar in their ability to alter cell proliferation and microtubule polymerization. Also, 7-epitaxol, an epimerization product of taxol, has been shown to be as active as taxol.[9] These observations suggested that the 2′- and 7-positions are suitable for structural modifications, the 2′-position as a site for reversible derivatization (prodrugs),[18] and the 7-position for analogue/prodrug modifications. Other prodrugs and analogues have also been reported recently.[19]

Results and Discussion

Chemistry. The hydroxyl group at the 2′-position of taxol is more reactive than the sterically hindered 7-hydroxyl group.[18,20] Therefore, it is possible to use this difference in reactivity to introduce several amino acid esters at the 2′-position of taxol. Reaction of taxol with N-protected amino acids gave 2′-amino acid esters of taxol in excellent yield. Different groups were used for the protection of the amino group, although only the acid-sensitive *t*-BOC example will be discussed. Deprotection of the *t*-BOC protecting group using reagents such as trifluoroacetic acid, *p*-toluenesulfonic acid, and other standard acidic conditions resulted in complex mixtures. The *t*-BOC group, however, was successfully deprotected using 99% formic acid. Reaction of taxol (**1**) with *N*-*t*-BOC-L-alanine gave 2′-(*N*-*t*-BOC-L-alanyl)taxol (**2**), which on deprotection using formic acid gave 2′-L-alanyltaxol (**3**). Methods to synthesize N,N-dialkylated amino acid esters of taxol were also explored. Thus, reaction of taxol with *N*,*N*-dimethylglycine in methylene chloride in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) gave 2′-(*N*,*N*-dimethylglycyl)-taxol (**4**) in good yield. The methanesulfonic acid salt of **4** (**4a**) was prepared, which had a water solubility of >2 mg/mL. Similarly, reaction of taxol with 3-(*N*,*N*-diethylamino)propionic acid hydrochloride salt in anhydrous methylene chloride and DCC gave 2′-[3-(*N*,*N*-diethylamino)propionyl]taxol (**5**) as the hydrochloride salt (**5a**). The water solubility of this salt was found to be less than 1 mg/mL. Therefore, the preparation of the methanesulfonic acid salt was attempted. The reaction of taxol with 3-(*N*,*N*-diethylamino)propionic acid methanesulfonate salt gave 2′-[3-(*N*,*N*-diethylamino)propionyl]-taxol methanesulfonate (**5b**) in 85–90% yield. The solubility of this salt in water was found to be >10 mg/mL. Due to stability limitations, **5** per se was never isolated. For all the 2′-esters, evidence for the site of esterification was obtained from proton NMR, wherein the C-2′ proton of taxol at 4.7 ppm appeared as a doublet at 5.5 ppm. This shift is in good agreement with the value reported for 2′-acetyltaxol.[17]



	R_1 (2′-position)	R_2 (7-position)
1	H	H
2	$COCH(CH_3)NHCOOC(CH_3)_3$	H
3	$COCH(CH_3)NH_2$	H
4	$COCH_2N(CH_3)_2$	H
4a	$COCH_2N(CH_3)_2.CH_3SO_3H$	H
5	$COCH_2CH_2N(C_2H_5)_2$	H
5a	$COCH_2CH_2N(C_2H_5)_2.HCl$	H
5b	$COCH_2CH_2N(C_2H_5)_2.CH_3SO_3H$	H
6	$COCH_2N(CH_3)_2$	$COCH_2N(CH_3)_2$
6a	$COCH_2N(CH_3)_2.CH_3SO_3H$	$COCH_2N(CH_3)_2.CH_3SO_3H$
7	$COOCH_2CCl_3$	H
8	$COOCH_2CCl_3$	$COCH_2N(CH_3)_2$
9	H	$COCH_2N(CH_3)_2$
9a	H	$COCH_2N(CH_3)_2.CH_3SO_3H$
10	$COCH(CH_3)NHCOOC(CH_3)_3$	$COCH(CH_3)NHCOOC(CH_3)_3$
11	$COCH(CH_3)NH_2$	$COCH(CH_3)NH_2$
12	H	$COCH(CH_3)NH_2$
12a	H	$COCH(CH_3)NH_2.CH_3SO_3H$

Although a few of the 2′-amino acid esters of taxol were sufficiently stable to be potentially used as prodrugs, other derivatives with increased chemical stability and enzymatic lability would be more desirable. It has been shown that modification at the 7-position did not appreciably affect the biological activity of taxol.[17] Therefore, the introduction of amino acid esters at the 7-position was attempted. Since the 2′-hydroxyl group is more reactive compared to the 7-hydroxyl, esterification at the 7-hydroxyl requires protection of the 2′-hydroxyl group. Two approaches were utilized to introduce substituents at the 7-position. In the first approach, the 2′-hydroxyl was protected using the [(2,2,2-trichloroethyl)oxy]carbonyl, or troc, protecting group as suggested by Magri and Kingston.[20] 2′-Troc-taxol (**7**) was allowed to react with N,N-dialkylated amino acids or N-protected amino acids to give the 7-substituted derivatives. This was followed by the removal of the protecting groups from the N-protected amino acid derivatives or just the troc group from the dialkylated amino acid derivatives. On the basis of this strategy, for example, 2′-troc-taxol was allowed to react with *N*,*N*-dimethylglycine in the presence of DCC and DMAP to give 2′-troc-7-(*N*,*N*-dimethylglycyl)taxol (**8**). The troc group was removed using Zn and acetic acid/methanol to yield the 7-(*N*,*N*-dimethylglycyl)taxol (**9**). In the second approach, taxol was allowed to react with more than 2 equiv of the N,N-dialkylated amino acids or N-protected amino acids to give the 2′,7-disubstituted derivatives. The compound 2′,7-bis(*N*,*N*-dimethylglycyl)-taxol (**6**) was prepared in this manner. For the N-protected amino acids, the amino group was deprotected to yield the 2′,7-diamino acid derivative. The amino acid substituent at the 2′-position, being more chemically labile, was selectively removed by simple hydrolysis to give the 7-monosubstituted taxol derivative. The reaction of taxol with *N*-*t*-BOC-L-alanine in the presence of DCC and DMAP gave 2′,7-bis(*N*-*t*-BOC-L-alanyl)taxol (**10**). The N-protected dialanyl derivative was deprotected using 99% formic acid to yield 2′,7-di-L-alanyltaxol (**11**). Reaction of **11** in the presence of phosphate buffer at pH 7.4 gave

