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Two novel series of allocolchicinoids with modified seven membered B-rings: design, synthesis, inhibition of tubulin assembly and cytotoxicity

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Abstract—Two new attractive series of allocolchicinoids were designed as inhibitors of tubulin assembly using the potent ketone **4** and the tetracyclic, pyrazole annulated NCME variant **7** (NCME = *N*-acetyl colchinol-*O*-methylether (**2**)) as lead structures. The first group of inhibitors of type **6** with novel oxepine and azepine B-ring structures belongs to the NCME-series and was synthesized via a multistep total synthesis starting from simple and cheap 3-methoxybenzaldehyde (**12**) and 3,4,5-trimethoxybenzaldehyde (**13**). Biaryl-coupling of the starting materials **12** and **13** was accomplished via Ziegler–Ullmann-reaction to furnish the biphenyl **11** equipped with two carbaldehyde functions. The subsequent Cannizzaro reaction of this dicarbaldehyde **11** proceeded with high regioselectivity to yield almost exclusively the key compound, the hydroxymethyl carboxylic acid **9**. Ring closure to the *o*,*o*′-bridged biphenyls was accomplished by two routes: on the one hand, treatment of **9** with aqueous hydrochloric acid yielded the lactone **15**. On the other hand, a four step sequence starting from the isomeric mixture **9/10** furnished the constitutionally isomeric lactams **23** and **24**; these could be converted to the corresponding thiolactams **25** and **26** and to the tetrazole annulated NCME-type derivatives **27** and **28**. The second series of bioactive compounds are congeners of allocolchicine (**3**). The well known desacetyl allocolchicine (**29**) was easily oxidized to the oxime **30**, which was further transformed to the corresponding ketone **31**. This served as key precursor for the syntheses of various tetracyclic allocolchicine modifications **33**–**36** annulated with a pyrazole, isoxazole, pyrimidine or 2-aminopyrimidine heterocycle, respectively. Unexpectedly, all the NCME-variants with a substituent in position 7 like in NCME (**2**) inhibited the tubulin assembly only moderately. In contrast, the new series of allocolchicine modifications proved to be highly potent antimicrotubule agents. Inhibition of tubulin assembly occurred at lower concentrations compared to those measured for the reference colchicine (**1**). Surprisingly, these promising results could not be confirmed in the cytotoxicity tests against the human MCF-7 breast cancer cell line, where an unexpected loss of effectiveness compared to the corresponding NCME-derivatives was observed.

1. Introduction

Drugs interfering with the tubulin-microtubule equilibrium are important and successfully employed anti-cancer medicaments. Although a variety of natural products were isolated and hundreds of compounds were synthesized, which bind to the colchicine site on tubulin and therefore inhibit tubulin assembly,[1,2] all efforts have failed up to now to establish one of them in standard cancer therapy.[3] It is anticipated that a very recently published X-ray structure of a tubuline-colchicine:RB3-SLD complex will be useful in the development of novel colchicinoid analogues.[4] However, some drug candidates of this family are undergoing clinical trials and showed selective damage of the tumour endothelium at low doses (less than one-tenth

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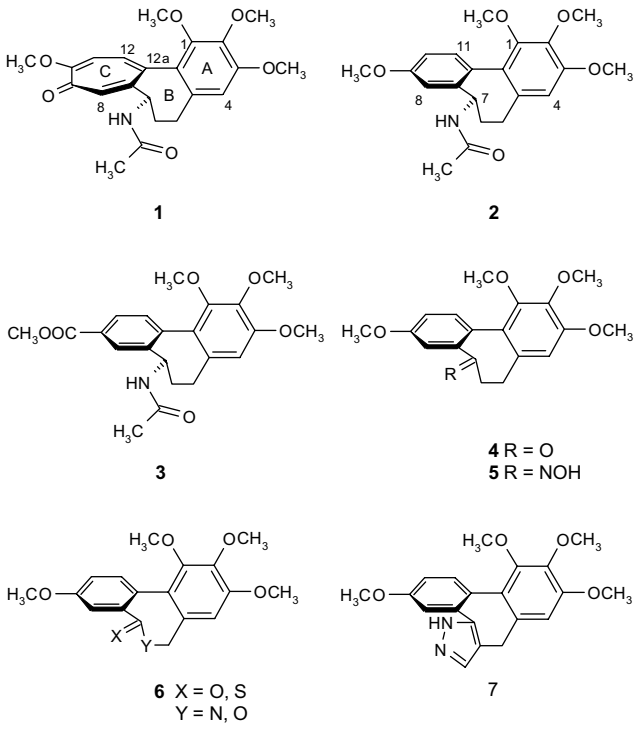
of the tolerated dose) and for that reason were categorized into the class of vascular disrupting agents.[3,5]

In continuation of our efforts[6] directed towards the development of hitherto unknown B-ring modified allocolchicinoids, we have focused our attention on the syntheses of novel variants of the highly potent *N*-acetyl colchinol-*O*-methylether (**2**, NCME) and allocolchicine (**3**). These two antitubulin agents are members of the allo series of the parent alkaloid (−)-(*aR*,7*S*)-colchicine (**1**) (see Ref. 7 for correct nomenclature) with a benzenoid rather than a tropolone C-ring (Scheme 1).

It is well accepted that the primary pharmacophore of the (allo)colchicinoids consists of the properly substituted A- and C-rings and only minor chemical modifications are possible in this part of the molecules without loosing activity.[2] In contrast to this, the B-ring does not seem to take part productively in the binding process to tubulin and its major function is only an entropic contribution by suppressing free rotation around the pivot bond between the A- and C-rings.[8] But B-ring modifications affecting the torsional angle between the least squares planes of the A- and C-rings cannot be freely made, because studies in structure–activity relationships resulting in a 'window' within high activity are possible. It spans from an angle of about 30° for some derivatives with a cyclohexene B-ring[9] and goes to 62° for thiocolchicone with a central cycloheptene moiety.[10]

As it was shown that ring expansion of (allo)colchicinoids to derivatives with an eight membered B-ring leads to less potent inhibitors,[6,11,12] we focused our interest on variants of allocolchicinoids with a modified seven membered B-ring. One attractive series of target compounds represent NCME-derivatives like **6** with substituted oxepine and azepine B-rings. They are similar to the highly potent ketone **4** and congeners of the less potent allocolchicinoids with a central azocine ring. On the other hand, we were interested in extending the development of hitherto unknown allocolchicinoid modifications comprised with heterocyclic ring systems annulated to the B-ring. These entities with a novel tetracyclic scaffold—as realized, for example, in the pyrazole fused NCME variant **7**, developed in our laboratories[6]—proved to be highly potent and are worth investigating more thoroughly. Therefore, we have changed the NCME-type methoxy substituent on the C-ring to a methyloxycarbonyl group to obtain derivatives of allocolchicine (**3**). To gain further information about structure–activity relationships (SAR), we additionally modified the ring size of the annulated heterocycles, their hetero atoms and their substitution pattern as well as the annulation side. On the synthetic route to these new antimicrotubule agents, novel allocolchicine congeners of the potent ketone **4** and oxime **5** could additionally be obtained. Syntheses and spectroscopic data of the novel B-ring modified allocolchicinoids are described together with an evaluation of their abilities to inhibit the assembly of tubulin and the growth of the human MCF-7 breast cancer cell line.

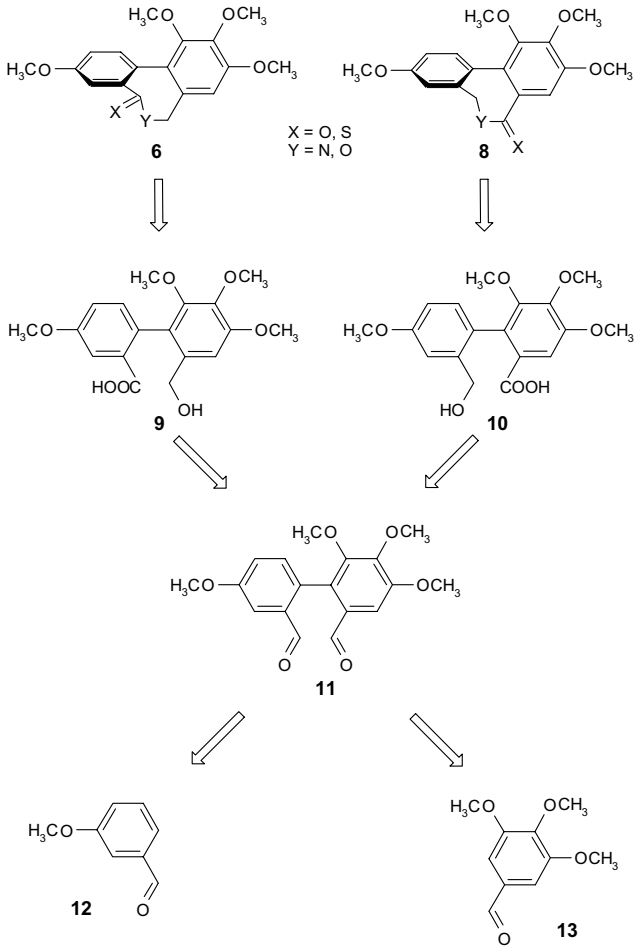


Scheme 1. (−)-(*aR*,7*S*)-colchicine (**1**), (−)-(*aR*,7*S*)-*N*-acetyl colchinol-*O*-methylether, NCME (**2**), allocolchicine (**3**), NCME–ketone **4** and –oxime **5**, target structures **6** and lead **7**.

2. Chemistry

To gain the novel NCME-variants of type **6** with substituted oxepine and azepine B-ring structures, it was necessary to develop a total synthesis. In Scheme 2, our retrosynthetic analysis is outlined.

