Structure-Antitubulin Activity Relationships in Steganacin Congeners and Analogues. Inhibition of Tubulin Polymerization in Vitro by (±)-Isodeoxypodophyllotoxin

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A new series of 23 synthetic analogues of the naturally occurring antitumor lignan steganacin was tested for the inhibition of microtubule assembly in vitro. Interestingly, (±)-isopicrostegane (I_{50} = 5 μM) was found to be almost as active as (±)-steganacin (I_{50} = 3.5 μM). On the other hand, racemic isodeoxypodophyllotoxin has an inhibiting activity of microtubule assembly comparable to that of (-)-podophyllotoxin, whereas (-)-isodeoxypodophyllotoxin is totally inactive.

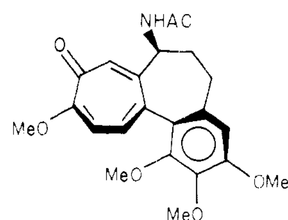
The spindle poisons inhibit the polymerization of tubulin (a dimeric protein) into microtubules and thus prevent spindle formation and cell division.[1] The first of these poisons known, i.e., colchicine (1),[2] binds to the dimeric tubulin at a specific site hence called the "colchicine site". Podophyllotoxin (2) and steganacin (4), two natural compounds respectively isolated from *Podophyllum peltatum* and *Steganotaenia araliacea*, were later shown to bind at the same site.[3,4] Some structure-antitubulin activity relationships in the steganacin series have recently been described, essentially studying the functional variations at C5 of the bis(benzocycloctadiene) (BBCOD) skeleton of steganacin (4).[5]

Several routes to the BBCOD skeleton are now known.[6-10] As far as we are concerned, we ourselves have developed a new synthetic pathway which led us to (±)-steganacin, as well as to a number of new analogues, having, for instance, a functional group at C8 and/or a cis lactone ring junction. These compounds present every possible configuration of the BBCOD skeleton, and a thorough study of their structure-antitubulin activity relationships allows us to get further insight into the usual structural requirements for the binding to the colchicine site of tubulin.

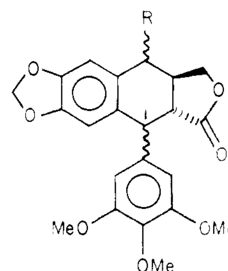
Experimental Section

The racemic compounds isodeoxypodophyllotoxin (3), steganone (5) and its analogues 6, 7, and 12-16, and the open chain analogues 19-22 of the BBCOD lignans were prepared as previously described, or following similar lines (Charts I and II).[9-11] Racemic steganacin (4), episteganacin (8), steganol (9), and episteganol (10) were prepared from (±)-steganone (5), according to the literature.[6] (±)-8α-(Trifluoroacetoxy)isopicrostegane (17), mp 204-208 °C, was obtained by treating the corresponding 8α-alcohol 16 with CF_3CO_2H containing catalytic amounts of $HClO_4$. (±)-8α-Acetoxyisopicrostegane (18), mp 186-187 °C, was obtained using acetic anhydride in pyridine for the acetylation of the same alcohol, 16. (±)-Stegane (11) was prepared from (±)-steganacin (4) in the following way. A solution of (±)-steganacin (4; 350 mg) in AcOEt (10 mL), containing $HClO_4$ (0.1 mL)

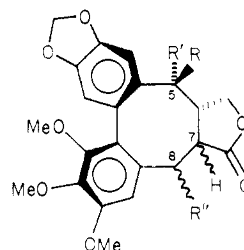
Chart I. Structural Formulas of Colchicine, Podophyllotoxin, and Steganacin and Its Derivatives



colchicine (1)



podophyllotoxin (2), R = OH; 1β-H
isodeoxypodophyllotoxin (3), R = H; 1α-H



"normal" series
steganacin (4), R = OAc; R' = R'' = H; 7α-H
steganone (5), R, R' = O; R'' = H; 7α-H
picrostegane (6), R = R' = R'' = H; 7β-H
8α-hydroxypicrostegane (7), R = R'' = H; R' = β-OH; 7β-H
episteganacin (8), R = R'' = H; R' = OAc; 7α-H
steganol (9), R = OH; R' = R'' = H; 7α-H
episteganol (10), R = R'' = H; R' = OH; 7α-H
stegane (11), R = R' = R'' = H; 7α-H
steg-7-en-8-ol (12), R = R' = H; R'' = OH; 7,8 double bond