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7-L-alanyltaxol (12) in 63% yield. For all the 7-substituted taxol derivatives, the C-7 proton at 4.33 ppm in taxol appeared as a doublet of doublets at ≈5.6.

Deutsch et al.[18] have also recently described attempts to prepare basic prodrugs of taxol using protected amino acids and the synthesis of a few water-soluble prodrugs. In attempting the deprotection of the *t*-BOC group, the same problems reported by Deutsch et al.[18] and Magri and Kingston[21] were observed in the present study. However, deprotection of the *t*-BOC group using 99% formic acid was found to effect deprotection with minimal side products. The deprotected amino acid derivatives of taxol could be purified using silanized silica gel. When regular silica gel was used for purification of the amino acid derivatives, substantial degradation of the taxol derivatives to taxol was observed. This degradation probably contributed to the difficulties encountered by Deutsch et al. in preparing amino acid ester prodrugs of taxol.

The synthesis of amino acid esters of taxol was met with various difficulties, especially in the deprotection of the amino groups. Epimerization and side-chain cleavage appeared to be the major problems. The direct condensation of taxol with N,N-dialkylated amino acids gave the amino acid esters in excellent yield.

The chemical stability of 2′-amino acid esters of taxol in aqueous solutions depends on the nature of the amino acids. For example, the methanesulfonate salt of 2′-(*N*,*N*-dimethylglycyl)taxol (**4a**) had a half-life of 96 h at pH 3.5, whereas **5b** showed a half-life of 438 h at 25 °C; however, at pH 7.4 in the presence of 0.02 M phosphate buffer, the half-lives were 6 and 0.25 h, respectively. The 2′-derivatives appeared to be substrates for enzymatic degradation; e.g., **4a** had half-lives of <3 min, 2 h, and 3 h, respectively, in the presence of rat, dog, and human plasma, whereas **5b** had a half-life of only 4 min in the presence of human plasma. In general, the 7-derivatives showed better chemical and enzymatic stabilities than the 2′-derivatives. For example, **9a** had a half-life of 378 h at pH 3.8 compared with 96 h at pH 3.5 for **4a** at 25 °C. At pH 7.4 in the presence of 0.02 M phosphate buffer the half-life for **9a** was 34 h. In the presence of rat and human plasma, the half-lives drop to <20 and <30 h, respectively. Compound **3** was found to be chemically very unstable in aqueous solution at any pH and was not extensively studied, whereas **12a** was found to be quite stable. Chemical stability studies, enzymatic hydrolysis studies, and activity studies on **12a** are ongoing.

Chemical stability is critical to the formulation of any water-soluble prodrug of taxol since partial degradation to the poorly soluble parent drug is likely to lead to precipitation of the taxol. The only derivative prepared in this study that appears to reasonably address this problem is **5b**. Five milligram per milliliter aqueous solutions of **5b** remain clear and precipitate-free for >4 h. Compound **5b** appears to show reasonable solid-state stability on storage. Further evaluation would be necessary to see if **5b** or a similarly behaving prodrug can be formulated for commercial utility.

Biological Evaluation. Some of the derivatives/prodrugs prepared in this study were tested in a variety of screens. Synthesis intermediates were not tested. The ability of the derivatives to effect microtubule assembly and B16 melanoma cell proliferation was examined in our laboratories at the University of Kansas. A MX-1 mammary tumor model screen was performed by the National Cancer Institute on some of the derivatives. Some derivatives, 2′-L-alanyltaxol, e.g., were not tested because of poor chemical stability.

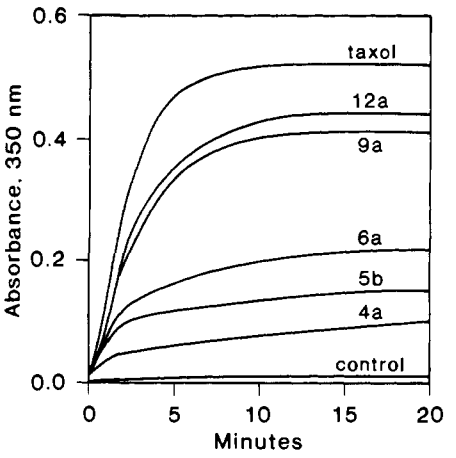

Figure 1. Effect of taxol derivatives on in vitro microtubule assembly. Tubulin (10 μM) was polymerized at 30 °C in the presence of 5 μM derivative and 0.5 mM GTP in PEM buffer. The increase in turbidity was monitored by the apparent absorbance at 350 nm.