First of all we decided to perform the biaryl coupling, that is, the creation of the pivot bond joining the latter A- and C-ring as the first step in order to create a variety of target compounds differing only in the B-ring and at the same time keeping the biaryl unit constant. The unsymmetrically substituted biaryl **11** provides the methoxy groups similarly positioned like in the lead NCME (**2**). Secondly, we have chosen from the great variety of biaryl coupling methods available[13] a modified Ullmann reaction, which can be carried out at room temperature[14] (also referred to as Ziegler–Ullmann reaction[15]). This reaction protocol includes the defined formation of unsymmetrically substituted biaryls in moderate to good yields, requires only cheap, less toxic reagents and tolerates the methoxy-substituents. The two carbaldehyde functions in the resulting biaryl **11** supply this key precursor with a high reactivity necessary to perform further reactions easily towards the target structures. Subsequent Cannizzaro reaction of **11** to the isomeric hydroxymethyl carboxylic acids **9** and **10** and further standard chemical procedures should result in the B-ring closure via formation of lactam and lactone bonds. This synthetic route yields two sets of constitutional isomers **6** and **8** at the same time. Besides the attractive derivatives **6** with substituents in position 7 (numbering like in NCME), the constitutional isomers



Scheme 2. Retrosynthetic analysis of heterocyclic B-ring NCME derivatives **6** and **8**.

8—expected to possess only low biological activity according to a study made by Brossi and co-workers[16]—could also be obtained.

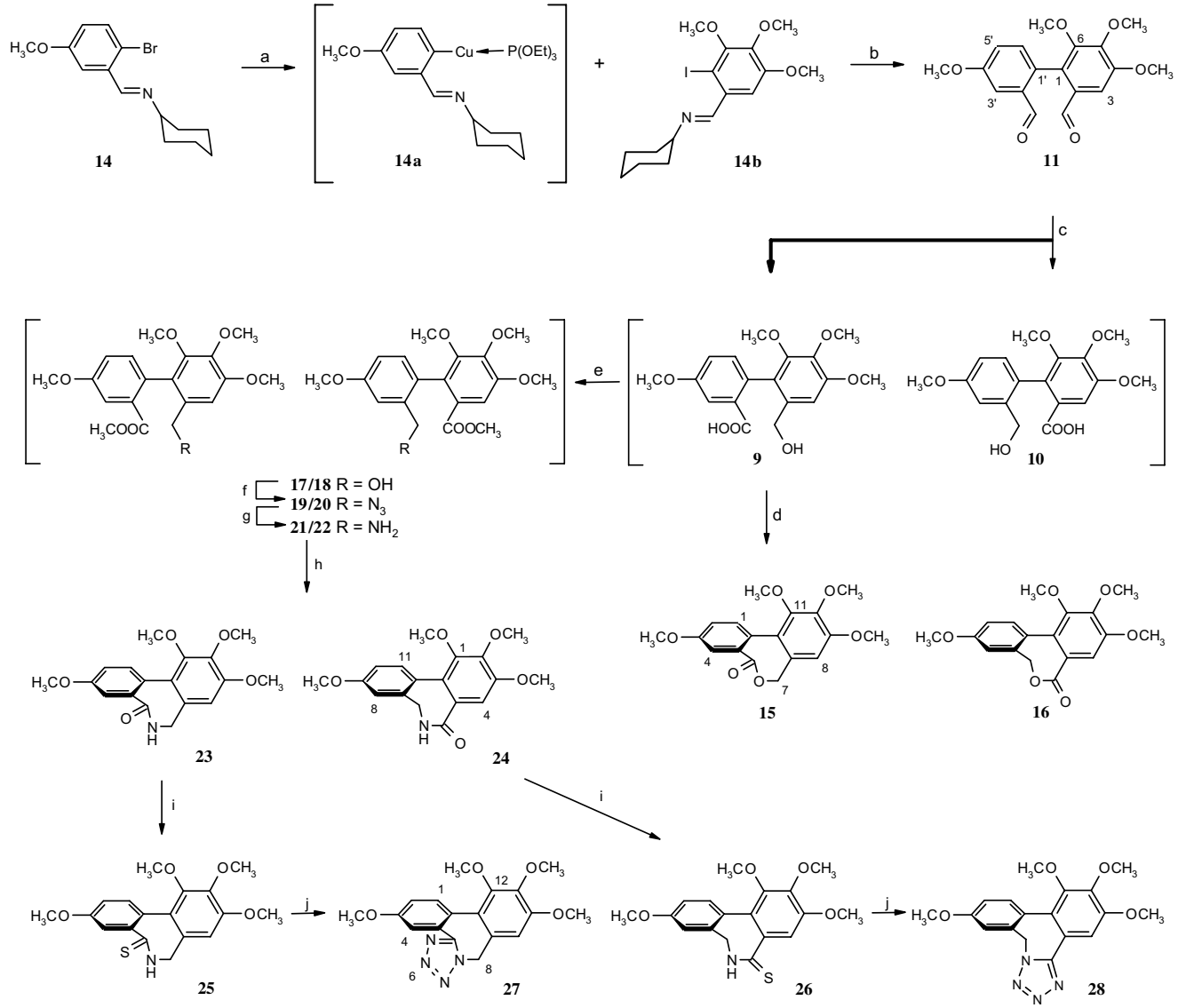
The synthetic route to the lactone **15** started with the Ziegler–Ullmann reaction of 2-bromo-5-methoxybenzaldehyde cyclohexylimine (**14**) and 2-iodo-3,4,5-trimethoxybenzaldehyde cyclohexylimine (**14b**), both easily available in two steps from 3-methoxybenzaldehyde (**12**)[17,18] and 3,4,5-trimethoxybenzaldehyde (**13**), respectively.[18,19] Lithiation of **14** with *n*-BuLi, transmetallation with $CuI{\cdot}P(OEt)_3$ to **14a** and subsequent treatment with the iodo-derivative **14b** resulted in the formation of the crude biaryl as di-cyclohexylimine derivative. This intermediate was not isolated and directly hydrolyzed in a one pot procedure to give the desired bibenzaldehyde **11** after flash chromatography in 66% yield (Scheme 3).

Subsequent Cannizzaro reaction of biaryl **11** in boiling aqueous sodium hydroxide solution furnished the hydroxymethyl carboxylic acids as a mixture of constitutional isomers **9** and **10**. Although being key intermediates in the further synthesis of the NCME-analogues **15** and **23**–**28**, these two constitutional isomers were not separated. On the one hand, complete separation seemed to be very laborious and the constitutional characterization of the isomers could be done at a later stage in the synthetic pathway. On the other hand, these biaryl species were not the target compounds because all similar non *o*,*o*′-bridged biaryl derivatives lacking the B-ring were found to be less active.[20]

The simplest method to obtain a ring closure starting from **9**/**10** obviously was a creation of a lactone. After treatment of the hydroxymethyl carboxylic acids **9** and **10** with aqueous hydrochloric acid at reflux temperature only one product, lactone **15**, could be isolated in excellent yield of 97% after flash chromatography, the constitution of which could be unambiguously determined by ^{1}H NMR spectroscopy using NOE-studies. After saturation of the signal for the aromatic proton at C-8, an increase in the intensities of the signals of the methylene protons at C-7 was observed. On the other hand, saturation of the proton signal at C-4 did not result in any enhancements of the signals of the methylene protons.

The strongly favoured formation of only one lactone can be rationalized with the regioselective course of the Cannizzaro reaction. The mechanism of this disproportionation involves an initial nucleophilic attack of the hydroxyl anion to one of the aldehyde functions. Consequently, two intermediate species are possible—which are not important in our examination—both leading via a hydride shift to the reduction of the second aldehyde function.[21] That means for our internal Cannizzaro reaction: The electron density at the aldehyde function attached to C-2 of biaryl **11** is much higher—it is a phenylogous formic acid methyl ester—than that attached to C-2′. That is why the initial attack of the nucleophilic hydroxyl anion to the aldehyde function at C-2′ is strongly favoured with the result of regioselective formation of the hydroxymethyl carboxylic acid **9** and lactone **15**. The isomeric lactone **16** could not be isolated, though there is evidence that the hydroxymethyl carboxylic acid **10** is generated (see below).

The formation of the corresponding NCME derivatives **23** and **24** with azepine B-ring structures is also outlined in Scheme 3. This synthetic pathway starting from biaryl precursor **11** up to the lactams **23** and **24** is based on a procedure published by Tichý et al.[22] This protocol used for the synthesis of axially chiral lactams was modified and improved according to our needs. Treatment of the mixture containing the hydroxymethyl carboxylic acids **9** and **10** twice with an excess of ethereal diazomethane solution gave the methyl esters **17** and **18** in 80% yield from **11**. These benzylic alcohol derivatives were converted to the corresponding azides directly by treatment with diphenyl phosphorazidate/DBU[23] furnishing the biaryls **19** and **20** in a moderate yield of 41%. Again, as in the case of the hydroxymethyl carboxylic acids **9**/**10** no attempt was undertaken to isolate the constitutionally isomeric intermediates. Conversion of the azides **19** and **20** to the corresponding primary amines was accomplished by treatment with triphenylphosphane and subsequent hydrolysis of the intermediate iminophosphoranes.[24] The resulting amines **21** and **22** could not be isolated because of spontaneous but incomplete



Scheme 3. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C, 15 min, CuI·P(Et)$_3$ −78 °C, 30 min; (b) rt, 15 h, then CH_2Cl_2, 15% CH_3COOH, rt, 24 h, 66%; (c) 5% aq NaOH, reflux, 4 h; (d) 0.1 N aq HCl, reflux 4 h, 97%; (e) 2 × ethereal CH_2N_2, 2 h, 80% from **11**; (f) $(PhO)_2P(O)N_3$, DBU, toluene, 0 °C 2 h, rt, 14 h, 41%; (g) PPh_3, THF, 6 h, rt, then H_2O, 14 h, rt; (h) $NaOCH_3$, CH_3OH, reflux 45 min, 18% for **23**, 1% for **24** (from **11**); (i) Lawesson's reagent, toluene, reflux 3.5 h, 92% for **25**, 80% for **26**; (j) $(CH_3)_3SiN_3$, $FeCl_3$, CH_2Cl_2, 24–40 h rt, 38% for **27**, 64% for **28**.

transformation to the lactams **23** and **24**. Completion of ring closure to the target NCME-type lactams was achieved by heating the mixture in methanol in the presence of sodium for a short time. Two products in different amounts could be obtained and separated by crystallization. Slow cooling of a hot solution of the lactams **23** and **24** in methanol exclusively gave isomer **24** as colourless crystals, whereas **23** stayed in solution. Differentiation of the two isomers was possible by two dimensional ^{1}H NMR TOCSY experiments with lactam **24**. In this ^{1}H NMR-spectrum, a coupling of the aromatic proton at C-8 with the protons of the methylene group at C-7 proved the constitution of the structure. A final confirmation of the structures by investigating lactam **23** by ^{1}H NMR experiments was not possible because of signal overlaps but was successfully undertaken at a later stage of the synthetic pathway. The 18:1 product ratio of **23**–**24** again was the outcome of the regioselective Cannizzaro reaction of dicarbaldehyde **11**.