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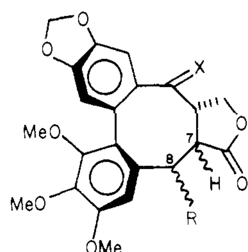
Chart II. Structural Formulas of Steganacin Analogues of the Iso Series



"iso series"

isosteganone (13), X = O; R = H
isostegane (14), X = H_2; R = H; 7α-H
isopicrostegane (15), X = H_2; R = H; 7β-H
8α-hydroxyisopicrostegane (16), X = H_2; R = α-OH; 7β-H
8α-(trifluoroacetoxy)isopicrostegane (17), X = H_2; R = α-$COCF_3$; 7β-H
8α-acetoxyisopicrostegane (18), X = H_2; R = α-OAc; 7β-H

and 5% Pd/C (400 mg), was hydrogenated in a Parr apparatus at 50 psi for 16 h. Workup in the usual manner afforded (±)-stegane (11; 71% yield) as prisms, mp 142–144 °C (MeOH). These three new compounds were characterized by their elemental analyses and their IR, NMR, and mass spectra.

Natural colchicine (1) and podophyllotoxin (2) were purchased from Aldrich Chemical Co. and from Sigma Chemical Co.

2-(N-Morpholino)ethanesulfonic acid, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA), and guanosine triphosphate (GTP) were obtained from Sigma. Activated charcoal (from Merck) was used at concentrations of 3 mg/mL. Bray's solution was from New England Nuclear, and ring C methoxy[^{3}H]colchicine (specific activity 2.8 Ci/mmol, batch 26) was from the Radiochemical Centre, LTD, Amersham.

All the compounds tested were dissolved in dimethyl sulfoxide (Me_2SO) at a concentration of 10^{-2} M and stored at −20 °C. In our experiments, the volume of Me_2SO used did not interfere with tubulin polymerization.

Porcine brain tubulin was purified following the method of Shelanski,[12] by three cycles of polymerization–depolymerization and was dissolved in the assembly buffer containing 0.1 M 2-(N-morpholino)ethanesulfonic acid, 0.5 mM $MgCl_2$, 1 mM EGTA, and 1 mM GTP, pH 6.6. This preparation (as controlled by acrylamide gel electrophoresis) contained 15% of microtubule associated proteins (MAPs) and 85% of tubulin.

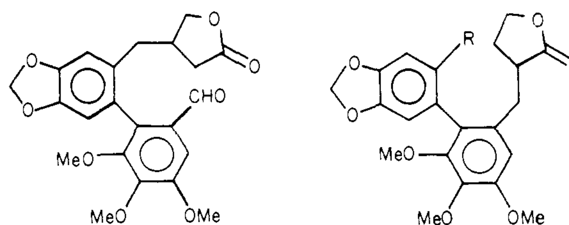
The concentation of protein was estimated by the method of Lowry et al.[13] with bovine serum albumin as a standard and was kept at ca. 1 mg/mL for the experiments.

Tubulin polymerization was monitored and recorded continuously by turbidimetry[14] at 350 nm with a Jobin-Yvon Duospac 203 UV spectrophotometer, equipped with a thermostated cell where the temperature of the sample was raised from 4 to 37 °C.

We determined for each drug the I_{50} value of its concentration which decreased by 50% the maximum polymerization rate of tubulin measured on the polymerization curve. The I_{50} value for each compound tested was compared with the I_{50} value for colchicine tested on the same day with tubulin from the same batch.[15]

It is known[16] that the interaction of colchicine with tubulin is slow, temperature dependent, and irreversible in the cold. Therefore, the samples containing the drug and the tubulin were preincubated for 1 h at 37 °C, so as to ensure maximum fixation of the drug on its receptor. Then, the samples were cooled to 4 °C in order to achieve complete depolymerization of the noninhibited tubulin and tested only 30 min later for polymerization, as described above. We found that I_{50} values for colchicine and all the other active compounds we tested were lower with incubation than without. No difference was observed in the case of the weakly active drugs for which a low affinity is the determining factor. We consider that the I_{50} values obtained in the case of preincubation are really representative of the affinity of the drug for the tubulin receptor.