Effect of Taxol Derivatives on Microtubule Assembly. The abilities of the taxol derivatives at a concentration of 5 μM to induce microtubule assembly in vitro were examined. As shown in Figure 1, analogues esterified at the C-7 hydroxyl group [7-(*N*,*N*-dimethylglycyl)taxol (**9a**) and 7-L-alanyltaxol (**12a**)] were more effective in promoting microtubule polymerization than those modified at the 2′-position (**4a** and **5b**) or both the 7- and 2′-positions [2′,7-bis(*N*,*N*-dimethylglycyl)taxol (**6a**)]. The percent of tubulin which had polymerized was determined to be 73% for taxol, 62% for **12a**, 56% for **9a**, 31% for **6a**, 26% for **5b**, and 18% for **4a**. HPLC analysis of the stability of these compounds demonstrated that the C-7 derivatives were completely stable for 20 min under assembly conditions. On the other hand, **6a** was degraded to 3% **9a**, while **4a** and **5b** were converted to 7% and 35% taxol, respectively. The degradation of the 2′-derivatives to taxol could account for the small activities observed in these cases. Thus, these studies confirm that a free hydroxyl group at the 2′-position but not at the 7-position is required for microtubule assembly stimulating activity in vitro. The results are consistent with other studies using various acetyltaxol derivatives which demonstrated that 2′-acetyl- or 2′,7-diacetyltaxol failed to support microtubule assembly, while 7-acetyltaxol demonstrated significant activity.[17]

Electron micrographs of the polymers showed that normal microtubules, some sheets of protofilaments, and a few ribbons and hoops were formed when assembly was done in the presence of taxol or any of the derivatives. In all cases, the polymers were depolymerized by 75–80% upon lowering the temperature to 5 °C. When Ca^{2+} was added to a final concentration of 5 mM to microtubules at steady state, little or no depolymerization occurred when taxol or any of the analogues were present. The same effects of Ca^{2+} and cold treatment were previously reported for taxol-induced assembly of pure tubulin.[22,23]

Effects of Taxol Analogues on B16 Melanoma Cell

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Table I. Effect of Taxol Derivatives on the Proliferation of B16 Melanoma Cells

compd	IC_{50}, nM[a]
taxol (1)	19
4a	21
5b	14
6a	42
9a	44
12a	43

[a] Concentration of compound which produced 50% inhibition of cell proliferation.

Proliferation. The effects of taxol derivatives on proliferation of B16 melanoma cells are presented in Table I. After a 40-h incubation with various concentrations of the derivatives, those esterified at the 2′-hydroxyl group were similar to taxol in their cytotoxicity, while **9a**, **12a**, and **6a** were about 50% as effective as taxol. Examination of the stability of the analogues under the cell-proliferation conditions showed that after a 40-h incubation, the derivatives were converted to different proportions of taxol and another compound which eluted after taxol on reversed-phase HPLC. This latter compound may be 7-epitaxol, an epimerization product of taxol which was previously reported to be formed in cell culture medium and to have similar activity as taxol in vivo and in vitro.[9] After 40 h, the 2′-derivatives were completely converted to taxol (70%) and the other degradation product (30%), which explains why their effects on proliferation of cells in culture were comparable to that of taxol. That is, the 2′-derivatives appear to behave as taxol prodrugs under these cell culture conditions. On the other hand, about 20–30% of **9a** and **12a** still remained under 40 h. The 2′,7-bis(*N*,*N*′-dimethylglycyl) derivative **6a** was partially degraded (35%) to a less potent inhibitor **9a**, which can account for its lower cytotoxicity compared to the 2′-analogues. Results obtained from a previous study demonstrated that the abilities of the 7-acetyl derivative and taxol to inhibit the growth of a macrophage-like cell line, J774.2, were similar, while the activities of 2′-acetyltaxol and 2′,7-diacetyltaxol were 3-fold and 10-fold less, respectively.[17] These studies therefore suggest that the cytotoxic properties of the 2′-taxol derivatives may be dependent on their conversion to taxol or an active taxol analogue or metabolite. In the case of the 7-derivatives, although these compounds promoted microtubule assembly in vitro to a similar extent as taxol, they were less cytotoxic than taxol. One possible explanation may be the differences in uptake of the derivatives and the parent compound by the cells.

To determine whether the effects of the taxol analogues on cell growth were reversible, cells were treated with a much higher concentration of the derivative (10^{-5} or 10^{-6} M) for 8 h, washed free of the derivative and its degradation products, and then allowed to grow in the medium. Cells, which had been treated with 10^{-6} M **5b**, **4a**, or **9a** recovered as quickly as cells treated with taxol (**Table II**). Proliferation after exposure to **6a** or **12a** was slightly slower than after treatment with taxol. However, at a 10-fold higher drug concentration, inhibition of cell growth was essentially not reversed; in fact, a fair amount of cell death occurred. Chromatin staining of cells incubated with 10^{-5} or 10^{-6} M taxol or any of the taxol analogues revealed fragmentation of the nuclei after 40 h of recovery. This morphological change is associated with cell death occurring by a process called apoptosis.[24] The degree of apoptosis was about 100% at 10^{-5} M and 20% at 10^{-6} M drug concentration.

Table II. Recovery of Cell Proliferation after an Acute Drug Treatment[a]

compd	percent change in cell number[b] 10^{-5} M	10^{-6} M
control[c]	832	832
taxol (1)[d]	75	252
4a	84	261
5b	−5	239
6a	−72	137
9a	−60	222
12a	−55	58

[a] B16 melanoma cells were treated with 10^{-5} or 10^{-6} M derivative for 8 h, washed free of the derivatives, and incubated in medium in the absence of the derivative for 48 h before measuring cell number. [b] Values are relative to time-zero cell number. [c,d] The average of three experiments.