A transformation towards hitherto unknown allocolchicinoids with thiolactam B-ring structures was now possible. Treatment of the lactams **23** and **24** with Lawesson's reagent[25] furnished the NCME variants **25** and **26** with thiolactam structures in 92% and 80% yields.

As NCME-derivatives with a tetrazole heterocyclus annulated to an azocine B-ring were found to be moderately active inhibitors of tubulin assembly,[6] it seemed to be attractive to synthesize analogous compounds comprising a seven membered B-ring. This opportunity was now given with the thiolactams **25** and **26** as starting materials using a methodology developed by Lehnhoff and Ugi.[26] Both compounds **25** and **26** were

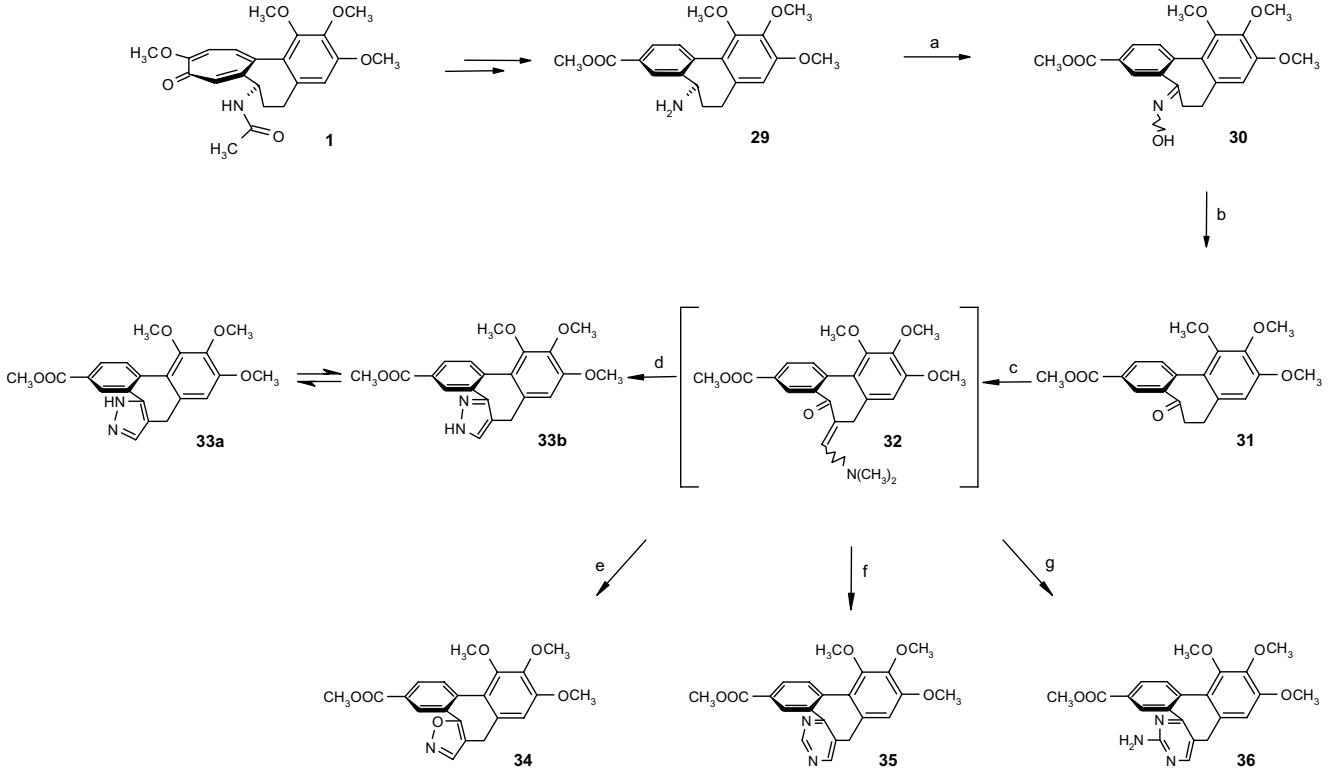
transformed with trimethylsilylazide/$FeCl_3$ to the tetrazole annulated NCME-derivatives **27** and **28** in moderate yields of 38% and 64%. To validate the assumed constitutions of the two isomeric species **27** and **28**, we examined the tetrazole annulated NCME-variant **27** by a ^{1}H NMR TOCSY experiment. The observed coupling between the aromatic proton signal at C-9 with the signals of the methylene protons at C-8 clearly confirmed the structure of tetrazole **27** and thereby also the constitutions of **25** and **23**.

For the syntheses of novel and promising allocolchicines and variants with various annulated heterocycles, the known desacetyl allocolchicine (**29**) was used as entry in the second part of our studies (Scheme 4). Desacetyl allocolchicine (**29**), equipped with the requisite primary amino function, is easily accessible from colchicine (**1**) in two steps.[27] Oxidation with hydrogen peroxide in the presence of Na_2WO_4 as catalyst[28] led to the formation of an insoluble amine:oxime adduct in a 1:1 ratio. After separation by flash chromatography, the desacetyl allocolchicine oxime (**30**) was obtained in 41% yield. In the ^{1}H NMR spectrum of **30**, a double set of signals in the ratio of 12:1 was observed. This was probably due to the presence of *E* and *Z* stereoisomers of the oxime. The analogous NCME-type oxime **5** (with a methoxy instead of a methyloxycarbonyl function at the C-ring) was found to exist as single *E*-isomer exhibiting only one set of signals in the ^{1}H NMR spectrum.[6]

For transformation of the oxime **30** to the corresponding ketone **31**, we utilized Maloney's protocol including pyridinium chlorochromate as oxidizing agent in non-aqueous medium.[29] The expected product was isolated in 50% yield. 7-Deacetamido-7-oxoallocolchicine (**31**) was synthesized recently in a different way by Vorogushin et al.[30] as intermediate in the total synthesis of allocolchicine (**3**). All spectroscopic data reported for **31** are in good agreement with our findings. However, no biological data for **31** have been published so far.

Once having ketone **31** at hand in appropriate amounts, it served as key precursor for the synthesis of allocolchicine variants annulated with various heterocycles as outlined in Scheme 4. Treatment of **31** with Bredereck's reagent (bis(dimethylamino)-*tert*-butoxymethane)[31] provided the enamino ketone **32** as the key intermediate, which due to instability was not isolated.

The reaction of **32** with hydrazine dihydrochloride[32] furnished the pyrazole annulated allocolchicines **33a/b** as a mixture of tautomers in 57% yield. As expected, all the signals of the protons of **33a/b** could be detected (except the N*H* signal) in the ^{1}H NMR spectrum in DMF-d_7. Surprisingly, in the ^{13}C NMR spectrum the carbon signals for the pyrazole moiety were not visible. This is due to transfer processes between the tautomers **a** and **b**, which cause significant line broadening of the quaternary signals. For that reason, a ^{13}C NMR spectrum of **33a/b** was run in DCOOD. The formic acid deuterates



Scheme 4. Reagents and conditions: (a) Na_2WO_4, H_2O_2, CH_3OH, H_2O, 15 °C → rt, 7 h, 41%; (b) pyridinium chlorochromate, CH_2Cl_2, 18 h, rt, 50%; (c) Bredereck's reagent, DMF, 65 °C, 16 h; (d) H_2N–NH_2·2HCl, CH_3OH, rt, 3 h, 57%; (e) NH_2OH·HCl, Na_2CO_3, CH_3COOH, CH_3OH, 100 °C, 2 h, 22%; (f) $NaOCH_3$, H_2NCHNH·CH_3COOH, CH_3OH, reflux, 19 h, 40%; (g) $NaOCH_3$, $H_2NC(NH)NH_2$·HCl, CH_3OH, reflux, 5.5 h, 67%.

one nitrogen of the pyrazole system and thus suppresses transfer processes so that all carbon signals were clearly visible.

Synthesis of the isoxazole annulated allocolchicinoid **34** was carried out by reaction of the enamino ketone **32** with hydroxylamine hydrochloride under acidic conditions[33] furnishing the product in only 22% yield. Two sets of signals in the NMR-spectra of **34** at room temperature in the ratio of 5:4 testify a somewhat higher energetic barrier for the interconversion of the corresponding atropoisomers.

Additionally, two attractive allocolchicine derivatives with an annulated six membered ring system were synthesized. On the one hand, the reaction of the enamino ketone **32** with formamidine acetate in the presence of sodium methanolate in methanol[34] furnished the pyrimidine annulated allocolchicinoid **35** in 40% yield. On the other hand, conversion of the enamino ketone **32** to the 2-amino-pyrimidine annulated allocolchicine variant **36** was carried out by refluxing precursor **32** in methanol in the presence of guanidine hydrochloride and sodium methoxide. This previously established method[35] provides the target compound in 67% yield.