Chart III. Open-Chain Analogues of Steganacin



19

"seco" series
secosteganone (20), R = CHO
secosteganol (21), R = CH_2OH
secosteganacin (22), R = CH_2OAc

Binding of [^{3}H]colchicine was measured by the method of Sherline.[17] [^{3}H]Colchicine (25 pCi/mol) was directly added to the tubulin at concentrations ranging from 2.5 to 20 mM colchicine, with and without the tested drug (50, 75, and 100 mM), and incubated at 37 °C for 1 h. The mixture was treated with charcoal, cooled in ice for 10 min, and then centrifuged at 9800g for an additional 10 min to effect the separation of the free colchicine from the water-soluble tubulin–colchicine complex. An aliquot of the supernatant was taken and diluted in 10 mL of Bray's solution and then counted in an Intertechnique SL 30 liquid scintillation counter.

The apparent inhibition constants (K_i) were obtained from the slopes of the double-reciprocal Lineweaver–Burk plot and assumed to represent the dissociation constants for the drugs.[18] Since colchicine shows an affinity for the dimeric form of tubulin[19] and not for the polymerized form (microtubules), it was important to use concentrations of drugs high enough to block completely the polymerization of tubulin, even at 37 °C, but not exceeding 10^{-4} M, in order to avoid a nonspecific precipitation of tubulin.

Results

The results of triplicate experiments are given in Table I, which compares the constants I_{50} and K_i of the drugs with the I_{50} and K_D of colchicine (1). The dissociation constant, K_D, of colchicine is consistent with results[20] showing that microtubule associated proteins (MAP), present in the Shelanski tubulin, lower the affinity of colchicine for tubulin. The Lineweaver–Burk plot obtained indicated that these drugs behave as competitive inhibitors of colchicine on tubuline.

The fact that the K_i values are always inferior to the K_D values is in agreement with the already noted[20] substoichiometric action of colchicine on tubulin polymerization. The products unable to inhibit at least 50% of the polymerization were considered as inactive, and those having a ratio $K_i/K_D > 100$ were beyond the accuracy of our method. Our results for colchicine (1), steganacin (4), steganone (5), and steganol (9) corroborate previous work.[4,5] We have also an original series of 9 compounds having the skeleton of bisbenzocyclooctadiene lignans, in which spatial, as well as functional, variations can be examined with respect to their activity on tubulin. Thus, the *spatial variations* affect the *biaryl junction*, which can

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Table I. Inhibition of Microtubule Assembly and Competition with [^{3}H]Colchicine of Some Bisbenzocyclooctadienes

compd	I_{50}, μM	I_{50}/I_{50} (CLC)[a]	K_i, μM	K_i/K_D (CLC)[b]
(–)-colchicine (1)[c]	2.5	1	5.75	1
(–)-podophyllotoxin (2)[c]	1.65	0.66	2.18	0.38
(±)-isodeoxypodophyllotoxin (3)	2.10	0.85	201	35[d]
(–)-isodeoxypodophyllotoxin (3)[c]	inact[e]		not tested	
steganacin (4)	3.5	1.4	10.5	1.8
secosteganacin (22)	inact		inact	
episteganacin (8)	inact		inact	
8α-acetoxyisopicrostegane (18)	450	180	not tested	
8α-(trifluoroacetoxy)isopicrostegane (17)	162	65	not tested	
stegane (11)	60	24	100	17.5
isostegane (14)	I_{20} = 70		inact	
picrostegane (6)	I_{30} = 700		inact	
isopicrostegane (15)	5	2	32	5.55
steganone (5)	125	50	178	31
isosteganone (13)	375	150	inact	
secosteganone (20)	inact		inact	
steganol (9)	110	44	185	32
episteganol (10)	inact		inact	
secosteganol (21)	inact		185	32
8α-hydroxypicrostegane (7)	inact		not tested	
8α-hydroxyisopicrostegane (16)	inact		not tested	
steg-7-en-8-ol (12)	450	180	not tested	
diphenyls (19)	inact		not tested	

[a] I_{50} (CLC) refers to colchicine. [b] K_D (CLC) refers to colchicine. [c] Optically active compounds. [d] K_i/K_D (CLC) = 13 when tubulin is preincubated with compound 3 prior to the addition of [^{3}H]colchicine (1). [e] inact = inactive.

be "normal" [as in stegane (**11**) and picrostegane (**6**)] or "iso" [as in isostegane (**14**)], and the *lactone ring*, which is normally trans [as in isostegane (**14**)] but is cis in the *picro* series [as in isopicrostegane (**15**)].