Table III. Antitumor Activity of Taxol Derivatives against Subrenal Capsule Human Mammary Carcinoma (MX-1) Xenograft in Mice[a]

compd	dose, mg/kg	% T/C[b]	average tumor weight initial, mg	final, mg	survivors after 11 days
control	0.00		0.75	12.79	11/12
taxol (1)	22.40	Tox[c]	0.60	0.00	3/6
	15.00	CR[d]	0.81	0.00	6/6
	10.00	−84	0.58	0.09	6/6
	6.67	CR	0.74	0.00	5/5
	4.45	CR	0.79	0.00	5/5
4a	30.00	Tox	0.68	0.00	1/6
	20.00	CR	0.56	0.00	4/6
	13.00	CR	0.64	0.00	4/6
	8.90	−53	0.77	0.36	5/6
	5.90	−78	0.61	0.13	6/6
5b	36.00	Tox	0.75	0.00	0/5
	18.00	CR	0.82	0.00	3/4
	9.00	CR	0.87	0.00	5/5
	4.50	CR	0.78	0.00	5/5
6a	42.0	144	0.66	4.35	4/5
	10.5	193	0.59	5.55	5/6
	5.25	70	0.71	2.51	6/6
9a	33.00	CR	0.74	0.00	4/5
	16.5	−93	0.59	0.04	3/4
	8.25	23	0.81	4.19	4/4
	4.13	34	0.74	5.66	4/5

[a] Studies performed at the National Cancer Institute according to procedures described on pp 23–24 of NIH Publication No. 84-2635 (February 1984). [b] Defined as (Δ tumor weight in treated animals/Δ tumor weight in controls) × 100 for those animals in which the tumor grows, and (Δ tumor weight in treated animals/initial tumor weight in controls) × 100 if the tumor regresses. [c] Tox refers to toxicity or when < 65% of the animals survive the test. [d] CR refers to complete remission or when the tumor is no longer detectable.

MX-1 Mammary Tumor Screen. The MX-1 mammary tumor screen results for various 2′- and 7-amino acid derivatives are summarized in Table III. The most active compound, other than taxol itself, was **5b**, which showed complete tumor remission at a dose of 4.5 mg/kg. The derivatives **4a** and **9a** showed activity less than that of taxol. In the case of the 7-derivative **9a**, the activity is probably due to incomplete reversion to taxol and/or lower intrinsic activity compared to taxol. In the case of **6a**, the activity is substantially reduced. This is in agreement with the activity reported for the 2′,7-diacetyl derivative of taxol.[17,21]

Summary. The solubility and biological testing data for the taxol derivatives used in this study are summarized in Table IV. The 2′- and/or 7-amino acid derivatives of

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Table IV. Summary of the Properties of Taxol Derivatives

compd	solubility in H_2O, μM (mg/mL)	rel effect on microtubule assembly[a]	rel effect on B16 melanoma cell proliferation[b]	rel antitumor activity[c]
taxol (1)	35[d,e] (0.03)	1.0	1.0	1
4a	4825 (5)	0.25	0.90	2
5b	>9420 (>10)	0.36	1.36	1
6a	>8110 (>10)	0.42	0.45	4
9a	>1870 (>2)	0.77	0.43	3
12a	>1890 (>2)	0.85	0.43	

[a] Tubulin polymerized by derivative/tubulin polymerized by taxol. [b] IC_{50} (taxol)/IC_{50} (derivative). [c] Antitumor activity of taxol and the derivatives against mammary carcinoma xenograft in mice with 1 being the most active (see Table III). [d] From Swindell, C. S.; Krauss, N. E. *J. Med. Chem.* **1991**, *34*, 1176–1184. [e] In our laboratory, the equilibrium solubility has been determined to be 0.77 μM or 0.7 μg/mL. The reason for the discrepancy with the Swindell et al. data is not known at this time.

taxol in their salt form showed increased water solubilty when compared to taxol. The 7-derivatives of taxol were effective in promoting microtubule assembly in vitro, while 2′-derivatives showed little activity. The derivatives **4a** and **5b** inhibited proliferation of B16 melanoma cells to an extent similar to that of taxol, while the other derivatives were about 50% as cytotoxic. In a mammary tumor screen, **5b** showed the greatest antitumor activity compared to the other analogues. The lower activities of the 7-derivatives in inhibiting tumor growth and melanoma cell proliferation (although they were almost as active as taxol in inducing microtubule assembly in vitro) may be due to differences in drug uptake by the cells. The similar cytotoxic and antitumor activities of the 2′-analogues and taxol can be explained by their probable conversion to taxol or an active taxol metabolite.

Experimental Section

Taxol was supplied by the National Cancer Institute. Melting points were recorded on a Thomas-Hoover melting point apparatus and are uncorrected. Preparative TLC and column chromatography were done on silanized silica gel (E. Merck) to reduce degradation of the esters. NMR spectra were recorded on Varian XL-300 and Brucker 500 spectrometers. Chemical shifts are in ppm relative to TMS (0.00). All derivatives showed a proton NMR spectral pattern identical to taxol except for the 2′- and 7-protons for those derivatives acylated at the 2′-hydroxyl and/or 7-hydroxyl positions. Mass spectra were recorded by The Midwest Center for Mass Spectrometry Laboratory, Lincoln, NE. Microanalyses were determined by the Medicinal Chemistry Department of the University of Kansas.

Synthesis. 2′-(*N*-*t*-BOC-L-alanyl)taxol (2). To a solution of taxol (0.18 g, 0.211 mmol) in methylene chloride (10 mL) was added *N*-*t*-BOC-L-alanine (0.04 g, 0.211 mmol). To this solution were added DCC (0.2 g, 0.97 mmol) and DMAP (0.02 g, 0.16 mmol), and the mixture was stirred at room temperature for 2 days and filtered. The filtrate was evaporated under nitrogen, and the residue was chromatographed over a silanized silica gel column and eluted successively with ethyl acetate–petroleum ether (1:1) and ethyl acetate. The ethyl acetate–petroleum ether fractions containing the product were concentrated to yield 0.24 g of compound **2**. ^1H NMR: (300 MHz, $CDCl_3$) δ 5.52 (d, 4 Hz, C_2-H), 4.5 (m, C_7-H). Mass spectrum *m/e* 1025 $(M + H)^+$.