3. Biological results and discussion

3.1. In vitro inhibition of tubulin assembly

All of the newly synthesized allocolchicinoids **15**, **23**–**28**, **30**, **31**, **33**–**36** and the biaryl dicarbaldehyde **11** were subjected to our standard assay conditions[6,36,37] for evaluation of tubulin assembly inhibition in vitro using calf brain tubulin. In order to allow a precise comparison between the novel ligands and both parent leads, NCME (**2**) and allocolchicine (**3**) were included in the evaluation. The IC_{50} values of all the allocolchicinoids under consideration were compared to that of colchicine (**1**) as the standard, measured within the same day with the same tubulin preparation. The data are compiled in Table 1, presented in terms of the relative IC_{50}/IC_{50Col} value determined from the IC_{50} values of the test compounds.

As can be seen from the data in Table 1a, the parent allocolchicinoids NCME (**2**) and allocolchicine (**3**) possess a 4–5 times higher inhibitory effect than the standard colchicine (**1**).

The biphenyl dicarbaldehyde **11** (Table 1b) lacking the B-ring proved to be inactive. Contrary to other tetramethoxybiphenyls, which were reported to have at least low activity,[20] this biaryl did not affect microtubule assembly.

One structure–activity relationship (SAR) of the new compounds (Table 1b) at first glance turns out that—judging from these experiments—all derivatives with a substituent at C-5 (numbering like in NCME (**2**)) are inactive, as in the case of **24**, **26** and **28**. This result is in full accordance with previous findings by Brossi and co-workers.[16]

Table 1. Inhibition of tubulin assembly

Compound	IC_{50}/IC_{50Col}
(a) Reference compounds	
Colchicine (**1**)	1
NCME (**2**)	0.25
Allocolchicine (**3**)	0.2
DPPT (**40**)	0.2
(b) Newly synthesized compounds	
11	Inactive
15	Inactive
23	3
24	Inactive
25	6
26	Inactive
27	1.7
28	Inactive
30	0.1
31	0.2
33a/b	0.4
34	1.1
35	1
36	0.5
(c) SAR studies: influence of the B-ring	
4	0.2
15	Inactive
23	3
37	Inactive
27	1.7
38	1.2
d) SAR studies: C-ring substituent	
30	0.1
5	0.17
31	0.2
4	0.2
33a/b	0.4
7	0.25
36	0.5
39	0.3

IC_{50} of Colchicine varies from 2 to 8 μM for the different experiments according to the tubulin preparation and concentration. Inactive: max 50% inhibition at 200 μM.

Surprisingly, the NCME-type lactone **15** was not active at all, although having the carbonyl group in the right position like the N-acetyl moiety in NCME (**2**). It is difficult to understand this result at the moment, because both parent NCME variants—the ketone **4** with a cycloheptene B-ring[6] and the related derivative with a nonsubstituted oxepine B-ring (results not published)—were found to be potent inhibitors of tubulin assembly, as well as the corresponding lactam **23** (Table 1c). An opening of the lactone B-ring during the assay leading to the hydroxymethyl carboxylic acid **9**—which is probably less active—seems very unlikely.

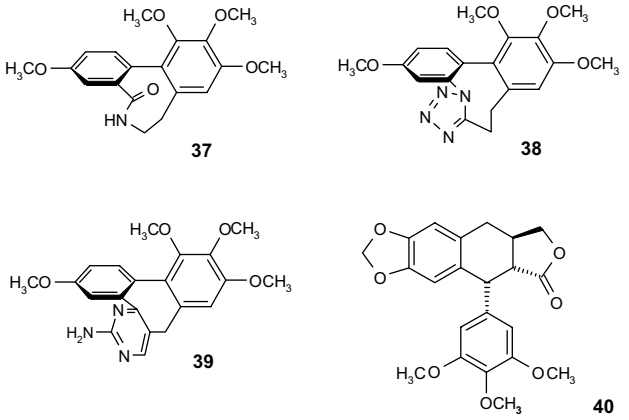
In the first part of our SAR studies, we focused our attention on the influence of the B-ring size to affect microtubule assembly. The two NCME-derivatives with a C-5 substituted azepine B-ring, as in the case of **23** and **25**, displayed good to moderate inhibitory effects towards tubulin assembly but do not surpass the activity of neither the lead NCME (**2**) nor the reference colchicine (**1**). However, comparison with the analogous NCME-derivative equipped with an eight membered

azocine B-ring gives another point of view (Table 1c). Switching from the eight membered azocine B-ring of **37**[6] (Scheme 5) to the B-ring contracted seven membered azepine **23** drastically increased the activity. This positive contribution to the inhibition of tubulin assembly is surely mainly due to the decrease of the torsional angle between the planes of the A- and C-rings. In this connection, it is worth mentioning that Berg et al. reported a thiocolchicinoid with an azocine B-ring structure. This was characterized by a torsional angle of 76° between the least squares planes of the A–C biaryl unit exhibiting an IC_{50Col} value of only ca. 10.[11]

Since bioisosterism serves as a valuable aid in SAR studies,[38] we replaced the lactam function of allocolchicinoid **23** by the bioisosteric thiolactam structure to yield the hitherto unknown NCME type modification **25**. Obviously, the thioxo instead of an oxo function at C-5 of **25** influences the binding of the ligands to tubulin in a negative way, thus accounting for the two times lower inhibitory effect (Table 1b).

Another SAR study allowed a comparison between the newly synthesized tetrazole annulated NCME-type allocolchicinoid **27** with an azepine B-ring and the recently described corresponding tetrazole annulated congener **38** with an azocine-B-ring[6] (Table 1c, Scheme 5). Both inhibitors possess the same potency, equal to that of colchicine (**1**). The torsional angle between the least squares planes of the A–C biaryl unit in **38** was reported to be 58°, which is within the range of all potent colchicinoids known so far.[6] The further reduction of the torsional angle as realized in **27** obviously does not have any consequences on the biologic activity.

In the second part of our SAR studies, we were interested in the influence of a methoxycarbonyl function as the C-ring substituent instead of the methoxy group as in NCME (**2**) and congeners. The newly synthesized allocolchicine derivatives, oxime **30** and ketone **31**, proved to be highly potent inhibitors of tubulin assembly with IC_{50} values in the range of that of allocolchicine (**3**) (Table 1b). In a direct comparison of two independent experiments, oxime **30** was shown to slightly surpass even the activity of deoxypodophyllotoxine DPPT (**40**), one of the most potent inhibitors known.



Scheme 5. NCME variants used for defining SAR **37**–**39**, deoxypodophyllotoxine (**40**).

The novel B-ring annulated tetracyclic allocolchicine variants **33**–**36** were found to be potent inhibitors of tubulin assembly. Their IC_{50} values are comparable to or smaller than that for colchicine (**1**), but they do not reach the inhibitory effect of the parent allocolchicine (**3**). Ring size and substitution pattern of the annulated heterocycles do not seem to have a remarkable impact on activity.

Table 1d allows a comparison of the relative IC_{50}/IC_{50Col} values of the new allocolchicine derivatives **30**, **31**, **33a/b** and **36** with the recently published data of the corresponding NCME-variants **4**, **5**, **7** and **39** differing only in the C-ring substituent.[6] The data reveal only very slight, not significant differences switching from a methoxy to a methoxycarbonyl function at C-3 of the allocolchicinoids under consideration. Thus, both substituents are equally well suitable for the design of potent antimicrotubule agents in the allocolchicinoid series.

It is worth mentioning that none of the newly synthesized active allocolchicinoids variants possesses a stereogenic centre at C-7—as in the case of the leads **1**–**3**. They therefore exist as a racemic mixture of two atropoisomers due to the hindered rotation around the pivot bond connecting the A- and C-rings. However, it is well known that only the *aR* enantiomer can bind to tubulin.[39] One should keep this in mind when comparing the test results with the derivatives with a chiral centre at C-7 as in colchicine (**1**) and NCME (**2**), which exclusively exist in the active *aR* conformation.[39]

3.2. In vitro inhibition of cell growth

The testing of (−)-(*aR*,7*S*)-colchicine (**1**) and selected allocolchicinoids was performed on the human MCF-7 breast cancer cell line. For the evaluation of the sensitivity of this cell line against the lead **1** and the newly synthesized allocolchicinoids, a computerized, kinetic chemosensitivity assay was used based on quantification of biomass by staining cells with crystal violet (see Experimental). The relationship between growth kinetics of the inhibitor-treated cell line and the plot of corrected *T*/*C* values versus time of inhibitor cell contact (icc) was recorded as described in Ref. 40. In order to yield detailed insights into the mode of action, for example, the inhibition profile reflecting cytostatic, transient cytotoxic or cytocidal inhibitor effects as well as development of resistance, the overall effects are presented as plots of corrected *T*/*C* values versus icc (Fig. 1). The test details are described in the Experimental.

In Figure 1 the cytotoxic effects of the novel, most promising allocolchicinoids **23**, **27**, **30**, **31** and **33**–**36** are compared with that of the parent natural alkaloid (−)-(*aR*,7*S*)-colchicine (**1**).