These spatial variations, if considered separately, are not determinant for activity; however, their combined effect is determinant; thus, stegane (**11**) and isopicrostegane (**15**) are active, whereas isostegane (**14**) and picrostegane (**6**) are inactive.

Functional variations affect the 5 or 8 positions. At C5, hydroxylation [steganol (**9**)] or ketonization [steganone (**5**)] hardly modify the activity of the stegane skeleton. A 5-acetoxy group [steganacin (**4**)] considerably increases the activity, contrary to a 5-epiacetoxy group which entails a complete loss of "activity". The C8-functionalized compounds are considerably less active, possibly due to the modification of the cyclooctadiene conformation or to a hindrance of the interaction of the lactone ring with the tubulin binding site.

The rigidity of the skeleton is lost in the C5–C6 or C7–C8 open-chain analogues **19–22** (Chart III). They cannot retain, unless statistically, the required configuration and are therefore inactive.

Racemic isodeoxypodophyllotoxin (**3**) inhibits polymerization of tubulin as much as (–)-podophyllotoxin (**2**), and both are competitive with colchicine. Since (–)-isodeoxypodophyllotoxin was inactive, the (+) enantiomer is therefore the active compound.

Discussion

The results given in Table I show that all the compounds tested, which belong to the series of colchicine, stegane, and podophyllotoxin, bind to the same site of the tubulin molecule. Indeed, the above three compounds exhibit a strong functional analogy, having a trimethoxyphenyl ring, an oxygen-substituted ring, and the same overall geometry.

The colchicine site is able to recognize the (+) enantiomer of isodeoxypodophyllotoxin, which bears some resemblance with the (–) enantiomer of podophyllotoxin, the H6 and H7 inversions being the only difference, as shown by molecular models. The configuration of (–)-podophyllotoxin (**2**) is unambiguously known.[21] In some ways,

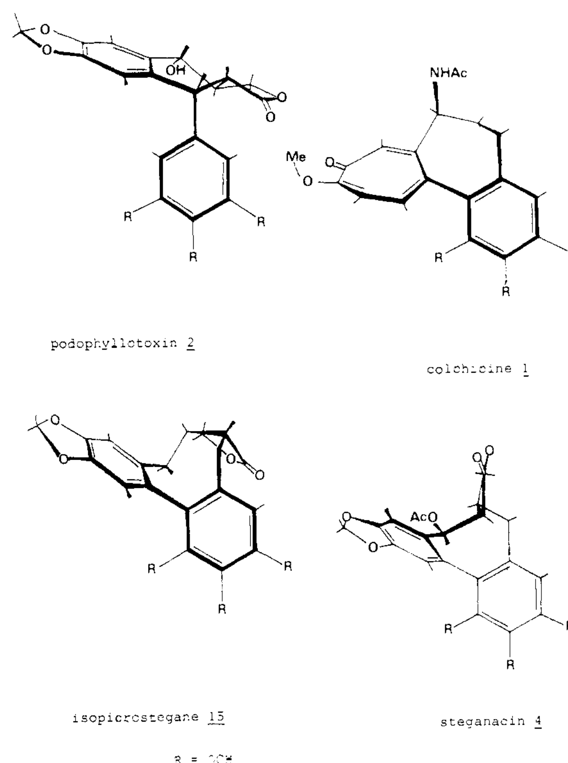

Figure 1. Perspective representations of some antitubulin compounds.

colchicine is very similar to podophyllotoxin; the trimethoxyphenyl group can have the same orientation in both compounds, and the acetamido group of colchicine might play the same role as the lactone ring of podophyllotoxin. Among the eight different stereoisomers of stegane (**11**), only one compound, the enantiomer of isopicrostegane (**15**) shown on Figure 1, is nearly superimposable to (–)-podophyllotoxin (**2**) and particularly so as