2′-L-Alanyltaxol (3). Sixty milligrams of **2** was allowed to react with formic acid (1 mL, Sigma 99%) for 30 min, and the acid was completely removed under nitrogen. The residue was taken up in water (10 mL); the pH was raised to 8 using a cold $NaHCO_3$ solution (0.05 M) and quickly extracted with methylene chloride. The methylene chloride extract was dried over anhydrous Na_2SO_4, and solvent was removed. The crude product was purified by preparative TLC over silanized silica gel plates and developed in ethyl acetate–petroleum ether (1:1). The band below taxol was removed by scraping and eluted with an ethyl acetate and methylene chloride mixture, and the solvent was removed to give 0.028 g of 2′-L-alanyltaxol: mp 160–165 °C dec. Mass spectrum: (FAB) *m/e* 925 $(M + H)^+$. Anal. ($C_{50}H_{56}N_2O_{15}$·$2H_2O$) C, N, H.

2′-(*N*,*N*-Dimethylglycyl)taxol (4). To a solution of taxol (0.21 g, 0.246 mmol) and *N*,*N*-dimethylglycine (0.0245 g, 0.246 mmol) in anhydrous methylene chloride (12 mL) were added DCC (0.15 g, 0.72 mmol) and DMAP (0.025 g, 0.2 mmol). The reaction mixture was stirred under anhydrous conditions for 1 day. Another 50 mg of DCC was then added, and the stirring was continued for an additional 6 h. The reaction mixture was filtered, and the filtrate was evaporated under nitrogen. The residue was chromatographed over silanized silica gel (35 g, 26 cm) and eluted successively with ethyl acetate–petroleum ether (1:1) and ethyl acetate. The ethyl acetate–petroleum ether fractions, upon slow evaporation, resulted in a solid, which was isolated by filtration. The mother liquor was further concentrated, petroleum ether was added until turbidity developed, and then it was set aside to obtain more compound. The total recovery was 0.14 g (61%): mp 168–171 °C dec. The NMR spectrum of **4** showed a shift in the 2′-proton resonance from 4.71 ppm in taxol to 5.51 ppm. This is consistent with the esterification at the 2′-position. All other resonances of the spectrum were in agreement with the assigned structure. HPLC purity 98–99%. Mass spectrum: (FAB) *m/e* 939. $(M + H)^+$. Anal. ($C_{51}H_{58}N_2O_{15}$) C, H, N.

Methanesulfonic Acid Salt of 2′-(*N*,*N*-Dimethylglycyl)taxol (4a). A 0.06-g sample of **4** (0.064 mmol) was dissolved in *tert*-butyl alcohol (2 mL) and water (1.5 mL). The mixture was cooled to ~5 °C and methanesulfonic acid (3.1 mL of a 2 mg/mL aqueous solution, 0.0645 mmol) was added dropwise. The mixture was stirred at 0–5 °C for 1 min and filtered through a 0.2-μm filter (Millipore) into a flask cooled in a dry ice–2-propanol mixture. The filtrate was freeze-dried to give 0.058 g of product (88%): mp 170–173 °C. Solubility in H_2O >2 mg/mL. Anal. ($C_{51}H_{58}N_2O_{15}$·CH_3SO_3H·$2H_2O$) C, H, N.

HCl Salt of 2′-[3-(*N*,*N*-Diethylamino)propionyl]taxol (5a). To a solution of taxol (0.12 g, 0.14 mmol) in methylene chloride (12 mL) containing 3-(*N*,*N*-diethylamino)propionic acid hydrochloride (0.025 g, 0.145 mmol) were added DCC (0.08 g, 0.38 mmol) and DMAP (0.01 g, 0.08 mmol). The resulting mixture was stirred at room temperature for 24 h. The reaction mixture was filtered, and the filtrate was evaporated under nitrogen. The crude product was chromatographed over a silanized silica gel column (18 g, 22 cm) and eluted successively with ethyl acetate–petroleum ether (1:2 followed by 1:1) and ethyl acetate. The ethyl acetate–petroleum ether fractions on slow evaporation gave a solid, which was isolated by filtration. The mother liquors containing product were combined and concentrated, petroleum ether was added to cloudiness, and it was set aside. Further solid was filtered to obtain 0.068 g of product (48%): mp 188–191 °C. ^1H NMR: (300 MHz, $CDCl_3$) δ 5.54 (d, 8 Hz, C_2′-H), 4.46 (m, C7-H), 1.05 (t, CH_3). Mass spectrum: (FAB) *m/e* 981 $(M + H)^+$. Solubility in H_2O <1 mg/mL. Anal. ($C_{54}H_{64}N_2O_{15}$·HCl) C, H, N.

Methanesulfonic Acid Salt of 2′-[3-(*N*,*N*-Diethylamino)propionyl]taxol (5b). To a solution of taxol (0.256 g, 0.3 mmol) and 3-(*N*,*N*-diethylamino)propionic acid methanesulfonate salt (0.076, 0.315 mmol) in methylene chloride (20 mL) was added DCC (0.3 g, 1.45 mmol). The mixture was stirred at room temperature for 24 h and filtered, and the filtrate was evaporated under nitrogen. The residue was chromatographed over a silanized silica gel column and eluted with ethyl acetate–petroleum ether (1:1) and ethyl acetate. The ethyl acetate–petroleum ether fractions on slow evaporation gave 0.27 g of product (85%): mp 170–174 °C. HPLC purity 99%. Solubility in H_2O ~10 mg/mL. Anal. ($C_{54}H_{64}N_2O_{15}$·CH_3SO_3H) C, H, N.