The reference compound **1** inhibited cell growth even at the lowest concentration of 0.01 μM leading to a constant hindrance of cell proliferation at a 30% level

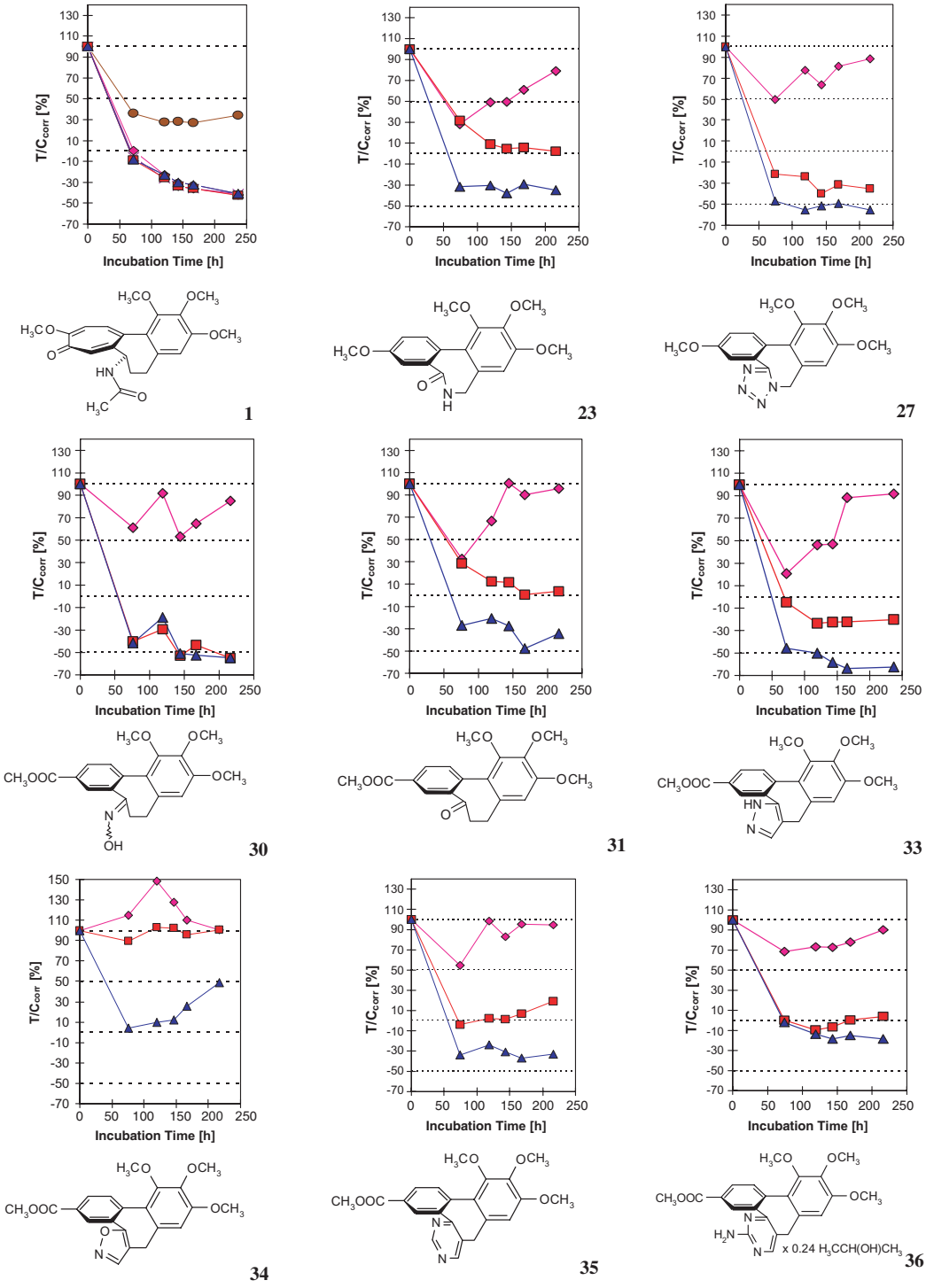


Figure 1. Effect of (−)-(*aR*,7*S*)-colchicine (**1**) and new allocolchicinoids on the MCF-7 breast cancer cell line at concentrations: ● 0.01 μM, X 0.05 μM, ♦ 0.1 μM, ■ 0.5 μM, ▲ 1 μM.

compared to nontreated control. Increase of the test concentration to 0.05 μM totally inhibits the growth of the MCF-7 cell line after 70 h. None of the new allocolchicinoids revealed comparable growth inhibition. Both of the new NCME-variants, lactam **23** and tetrazole **27**, caused only transient cell growth inhibition at a concentration of 0.1 μM. Whereas lactam **23** exhibited cytostatic effects at 0.5 μM ($T/C = 0\%$) after 70 h, the tetrazole **27** at this concentration already revealed cytocidal activity ($T/C < 0\%$). The C-5 modified allocolchicine derivatives **30** and **31** influenced the cell growth in a similar way. Both compounds were found to have only temporary inhibitory effect at 0.1 μM. In contrast, oxime **30** reached the cytocidal level already at a concentration of 0.5 μM after 70 h, whereas ketone **31** showed complete cytostase at the same concentration after 160 h. Three of the four heterocyclic annulated allocolchicine derivatives, **33**, **35** and **36**, inhibited the

MCF-7 cell growth at similar concentrations. All of these inhibitors needed concentrations of 0.5 μM to perform cytostatic (**35**, **36**) or cytocidal (**33a/b**) effects, at the lower concentration of 0.1 μM only transient influence on the cell growth was observed. Isoxazole **34** proved to be the weakest inhibitor of cell growth in the set of compounds under consideration, obvious from the poor effects at the 0.5 μM concentration ($T/C = 90\%$ after 75 h icc). Even at the highest concentration employed (1 μM), this substance had only transient cytostatic effects.

All new allocolchicinoids, which inhibited the tubulin assembly stronger than colchicine (**1**), that is, which had an $IC_{50Col} < 1$, proved to be less active in the cytotoxicity assay against the MCF-7 breast cancer cell line compared to the natural alkaloid. Furthermore, a comparison of NCME-type allocolchicinoids equipped with a methoxy group at the C-ring (data in Ref. 6) with the corresponding allocolchicine variants with a methoxycarbonyl function at this position reveals a general negative influence of the methyl ester function towards the inhibition of cell growth of the MCF-7 cancer cell line. The NCME-variants **5**, **7** and **39** required a concentration as low as 0.05 μM to completely inhibit the cell growth.[6] In contrast, for the allocolchicine variants **30**, **33** and **36** a concentration that was 10 times higher was necessary to reach the same effect.

4. Conclusion

We have presented herein the total syntheses of four novel NCME-type allocolchicinoids **23**–**26** comprising C-5 and C-7 substituted (numbering like in NCME (**2**)) oxepine and azepine B-ring structures, as well as that of two tetrazole annulated variants **27** and **28** belonging to the same series. The second class of new tubulin ligands represent the allocolchicine derivatives **33**–**36** equipped with different B-ring annulated heterocycles. These were obtained from colchicine (**1**) as starting material in multistep syntheses with desacetyl allocolchicine (**29**), its corresponding oxime **30** and ketone **31** as key intermediates. Investigation of the biologic activities of the new tubulin inhibitors revealed—in comparison with related allocolchicinoids, previously published by our group[6]—two major results: firstly, allocolchicinoids with heterocycle fused seven membered B-rings proved to be highly potent inhibitors of tubulin assembly, independently of ring size and hetero atoms employed in the annulated heterocycles. Secondly, a significant decrease of biologic activity against the MCF-7 cell line was observed by switching from allocolchicinoids of the NCME- to those of the allocolchicine-series, in spite of nearly identical relative IC_{50}/IC_{50Col} values of corresponding tubulin ligands in both series.

5. Experimental

5.1. General procedures

Standard vacuum techniques were used in the handling of air sensitive materials. Melting points are uncorrected: 'Leitz-Heiztischmikroskop' HM-Lux. Solvents were dried and freshly distilled before use according to the literature procedures. IR: FT-IR spectrometer 510-P (Nicolet). Liquids were run as films, solids as KBr pellets. ^1H NMR and ^{13}C NMR: Jeol JNM-GX 400 and LA 500; δ/ppm = 0 for tetramethylsilane, 7.24 for chloroform. MS: Vacuum Generators 7070 (70 eV; ^{11}B). Column chromatography: Purifications were carried out on Merck silica gel 40 (40–60 mesh), flash chromatography. Reactions were monitored by thin-layer chromatography (TLC) by using plates of silica gel (0.063–0.200 mm, Merck) or silicagel-60F_{254} microcards (Riedel de Haen). Optical rotations: Mod. Dip-370 polarimeter (Jasco). UV: Spekol UV VIS (Jena Analytik AG).

5.1.1. 4,5,6,4′-Tetramethoxy-biphenyl-2,2′-dicarbaldehyde (11). To a solution of 503 mg (1.7 mmol) of 2-bromo-5-methoxybenzaldehyde-cyclohexylimine (**14**) in 40 mL of dry THF at −78 °C was added 1.2 mL (1.9 mmol) of *n*-butyllithium (15% in *n*-hexane) forming a yellow solution, which was stirred for 15 min. CuI·$P(OEt)_3$ (910 mg, 2.56 mmol) was added in one portion and the solution was stirred for 30 min. The resulting deep red solution was treated with 685 mg (1.7 mmol) of 2-iodo-3,4,5-trimethoxy-benzaldehyde-cyclohexylimine (**14b**) and the reaction mixture was allowed to warm slowly to room temperature and stirred for an additional 15 h. The green-yellow mixture was diluted with 40 mL of dichloromethane and 30 mL of aqueous acetic acid (15%) and stirred vigorously for 24 h. After separation of the layers the organic phase was washed with 10% HCl (3 × 50 mL), saturated Na_2CO_3 solution (8 × 30 mL) and finally with brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography (silica gel, ethyl acetate/*n*-hexane 1:3) and the third fraction contained the crude biphenyl (**11**) (373 mg, 1.13 mmol), which could be crystallized from *n*-hexane. Colourless crystals, yield 66%, mp 74–75 °C. UV (MeOH): λ_{max} ($\log\varepsilon$) 204 nm (4.68), 229 (4.64). IR (KBr): ν (cm^{-1}) 2941 (CH), 1679 (CO), 1586 (C=C), 1103, 1080 (COC). ^1H NMR (500 MHz, CD_2Cl_2): δ 3.50 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3); 3.94 (s, 3H, OCH_3); 3.95 (s, 3H, OCH_3); 7.21 (m, 2H, 3′-H* and 6′-H*); 7.37 (s, 1H, 3-H); 7.53 (m, 1H, 5′-H*); 9.59 (s, 1H, C*H*O); 9.73 (s, 1H, C*H*O). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 55.6 (O*C*H_3); 56.1 (O*C*H_3); 60.7 (O*C*H_3); 61.1 (O*C*H_3); 105.8 (CH, C-3*); 110.9 (CH, C-5′*); 120.5 (CH, C-6′*); 128.4 (C-1*); 129.5 (C-2*); 130.3 (C-2′*); 133.9 (CH, C-3′*); 136.0 (C-1′*); 147.2 (C-5); 151.1 (C-6*); 153.7 (C-4*); 159.8 (C-4′*); 190.1 (*C*HO); 191.0 (*C*HO). *Assignments not confirmed. MS (70 eV): *m*/*z* (%) 330 (M^+, 34), 315 (6), 301 (100), 286 (11), 271 (14). HRMS calcd for $C_{18}H_{18}O_6$ (330.34): 330.1103, found: 330.1107. $C_{18}H_{18}O_6$ calcd: C 65.45, H 5.49, found: C 65.65, H 5.36.