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regards the lactone ring. This might account for the strong activity observed for racemic isopicrostegane (15). The enantiomer of isopicrostegane (15) shown in Figure 1 also bears some similarities with an enantiomer of stegane (11) (which is active in the racemic form). The fact that the lactone ring of this enantiomer of stegane occupies a position different from that of (–)-podophyllotoxin (2) might account for the comparatively low activity of stegane (11). The configuration of isopicrostegane (15) in Figure 1 is opposite to that of natural steganacin (4). Indeed, the latter is described as having an absolute configuration opposite to those of colchicine (1) and podophyllotoxin (2) on the basis of X-ray diffraction studies (without heavy atom). In our view, this is contradictory to the fact that steganacin (4) has a strong antitubulin activity.

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Synthesis and Antitumor Activity of Analogues of the Antitumor Antibiotic Chartreusin[1,2]

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First isolated in 1953 from a fermentation broth, chartreusin (1) has received renewed interest as a result of substantial antitumor activities recently demonstrated in several murine test systems. Poor water solubility frustrated formulation attempts, and rapid biliary excretion observed in mice made 1 an improbable candidate for clinical development but an excellent candidate for an analogue synthesis program. From a common intermediate, which was prepared from 1, three analogues were synthesized wherein the disaccharide moiety of 1 was systematically replaced with fucose (6), glucose (7), and the disaccharide maltose (8). Each of the three analogues had a cytotoxic potency against cultured L1210 cells which was equal to, or better than, that shown by 1. Based on the structural similarity with the parent, an improved water solubility, and a favorable accessibility through synthesis, maltoside 8 was chosen for further antitumor evaluation with an in vivo test system. Versus murine P388 leukemia, 8 showed reproducible activity comparable to chartreusin at similar dose levels. Although 8 caused no observable toxic effects at therapeutic dose levels when given ip, neither 1 nor 8 produced active indications when administered subcutaneously.

In 1953 the isolation of chartreusin (1) from the culture broth and mycelial cake of *Streptomyces chartreusis* was reported.[3] The assigned generic name was appropriate for the greenish-yellow crystalline antibiotic which exhibited a limited spectrum of antibacterial properties.[4] In a series of communications[5] from the University of Zurich the structure elucidation of chartreusin was set forth. However, the synthesis of 1 has not appeared in the literature.[6] Although no antineoplastic properties were originally found for the fermentation product, a recent reevaluation[7] of 1 using current antitumor test systems revealed significant activity in the murine L1210 and P388 leukemias, as well as in the B16 melanoma system. The latter can be considered a solid tumor model. In spite of the good activity shown in these three signal tumor systems, it is unlikely that chartreusin will receive further preclinical development because of two adverse factors. Although substantial activity was observed when tumor cells were inoculated intraperitoneally (ip) and when drug was also given ip, a rapid biliary excretion of 1 after intravenous (iv) administration resulted in plasma and tissue concentrations of drug below the necessary therapeutic level.[7] Oral (po) as well as subcutaneous (sc) administration of 1 failed to produce a positive antitumor response. In addition to the dependence of activity on the administration route, a second difficulty was encountered when a clinical formulation of the poorly soluble antibiotic (15 μg/mL H_2O) was attempted. Although formulation techniques resulted in a 300-fold enhancement of aqueous solubility, an acceptable clinical formulation was not achieved.[8]

Although chartreusin itself is an improbable candidate for clinical trials, its novel structure in combination with impressive activities in three experimental mouse tumor systems suggested the synthesis of chartreusin analogues. With the intention of using 1 as a synthetic starting material, a program was initiated to synthesize analogues of 1 which might possibly have (i) more favorable pharmacodynamic properties and (ii) improved water solubilities. Chartreusin, as indicated in structure 1, has a planar pentacyclic ring system which is linked by a glycosidic bond to a disaccharide moiety (2-*O*-α-D-digitaloxy-β-D-fucosyl). For initial structure modifications of 1, the approach selected required holding constant the aglycon of the glycoside (9, chartarin) while varying the carbohydrate

(1) Presented before the Division of Medicinal Chemistry at the 178th National Meeting of the American Chemical Society, Washington, D.C., Sept. 1979, Abstr MEDI 48.
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