2′,7-Bis(*N*,*N*-dimethylglycyl)taxol (6). Taxol (0.06 g, 0.0702 mmol) was dissolved in anhydrous methylene chloride (5 mL), and *N*,*N*-dimethylglycine (0.015 g, 0.145 mmol), DCC (0.08 g, 0.387 mmol), and DMAP (0.008 g, 0.065 mmol) were added. The reaction mixture was stirred at room temperature for 24 h and filtered. The solvent was removed from the filtrate, and the residue was purified by preparative TLC on silanized silica gel plates and developed in ethyl acetate–petroleum ether (1:1). A band (R_f 0.17) above DMAP was removed, eluted with an ethyl acetate/ethanol mixture, and filtered, and the solvent was removed. The residue was recrystallized from ethyl acetate–pe-

troleum ether to give 0.046 g of product (64%): mp 194–198 °C. Mass spectrum: (FAB) m/e 1024 $(M + H)^+$. In the NMR spectrum (300 MHz, $CDCl_3$) the 2′-proton (4.71 ppm) and C7-proton (4.33 ppm) shifted to 5.5 and 5.6 ppm, respectively, showing esterification at both the 2′- and 7-positions. Also, the N-CH_3 proton appeared as a singlet at 2.3 ppm. Anal. ($C_{55}H_{65}N_3O_{16}$·$2H_2O$) C, H, N.

Dimethanesulfonic Acid Salt of 2′,7-Bis(*N*,*N*-dimethylglycyl)taxol (6a). To a solution of **6** (0.06 g, 0.0585 mmol) in *tert*-butyl alcohol (2 mL) and water (1 mL) cooled to 0–5 °C was added methanesulfonic acid (0.0114 g, 3.79 mL, 0.117 mmol). The mixture was stirred at 0–5 °C for 2 min and filtered through a Millipore filter (0.2 μm), and the filtrate was freeze-dried to give 0.062 g of product: mp 160–163 °C. Solubility in H_2O >10 mg/mL.

2′-(Troc)taxol (7). Compound **7** was prepared following the procedure described by Magri and Kingston.[20] Taxol (0.27 g, 0.316 mmol) was dissolved in methylene chloride (10 mL) and pyridine (1.5 mL). The reaction mixture was cooled to −20 °C, 2,2,2-trichloroethyl chloroformate (80 μL) was added, and the mixture was stirred for 3 h, after which an additional 25 μL of the chloroformate was added and stirring continued overnight. The reaction mixture was then diluted with methylene chloride (50 mL) and washed successively with 0.1 M HCl (25 mL twice), 0.05 M cold $NaHCO_3$ (25 mL), and water. The organic phase was dried over anhydrous $MgSO_4$, and the solvent was removed. The crude material was purified by preparative TLC over silanized silica and developed in ethyl acetate–petroleum ether (1:3). The band above taxol was cut, eluted with ethyl acetate, and the solvent was removed to give 0.32 g (97%): mp 221–226 °C (dec, softens ~160 °C).

2′-Troc-7-(*N*,*N*-dimethylglycyl)taxol (8). A mixture of 2′-(troc)taxol (0.27 g, 0.262 mmol) and *N*,*N*-dimethylglycine (0.054 g, 0.524 mmol) was dissolved in methylene chloride (15 mL). To this solution were added DCC (0.215 g, 1.04 mmol) and DMAP (0.025 g, 0.20 mmol), and the mixture was stirred at room temperature for 2 days. The reaction mixture was filtered, and the solvent was removed. The crude material was purified by prepative TLC over silanized silica gel plates and developed in ethyl acetate–petroleum ether (1:1). A band below taxol [R_f 0.47, ethyl acetate–petroleum ether (1:1)] was scraped and eluted with ethyl acetate, and the solvent was removed to yield 0.26 g of product (89%): mp 176–180 °C dec. Mass spectrum: (FAB) m/e 1118 $(M + H)^+$, m/e 1124 $(M + Li)^+$. Anal. ($C_{54}H_{59}Cl_3N_2O_{17}$) C, H, N.

7-(*N*,*N*-Dimethylglycyl)taxol (9). To a solution of **8** (0.335 g, 0.30 mmol) in 9:1 methanol–acetic acid (12 mL) was added zinc dust (0.275 g), and the mixture was stirred at room temperature for 25 min and filtered. The filtrate was concentrated to ~1 mL, diluted with methylene chloride (35 mL), and washed successively with 0.01 M HCl (20 mL, twice), 0.01 M cold $NaHCO_3$, and water. The organic extract was dried over anhydrous Na_2SO_4 and filtered, and the solvent was removed to give 0.24 g of product. This compound was purified by preparative TLC with silanized silica gel plates (20 × 20 cm, 3 Nos) and developed in methylene chloride–ethyl acetate (7:1). The band corresponding to 7-(*N*,*N*-dimethylglycyl)taxol (R_f 0.35) was cut and eluted with ethyl acetate and ethanol, and the solvent was removed to yield 0.19 g of product (68%): mp 180–185 °C (softens at 140 °C). Mass spectrum: (FAB) m/e 939 $(M + H)^+$. In the ^1H NMR spectrum (300 MHz, $CDCl_3$) the resonances of the C7-H at 4.33 ppm in taxol appeared as a doublet at 5.65 ppm. The N-$(CH_3)_2$ resonance appeared as a singlet at 2.35 ppm. The methylene group of the glycinate appeared at 3.16 ppm.