5.1.2. 3,9,10,11-Tetramethoxy-7*H*-dibenzo[*c,e*]oxepine-5-one (15). A solution of 500 mg (1.51 mmol) of dicarbaldehyde **11** in 25 mL of 5% NaOH was refluxed for 4 h. After cooling to room temperature the pH of the

solution was adjusted with diluted aqueous HCl to 1–2. The resulting precipitate was extracted with $CHCl_3$ (4×20 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo. This mixture of isomers **9** and **10** (268 mg, 0.77 mmol) was refluxed in 20 mL of 0.1 N HCl for 4 h. The aqueous layer was extracted with CH_2Cl_2 (4×10 mL) and the combined organic layers were dried (Na_2SO_4) and the solvent was evaporated in vacuo. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH 9.8:0.2) and yielded 247 mg (0.75 mmol) of the crude lactone **15**, which was crystallized from cyclohexane. Colourless crystals, ratio of conformers: 15:1, yield 97%, mp 123 °C. UV (MeOH): λ_{max} ($\log\varepsilon$) 206 nm (4.58), 272 (4.28), 313 (3.72). IR (KBr): ν (cm^{-1}) 2944 (CH), 1719 (CO), 1609 (C=C), 1104, 1081 (COC). ^{1}H NMR (500 MHz, $CDCl_3$): major conformer: δ 3.59 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 3.89 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3); 4.81 (d, 1H, 7-H, ^2J = 12.2 Hz); 4.90 (d, 1H, 7-H, ^2J = 12.2 Hz); 6.75 (s, 1H, 8-H); 7.11 (dd, 1H, 2-H, ^3J = 8.8 Hz, ^4J = 2.9 Hz); 7.36 (d, 1H, 4-H, ^4J = 2.9 Hz); 7.73 (d, 1H, 1-H, ^3J = 8.7 Hz), minor conformer: δ 3.84 (s, 3H, OCH_3); 3.92 (s, 3H, OCH_3); 3.96 (s, 3H, OCH_3); 5.00 (d, 1H, 7-H, ^2J = 12.1 Hz); 6.95 (d, 1H, 4-H, ^4J = 2.6 Hz); 6.96 (dd, 1H, 2-H, ^3J = 8.5 Hz, ^4J = 2.7 Hz); 7.20 (s, 1H, 8-H). ^{13}C NMR (125.8 MHz, $CDCl_3$) major conformer: δ 55.5 (OCH$_3$); 56.1 (OCH$_3$); 61.1 (OCH$_3$); 61.1 (OCH$_3$); 69.7 (CH_2, C-7); 107.7 (CH, C-8); 114.2 (CH, C-2); 118.7 (CH, C-4); 124.5 (C-11a*); 125.9 (C-11b*); 131.0 (C-7a*); 131.9 (C-4a*); 131.9 (CH, C-1); 143.7 (C-10); 151.9 (C-11*); 152.9 (C-9*); 158.7 (C-3); 170.3 (C-5), minor conformer: δ 55.4 (OCH$_3$); 60.9 (OCH$_3$); 69.4 (CH_2, C-7); 109.9 (CH, C-8); 113.4 (CH, C-2); 114.7 (CH, C-4); 126.5 (C-11b*); 132.4 (CH, C-1); 143.7 (C-7a*); 146.0 (C-10); 150.6 (C-11*); 152.7 (C-9*); 159.1 (C-3); 170.0 (C-5). *Assignments not confirmed. The signals of the minor isomer could not be fully identified. MS (70 eV): *m/z* (%) 330 (100, M^+), 302 (3), 285 (4), 271 (14), 256 (5). HRMS calcd for $C_{18}H_{18}O_6$ (330.34): 330.1103, found: 330.1089. $C_{18}H_{18}O_6$ calcd: C 65.45, H 5.49, found: C 65.35, H 5.48.

5.2. Synthesis of the lactams 23 and 24

A solution of 4.99 g (15.1 mmol) of dicarbaldehyde **11** in 100 mL of 5% aqueous NaOH was refluxed for 4 h. After cooling to room temperature the pH of the solution was adjusted to 1–2 with diluted aqueous HCl. The precipitate was extracted with $CHCl_3$ (4×30 mL). The combined organic layers were dried (Na_2SO_4) and the volume of the solution was reduced to ca. 30 mL. This solution was treated twice with an excess of ethereal CH_2N_2 (generated from 3.88 g (18 mmol) Diazald) and the reaction mixture was stirred for 2 h at rt. (CAUTION! Diazomethane is toxic and potentially explosive. Special precautions have to be taken when working with this reagent. See Ref. 41 for more information.) After evaporation of the solvent in vacuo the purification of the residue by column chromatography (silica gel, CH_2Cl_2/MeOH 9.9:0.1) furnished 4.34 g of a mixture of the isomeric methyl esters **17** and **18** as light yellow solid.

This mixture (3.96 g, 10.2 mmol) was dissolved in 20 mL of dry toluol and 2.6 mL (12.2 mmol) of diphenyl phosphorazidate was added. The mixture was cooled to 0 °C and 1.8 mL of DBU was added. The reaction mixture was stirred for 2 h at 0 °C and then for 14 h at room temperature. After addition of 20 mL of CH_2Cl_2, the mixture was washed one time with water (30 mL) and one time with 5% aqueous HCl (30 mL). The organic layer was concentrated in vacuo and purified by column chromatography (silica gel, ethyl acetate/*n*-hexane 1:4) to afford 1.63 g (4.2 mmol) of a mixture of the isomeric azides **19** and **20** as light yellow oil.

To a solution of 1.76 g (4.5 mmol) of this mixture in dry THF was added 1.31 g (5.0 mmol) of triphenylphosphane in portions and the reaction mixture was stirred at room temperature for 6 h. After addition of seven drops of water the mixture was stirred for 14 h at room temperature. The solvent was evaporated in vacuo and the residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH 9.8:0.2 → 9.0:1.0). The isolated light yellow solid was dissolved in 30 mL of dry MeOH, 50 mg of NaOMe was added and the mixture was refluxed for 45 min. After addition of 20 mL of water the aqueous layer was extracted with CH_2Cl_2 (3×15 mL). The combined organic layer was dried (Na_2SO_4) and concentrated in vacuo. Crystallization from MeOH afforded 50 mg (0.15 mmol) of lactam **24** as crystals. The isomeric lactam **23** (880 mg, 2.67 mmol) stayed in solution and was separated by filtration and recrystallized from ethyl acetate/*n*-hexane.

5.2.1. 3,9,10,11-Tetramethoxy-6,7-dihydro-dibenzo-[*c,e*]azepine-5-one (23). Colourless crystals, yield 18% (from **11**), mp 142 °C. UV (CF_3CH_2OH): λ_{max} ($\log\varepsilon$) 210 nm (4.47), 268 (4.17), 309 (3.58). IR (KBr): ν (cm^{-1}) 3186 (NH), 2934 (CH), 1672 (CO), 1607 (C=C), 1100, 1060 (COC). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.51 (s, 3H, OCH_3); 3.82 (m, 1H, 7-H); 3.86 (s, 3H, OCH_3); 3.88 (s, 3H, OCH_3); 3.88 (s, 3H, OCH_3); 4.05 (dd, 1H, 7-H, ^2J = 14.6 Hz, ^3J = 6.0 Hz); 6.60 (s, 1H, 8-H); 7.05 (dd, 1H, 2-H, ^3J = 8.8 Hz, ^4J = 2.9 Hz); 7.42 (d, 1H, 4-H, ^4J = 2.9 Hz); 7.67 (d, 1H, 1-H, ^3J = 8.8 Hz); 7.85 (br s, 1H, NH). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 45.5 (CH_2, C-7); 55.4 (OCH$_3$); 56.1 (OCH$_3$); 61.0 (OCH$_3$); 61.2 (OCH$_3$); 106.1 (CH, C-8); 112.6 (CH, C-2); 117.6 (CH, C-4); 124.2 (C-11a); 126.2 (C-11b); 132.3 (CH, C-1); 134.9 (C-7a*); 135.8 (C-4a*); 142.5 (C-10); 152.4 (C-11*); 152.6 (C-9*); 158.7 (C-3); 171.2 (C-5). *Assignments not confirmed. MS (70 eV): *m/z* (%) 329 (100, M^+), 314 (4), 300 (10), 286 (4), 271 (11). HRMS calcd for $C_{18}H_{19}N_1O_5$ (329.35): 329.1263, found: 329.1265. $C_{18}H_{19}N_1O_5$. calcd: C 65.64, H 5.81, N 4.25, found: C 65.72, H 5.69, N 4.58.