Methanesulfonic Acid Salt of 7-(*N*,*N*-Dimethylglycyl)taxol (9a). Compound **9** (0.065 g, 0.069 mmol) was dissolved in *tert*-butyl alcohol (2.5 mL) and water (1 mL). The solution was cooled to 5–10 °C, and methanesulfonic acid (3.36 mL of a 2 mg/mL solution, 0.0697 mmol) was added. The mixture was stirred for 2 min and filtered through a Millipore filter (0.2 μm) to a flask cooled in ice. The filtrate was freeze-dried to give 0.066 g of product (94%): mp 164–168 °C dec. Solubility in H_2O >2 mg/mL. Anal. ($C_{51}H_{58}N_2O_{15}$·CH_3SO_3H·$2H_2O$) C, H, N.

2′,7-Bis(*t*-BOC-L-alanyl)taxol (10). To a solution of taxol (0.21 g, 0.246 mmol) and *N*-*t*-BOC-L-alanine (0.14 g, 0.739 mmol) in methylene chloride (15 mL) were added DCC (0.25 g, 1.21 mmol) and DMAP (0.025 g, 0.20 mmol). The mixture was stirred at room temperature for 24 h and filtered. The residue was chromatographed over silanized silica gel (20 g, 14 cm) and eluted with ethyl acetate–petroleum ether (1:1) and ethyl acetate. The ethyl acetate–petroleum ether fractions containing the disubstituted derivative were pooled, and the solvent was removed to yield 0.27 g of compound **10** (92%): mp 158–161 °C dec.

7-L-Alanyltaxol (12). Compound **10** (0.29 g, 0.242 mmol) and formic acid (2 mL) were mixed, the mixture was stirred at room temperature for 90 min, and excess formic acid was removed under nitrogen. The residue was dissolved in ethanol, petroleum ether was added, and the solid formed was filtered to yield 0.27 g of 2′,7-di-L-alanyltaxol (**11**). Mass spectrum: (FAB) m/e 996 $(M + H)^+$. Crude **11** was taken up in acetonitrile (4 mL) and phosphate buffer (0.02 M, pH 7.4, 50 mL), and the mixture was stirred at room temperature for 12 h. The pH of the solution was raised to 6.8 using a few milliliters of 5% Na_2HPO_4. The cloudy solution was stirred at room temperature for an additional 8 h. The reaction mixture was diluted with methylene chloride (50 mL), and cold $NaHCO_3$ (0.05 M, 50 mL) was added. The reaction mixture was immediately extracted with methylene chloride (50 mL, three times), and the organic extract was washed once with water and dried over anhydrous sodium sulfate. After decanting, the solvent was removed to give 0.24 g of compound **12**. This compound was purified by column chromatography over silanized silica gel to yield 0.135 g of compound **12** (63%): mp 159–163 °C. Mass spectrum: (FAB) m/e 925 $(M + H)^+$. In the NMR spectrum (300 MHz, $CDCl_3$), the proton at C-7 at 4.33 ppm in taxol appeared as a doublet of doublets at 5.65 ppm. The CH_3 group on the alanine moiety appeared as a doublet at 1.27 ppm. Anal. ($C_{50}H_{56}N_2O_{15}$·$2.5H_2O$) C, H, N.

Methanesulfonic Acid Salt of 7-L-Alanyltaxol (12a). To a solution of **12** (0.062 g, 0.0658 mmol) in *tert*-butyl alcohol (2 mL) and water (1 mL) cooled to 0–5 °C was added methanesulfonic acid (6.39 mg, 2.13 mL of a 3 mg/mL H_2O solution), and the mixture was stirred for 2 min and filtered through a Millipore filter (0.2 μm). The filtrate was freeze-dried to obtain 0.06 g of product: mp 180–184 °C. Solubility in H_2O >2 mg/mL. Anal. ($C_{50}H_{56}N_2O_{15}$·CH_3SO_3H·$2H_2O$) C, H, N.

In Vivo and in Vitro Testing. Various in vivo and in vitro tests were performed on a limited number of the derivatives/prodrugs of taxol prepared in this study. Some of the derivatives were tested by the National Cancer Institute. Other tests were microtubule assembly kinetics and B16 melanoma cell proliferation studies, which were performed in our laboratories.

Tubulin Preparation. Microtubule protein was isolated from bovine brain by the assembly–disassembly method of Shelanski et al.[25] Tubulin was purified from microtubule-associated proteins by chromatography on a Biogel P-10–phosphocellulose piggyback column as described previously.[26]

Microtubule Assembly. Tubulin (10 μM) was assembled at 30 °C in the presence of 5 μM taxol or a taxol analogue and 0.5 mM GTP in PEM buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA and 1 mM $MgSO_4$). The assembly reaction was monitored by the increase in apparent absorbance at 350 nm using a temperature-controlled spectrophotometer. To determine the amount of assembled tubulin, the samples were centrifuged in a Beckman TL-100 ultracentrifuge at 37 °C and 200000g for 4 min. The pellet was dissolved in cold PEM buffer and allowed to depolymerize on ice for 15 min. The protein concentration in the supernatant after centrifugation at 4 °C was determined by the method of Bradford.[27]

B16 Melanoma Cell Proliferation. Cells were seeded on glass coverslips at a concentration of 2.5×10^4 cells/mL in cluster dishes and grown in Dulbecco's modified minimal essential medium

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(MEM) containing 10% bovine calf serum at 37 °C for 24 h in a 97% humidified atmosphere of 5.5% CO_2. The medium was then replaced with fresh medium containing taxol or its derivatives in concentrations ranging from 5×10^{-9} M to 75×10^{-9} M. The final concentration of DMSO in the cell medium was 0.5% or less. This amount of DMSO did not have any effect on cell proliferation as determined from control experiments. After 40 h, the cells were released by trypsinization and counted in a Coulter counter. To study cell recovery after an acute treatment of the drug, cells were incubated with 10^{-5} and 10^{-6} M taxol or its analogue for 8 h. The medium was removed, and the cells were rinsed three times with fresh medium and allowed to grow in medium free of drug for 48 h.

Chromatin Fluorescence Staining. Following drug treatment and recovery, the medium was removed, and the cells were fixed with 4% formaldehyde in 0.05 M phosphate buffer, pH 7.3, and permeabilized with cold 95% ethanol for 30 min at 0 °C. Between each step the cells were washed once with PBSA (phosphate-buffered saline, without Ca^{2+} and Mg^{2+}) and twice with PBS (phosphate-buffered saline, with Ca^{2+} and Mg^{2+}). Chromosome staining was done using 1 mL of Hoechst 33342 DNA stain (0.2 μg/mL in PBS) per coverslip for 1 h in the dark at room temperature. The coverslips were mounted on glass slides and viewed under a Leitz fluorescence microscope.

Stability of Drugs during Microtubule Assembly and Cell Growth Studies. The stability of the taxol derivatives was examined under the same conditions used for assembly (PEM buffer, pH 6.9, 30 °C) and for cell growth (MEM with 10% serum, 37 °C) by chromatography on a C-8 Synchropak reversed-phase HPLC column. The compounds were eluted with 0.02 M ammonium acetate, pH 7-acetonitrile (50:50) and detected by their absorbance at 254 nm.

Electron Microscopy. For negative staining, aliquots of the assembly mixture diluted 15-fold in PEM buffer containing 0.25% glutaraldehyde were placed on Formvar- and carbon-coated grids, washed three times with H_2O, and stained with 2% uranyl acetate. The grids were viewed on a Phillips 300 transmission electron microscope.

In Vivo MX-1 Mammary Screen. A study of a limited number of derivatives in the subrenal capsule human mammary carcinoma (MX-1) xenograft screen in mice was performed by the National Cancer Institute. The study was performed according to procedures described in NIH Publication No. 84-2635 (February 1984). All the derivatives were administered ip as aqueous solutions except for taxol.

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Registry No. **2**, 137234-34-5; **3**, 131966-80-8; **4**, 117527-60-3; **4a**, 132073-89-3; **5**, 132073-90-6; **5a**, 131966-75-1; **5b**, 132073-91-7; **6**, 131966-78-4; **6a**, 132073-93-9; **7**, 100431-55-8; **8**, 131966-76-2; **9**, 131966-77-3; **9a**, 132073-92-8; **10**, 131966-83-1; 11, 137234-35-6; **12**, 131966-79-5; **12a**, 132073-94-0; L-$HO_2CCH(CH_3)NHCOOC(CH_3)_3$, 15761-38-3; $HO_2CCH_2N(CH_3)_2$, 1118-68-9; $HO_2C(CH_2)_2N(C_2H_5)_2 \cdot HCl$, 15674-67-6; $HO_2C(CH_2)_2N(C_2H_5)_2 \cdot CH_3SO_3H$, 131919-23-8; $ClCOOCH_2CCl_3$, 17341-93-4.

Preparation and Biological Activities of Potential Vasopressin Photoaffinity Labels

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Several potential photoaffinity analogues of the peptide hormone vasopressin (VP) were prepared by classical solid-phase peptide synthesis using two different pathways. Peptide sequences were built by introduction of (a) $N^{\alpha r}$-protected aminophenylalanine or (b) nitrophenylalanine in the photolabeling position. Conversion to the azido peptide was completed in pathway a after cleavage and before purification and in pathway b from small quantities of purified nitrophenylalanine-containing precursor peptides. V_1 receptor binding properties were measured using membranes prepared from rat liver cells. The binding potential of agonistic VP structures was abolished by the introduction of an azido or a nitro group into the aromatic side chain at position 3. Cyclo desamino-β,β-dialkyl-Cys[1]-type VP antagonist structures were prepared with the photoactivable moiety in position 2 and an iodination residue in position 9. One particular compound, [Dmpa[1],Phe(N_3)[2],Val[4],Lys[8],D-Tyr[9]]VP (**8**), containing β,β-dimethyl-β-mercaptopropionic acid in position 1, had excellent binding properties, both in the radioiodinated ($K_d = 4.8 \pm 1.9 \times 10^{-10}$ M) and noniodinated form ($K_d = 6.4 \pm 0.98 \times 10^{-10}$ M). The analogues with long-chain β-alkylation (diethyl and pentamethylene) and the linear antagonist photolabel showed significantly less affinity. Optimal binding properties were obtained within a very narrow range of hydrophobicity; greater or lesser hydrophobicity was correlated to less potent binding. The precursor analogues, containing nitrophenylalanine, displayed a structure–activity relationship similar to that of the azido peptides. The most potent analogues will be used for receptor labeling studies. A linear antagonist structure having a photosensitive group in position 1 has also been prepared, but this compound displayed much less affinity than the cyclic antagonists. The most potent compounds were also highly selective for the V_1 receptor and did not recognize the V_2 receptor from other preparations.

Introduction

Peptide hormone receptors have been a favored research target, both in the past and today. Many efforts have been made to prepare highly specific ligands for labeling and, eventually, for biochemical isolation of their respective receptors. In particular, the receptors for the peptide hormone vasopressin (VP) have been investigated with selective ligands, but both the vascular receptor V_1 and the renal receptor V_2 have so far eluded isolation. In the past, several studies have been carried out with photosensitive analogues of VP and the other neurohypophyseal hormones, but all studies with photoaffinity analogues of VP produced unsatisfactory results because the molecular

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