5.2.2. 1,2,3,9-Tetramethoxy-6,7-dihydro-dibenzo[*c,e*]azepine-5-one (24). Colourless crystals, yield 1% (from **11**), mp 231 °C. UV (CF_3CH_2OH): λ_{max} ($\log\varepsilon$) 214 nm (4.46), 247 nm (4.47), 308 nm (3.66). IR (KBr): ν (cm^{-1}) 3178 (NH), 2926 (CH), 1653 (CO), 1609, 1594 (C=C), 1098, 1072 (COC). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.55 (s, 3H, OCH_3); 3.81–3.86 (m, 4H, OCH_3, 7-H); 3.93 (s, 3H, OCH_3); 3.95 (s, 3H, OCH_3);

4.15 (dd, 1H, 7-H, ^2J = 14.4 Hz, ^3J = 6.1 Hz); 6.79 (d, 1H, 8-H, ^4J = 2.7 Hz); 6.88 (dd, 1H, 10-H, ^3J = 8.8 Hz, ^4J = 2.7 Hz); 7.12 (t, 1H, N*H*, ^3J = 6.2 Hz); 7.26 (s, 1H, 4-H); 7.63 (d, 1H, 11-H, ^3J = 8.6 Hz). ^{13}C NMR (100.5 MHz, DMSO-d_6): δ 44.6 (CH_2, C-7); 55.8 (O*C*H$_3$); 56.4 (O*C*H$_3$); 61.1 (O*C*H$_3$); 61.2 (O*C*H$_3$); 108.7 (CH, C-4*); 112.5 (C-10*); 113.1 (CH, C-8*); 124.4 (C-11a*); 126.1 (C-11b*); 131.3 (C-7a*); 132.9 (CH, C-11); 142.3 (C-4a*); 144.5 (C-2*); 150.8 (C-1*); 152.6 (C-3*); 159.0 (C-9); 169.1 (C-5). *Assignments not confirmed. MS (70 eV): *m/z* (%) 329 (100, M^+), 314 (4), 300 (6), 285 (9), 270 (13). HRMS calcd for $C_{18}H_{19}N_1O_5$ (329.36): 329.1263, found: 329.1279.

5.2.3. 3,9,10,11-Tetramethoxy-6,7-dihydro-dibenzo[*c,e*]-zepine-5-thione (25). Lactam **23** (550 mg, 1.67 mmol) and 675 mg (1.67 mmol) of Lawesson's reagent were dissolved in 9 mL of dry toluene and the mixture was refluxed for 3.5 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH 9.9:0.1) to afford 530 mg (1.54 mmol) of the thiolactam **25**, which could be crystallized with ethyl acetate/*n*-hexane. Light yellow crystals, yield 92%, mp 140 °C. UV (CF_3CH_2OH): λ_{max} (log ε) 204 nm (4.55), 220 (4.52), 249 (4.37), 274 (4.25), 333 (3.91). IR (KBr): ν (cm^{-1}) 3146 (NH), 2937 (CH), 1599 (C=C), 1535 (C=S). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.53 (s, 3H, OC*H*$_3$); 3.86 (s, 3H, OC*H*$_3$); 3.88 (s, 3H, OC*H*$_3$); 3.89 (s, 3H, OC*H*$_3$); 3.95 (m, 1H, 7-H); 4.07 (m, 1H, 7-H); 6.57 (s, 1H, 8-H); 7.02 (dd, 1H, 2-H; ^3J = 8.8 Hz, ^4J = 2.9 Hz); 7.60 (d, 1H, 1-H, ^3J = 8.8 Hz); 7.71 (d, 1H, 4-H, ^4J = 2.7 Hz); 9.49 (t, 1H, N*H*, ^3J = 5.9 Hz). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 49.3 (CH_2, C-7); 55.5 (O*C*H$_3$); 56.2 (O*C*H$_3$); 61.6 (O*C*H$_3$); 61.2 (O*C*H$_3$); 106.0 (CH, C-8); 114.8 (CH, C-2); 117.5 (CH, C-4); 124.0 (C-11a*); 124.3 (C-11b); 132.0 (CH, C-1); 134.3 (C-7a*); 139.6 (C-4a*); 142.8 (C-10*); 152.5 (C-11*); 152.9 (C-9*); 158.3 (C-3); 199.1 (C-5). *Assignments not confirmed. MS (70 eV): *m/z* (%) 345 (100, M^+), 312 (42), 297 (10), 281 (77). HRMS calcd for $C_{18}H_{19}N_1O_4S_1$ (345.41): 345.1035, found: 345.1051.

5.2.4. 1,2,3,9-Tetramethoxy-6,7-dihydro-dibenzo[*c,e*]azepine-5-thione (26). Lactam **24** (322 mg, 0.98 mmol) and 395 mg (0.98 mmol) of Lawesson's reagent were dissolved in 7 mL of dry toluene and the mixture was refluxed for 3.5 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH 9.9:0.1) to afford 270 mg (0.78 mmol) of the thiolactam **26**, which could be crystallized with CH_2Cl_2/*n*-hexane. Light yellow crystals, yield 80%, mp 191 °C. UV (CF_3CH_2OH): λ_{max} (log ε) 257 nm (4.48), 329 (3.90). IR (KBr): ν (cm^{-1}) 3236 (NH), 2939 (CH), 1608 (C=C), 1522 (C=S). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.52 (s, 3H, OC*H*$_3$); 3.81 (s, 3H, OC*H*$_3$); 3.94 (s, 3H, OC*H*$_3$); 3.95 (s, 3H, OC*H*$_3$); 3.96 (m, 1H, 7-H), 4.15 (dd, 1H, 7-H, ^2J = 13.8 Hz, ^3J = 6.8 Hz); 6.76 (d, 1H, 8-H, ^4J = 2.7 Hz); 6.89 (dd, 1H, 10-H, ^3J = 8.7 Hz, ^4J = 2.7 Hz); 7.61 (s, 1H, 4-H); 7.63 (d, 1H, 11-H, ^3J = 8.7 Hz); 9.13 (br s, 1H, N*H*). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 49.2 (CH_2, C-7); 55.3 (O*C*H$_3$); 56.1 (O*C*H$_3$); 60.7 (O*C*H$_3$); 61.0 (O*C*H$_3$); 110.8 (CH, C-4*); 111.7 (CH, C-8*); 113.1 (CH, C-10*); 123.1 (C-11a*); 125.4 (C-11b*); 133.1 (CH, C-11); 134.3 (C-7a*); 139.1 (C-4a*); 144.9 (C-2); 150.1 (C-1*); 152.0 (C-3*); 159.2 (C-9); 198.9 (C-5). *Assignments not confirmed. MS (70 eV): *m/z* (%) 345 (100, M^+), 330 (6), 312 (6). HRMS calcd for $C_{18}H_{19}N_1O_4S_1$ (345.41): 345.1035, found: 345.1046.

5.2.5. 3,10,11,12-Tetramethoxy-8*H*-5,6,7,7a-tetraaza-dibenzo[*e,g*]azulen (27). To a solution of 270 mg (0.78 mmol) of thiolactam **25** in 15 mL of dry dichloromethane was added 0.2 mL (1.56 mmol) of trimethylsilylazide and the reaction mixture was stirred at rt for 5 min. $FeCl_3$ (253 mg, 0.94 mmol) was added and the mixture was stirred under argon at rt for 24 h. After washing once with saturated $NaHCO_3$ and drying with Na_2SO_4 the solvent was evaporated in vacuo. Flash chromatography of the residue (silica gel, ethyl acetate/*n*-hexane 1:3) gave 106 mg (0.3 mmol) of tetrazole (**27**), which was recrystallized from dichloromethane/*n*-hexane to give light yellow crystals. Yield: 38%. Mp 159 °C. UV (CF_3CH_2OH): λ_{max} (log ε) = 218 nm (4.58), 270 nm (4.30), 309 nm (3.77). IR (KBr): ν (cm^{-1}) = 2948 (CH), 1610 (C=C), 1106, 1084 (CO). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.42 (s, 3H, OC*H*$_3$); 3.87 (s, 3H, OC*H*$_3$); 3.90 (s, 3H, OC*H*$_3$); 3.91 (s, 3H, OC*H*$_3$); 4.91 (d, 1H, 8-H, ^2J = 13.7 Hz); 5.56 (d, 1H, 8-H, ^2J = 14.3 Hz); 6.77 (s, 1H, 9-H); 7.14 (dd, 1H, 2-H, ^3J = 8.9 Hz, ^4J = 2.8 Hz); 7.53 (d, 1H, 4-H, ^4J = 2.7 Hz); 7.82 (d, 1H, 1-H, ^3J = 9.0 Hz). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 50.9 (CH_2, C-8); 55.7 (O*C*H$_3$); 56.1 (O*C*H$_3$); 60.9 (O*C*H$_3$); 61.1 (O*C*H$_3$); 107.7 (CH, C-9); 112.7 (CH, C-2); 117.8 (CH, C-4); 123.6 (C-12a*); 124.0 (C-12b*); 125.8 (C-8a*); 130.5 (C-4a*); 134.2 (CH, C-1); 143.5 (C-11); 152.7 (C-12*); 153.0 (C-4b*); 153.3 (C-10*); 159.1 (C-3). *Assignments not confirmed. MS (70 eV): *m/z* (%) 354 (100, M^+), 325 (26), 311 (24), 296 (6), 295 (6). HRMS calcd for $C_{18}H_{18}N_4O_4$ (354.36 g/mol): 354.1328; found 354.1345.

5.2.6. 1,2,3,10-Tetramethoxy-8*H*-5,6,7,7a-tetraaza-dibenzo[*e,g*]azulen (28). To a solution of 134 mg (0.39 mmol) of thiolactam **26** in 10 mL of dry dichloromethane was added 0.1 mL (0.78 mmol) of trimethylsilylazide and the reaction mixture was stirred at rt for 5 min. $FeCl_3$ (126 mg, 0.47 mmol) was added and the mixture was stirred under argon at rt for 40 h. After washing once with saturated $NaHCO_3$ and drying with Na_2SO_4 the solvent was evaporated in vacuo. Flash chromatography of the residue (silica gel, ethyl acetate/*n*-hexane 1:2) gave 89 mg (0.25 mmol) of tetrazole **28**, which was recrystallized from dichloromethane/*n*-hexane to give light yellow crystals. Yield: 64%, mp 196 °C. UV (CF_3CH_2OH): λ_{max} (log ε) = 217 nm (4.34), 249 nm (4.49), 309 nm (3.65). IR (KBr): ν (cm^{-1}) 2945 (CH), 1612, 1601 (C=C), 1086, 1077 (COC). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.64 (s, 3H, OC*H*$_3$); 3.83 (s, 3H, OC*H*$_3$); 3.96 (s, 3H, OC*H*$_3$); 4.00 (s, 3H, OC*H*$_3$); 4.99 (d, 1H, 8-H, ^2J = 14.1 Hz); 5.62 (d, 1H, 8-H, ^2J = 14.1 Hz); 6.91 (dd, 1H, 11-H, ^3J = 8.8 Hz, ^4J = 2.7 Hz); 6.97 (d, 1H, 9-H, ^4J = 2.7 Hz); 7.38 (s, 1H, 4-H); 7.61 (d, 1H, 12-H, ^3J = 8.6 Hz). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 50.8 (CH_2, C-8); 55.4 (O*C*H$_3$);

56.3 (O*C*H$_3$); 61.2 (O*C*H$_3$); 61.3 (O*C*H$_3$); 108.2 (CH, C-4*); 113.3 (CH, C-11*); 114.3 (CH, C-9*); 118.5 (C-8a*); 125.1 (C-12a*); 125.4 (C-12b*); 134.3 (CH, C-12); 135.2 (C-4a*); 145.2 (C-2); 152.2 (C-4b*); 152.9 (C-1*); 153.3 (C-3*); 159.6 (C-10). *Assignments not confirmed. MS (70 eV): *m/z* (%) 354 (100, M$^+$), 311 (39), 268 (33), 253 (14). HRMS calcd for $C_{18}H_{18}N_4O_4$ (354.36 g/mol): 354.1328; found: 354.1333.

5.2.7. 5-Hydroxyimino-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a,c*]cyclohepten-3-carboxylic acid methyl ester (30). To a solution of 724 mg (2.03 mmol) of desacetyl allocolchicine (**29**) in 21 mL of methanol was added a solution of 70 mg (0.21 mmol) of Na_2WO_4 in 28 mL of water. The reaction mixture was cooled to 15–17 °C and 1.5 mL of hydrogen peroxide (30% solution) was added. After stirring for 1 h an additional 0.75 mL of hydrogen peroxide (30% solution) was added and the mixture was allowed to warm to rt. After ca. 6 h the formed precipitate was extracted with chloroform (6 × 10 mL). The collected organic phase was dried with Na_2SO_4, evaporated under reduced pressure and the residue was purified by flash chromatography (silica gel, dichloromethane/methanol 9.8:0.2). Fraction 1 furnished 309 mg (0.83 mmol) of oxime **30**, fraction 2 contained 355 mg (49%) of starting material **29**. The product was recrystallized from dichloromethane/*n*-hexane. Isomeric ratio: 12:1. Light yellow crystals, yield: 41%; mp 160 °C. UV ($CHCl_3$): λ_{max} (log ε) 244 nm (4.21), 305 nm (4.15). IR (KBr): ν (cm^{-1}) 3367 (OH), 2944 (CH), 1715 (CO), 1597 (CN), 1135, 1093 (COC). ^{1}H NMR (500 MHz, $CDCl_3$): major isomer: δ 2.58 (m, 1H, 7-H); 2.67 (m, 1H, 7-H); 2.82 (m, 1H, 6-H); 3.27 (m, 1H, 6-H); 3.49 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 3.88 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3); 6.56 (s, 1H, 8-H); 7.61 (d, 1H, 1-H, ^3J = 8 Hz); 8.06 (dd, 1H, 2-H, ^3J = 8 Hz, ^4J = 1.9 Hz); 8.09 (d, 1H, 4-H, ^4J = 1.9 Hz); minor isomer: δ 3.45 (s, 3H, OCH_3); 3.89 (s, 3H, OCH_3); 7.65 (d, 1H, 1-H, ^3J = 8.2 Hz); 8.01 (d, 1H, 4-H, ^4J = 1.4 Hz). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ major isomer: δ 29.9 (CH_2, C-7); 34.7 (CH_2, C-6); 52.1 (O*C*H$_3$); 56.0 (O*C*H$_3$); 61.0 (O*C*H$_3$); 61.1 (O*C*H$_3$); 107.2 (CH, C-8); 123.7 (C-11a*); 128.8 (C-3*); 129.5 (CH, C-2*); 130.1 (CH, C-4*); 131.4 (CH, C-1*); 135.0 (C-7a*); 136.4 (C-4a*); 140.0 (C-11b*); 141.3 (C-10); 151.8 (C-11*); 153.4 (C-9*); 160.7 (C-5); 166.6 (CO). Minor isomer: δ 30.1 (CH_2, C-7); 39.4 (CH_2, C-6); 61.2 (O*C*H$_3$); 61.3 (O*C*H$_3$); 108.0 (CH, C-8); 128.6 (CH, C-4); 129.3 (CH, C-2*); 130.9 (CH, C-1*); 157.4 (C-5*). The signals of the minor isomer could not be completely determined. *Assignments not confirmed. MS (70 eV): *m/z* (%) 371 (100, M$^+$), 356 (15), 340 (18), 324 (6), 310 (8). HRMS calcd for $C_{20}H_{21}N_1O_6$ (371.39 g/mol) calcd: 371.1369; found: 371.1365.

5.2.8. 9,10,11-Trimethoxy-5-oxo-6,7-dihydro-5*H*-dibenzo[*a,c*]cyclohepten-3-carboxylic acid methyl ester (31). To a suspension of 269 mg (1.25 mmol) of pyridinium chlorochromate in 5 mL of dichloromethane was added a solution of 233 mg (0.62 mmol) of desacetyl allocolchicine oxime (**30**) in 5 mL of dichloromethane. The mixture was stirred at rt for 18 h. After addition of 30 mL of diethyl ether the resulting precipitate was filtered off. The filtrate was evaporated in vacuo and the residue purified by flash chromatography (silica gel, dichloromethane/methanol 9.9:0.1). The crude product (110 mg, 0.31 mmol) was recrystallized from ethyl acetate/*n*-hexane. Light yellow crystals, yield: 50%; mp: 116 °C. UV ($CHCl_3$): λ_{max} (log ε) 258 nm (4.16), 290 nm (4.15). IR (KBr): ν (cm^{-1}) 2960 (CH), 1720 (CO), 1687 (CO), 1594 (C=C), 1137, 1099 (COC). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.65 (m, 1H, 7-H); 2.85–3.05 (m, 3H, 7-H, 2 × 6-H); 3.50 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 3.89 (s, 3H, OCH_3); 3.92 (s, 3H, OCH_3); 6.59 (s, 1H, 8-H); 7.63 (d, 1H, 1-H, ^3J = 8.2 Hz); 8.13 (dd, 1H, 2-H, ^3J = 8 Hz, ^4J = 1.9 Hz); 8.19 (d, 1H, 4-H, ^4J = 1.9 Hz). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 30.0 (CH_2, C-7); 47.7 (CH_2, C-6); 52.2 (O*C*H$_3$); 56.0 (O*C*H$_3$); 61.1 (2 × O*C*H$_3$); 107.1 (CH, C-8); 123.4 (C-11a); 129.0 (CH, C-2*); 129.1 (C-11b*); 131.2 (CH, C-4*); 131.6 (CH, C-1*); 135.8 (C-7a*); 138.5 (C-3*); 139.6 (C-4a*); 141.6 (C-10); 152.3 (C-11); 153.7 (C-9); 166.2 (*C*OOCH$_3$); 205.9 (C-5). *Assignments not confirmed. MS (70 eV): *m/z* (%) 356 (100, M$^+$), 341 (6), 328 (6), 325 (3). HRMS calcd for $C_{20}H_{20}O_6$ (356.37 g/mol): 356.1260; found: 356.1266. $C_{20}H_{20}O_6$ calcd: C 67.41, H 5.66; found: C 67.47, H 5.54.

5.2.9. Typical procedure for the preparation of enamino ketone (32). A solution of 1 equiv of ketone **31** in dry DMF (0.1 mmol/mL) was warmed under argon to 65 °C. Two equivalents of bis-(dimethylamino)-*tert*-butoxymethane (Bredereck's reagent) was added and the reaction mixture was stirred at 65 °C for 16 h. The solvent was removed in vacuo and the resulting yellow solid was used without further purification and characterization.

5.2.10. 10,11,12-Trimethoxy-5,8-dihydro-dibenzo-[4,5;6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxylic acid methyl ester (33a) and 10,11,12-trimethoxy-6,8-dihydro-dibenzo[4,5;6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxylic acid methyl ester (33b). The enamino ketone **32** (prepared from 167 mg (0.47 mmol) of ketone **31**) was dissolved in 8 mL of methanol and 52 mg (0.5 mmol) of hydrazine-dihydrochloride was added. The reaction mixture was stirred for 3 h at rt and the solvent was evaporated under reduced pressure. Flash chromatography of the residue gave 102 mg (0.27 mmol) of pyrazole (**33a/b**), which could be recrystallized from ethyl acetate. Yellowish crystals, yield: 57%; mp 254 °C. UV (HCOOH): λ_{max} (log ε) 262 nm (4.35), 299 nm (4.10). IR (KBr): ν (cm^{-1}) 3351 (NH), 2954 (CH), 1717 (CO), 1594 (C=C), 1106, 1088 (COC). ^{1}H NMR (500 MHz, DMF-d_7): δ 3.31 (d, 1H, 8-H, ^2J = 14 Hz); 3.40 (s, 3H, OCH_3); 3.74 (s, 3H, OCH_3); 3.76 (d, 1H, 8-H, ^2J = 15 Hz); 3.88 (s, 3H, OCH_3); 3.92 (s, 3H, OCH_3); 6.90 (s, 1H, 9-H); 7.63 (s, 1H, 7-H); 7.79 (d, 1H, 1-H, ^3J = 8.2 Hz); 7.93 (dd, 1H, 2-H, ^3J = 8.2 Hz, ^4J = 1.8 Hz); 8.41 (d, 1H, 4-H, ^4J = 1.6 Hz). ^{13}C NMR (125.8 MHz, DMF-d_7): δ 52.2 (O*C*H$_3$); 56.1 (O*C*H$_3$); 60.7 (O*C*H$_3$); 60.9 (O*C*H$_3$); 107.7 (CH, C-9); 122.5 (C-12a*); 123.8 (C-12b*); 126.3 (CH, C-2*); 128.8 (C-3*); 128.8 (CH, C-4*); 133.7 (CH, C-1*); 138.3 (C-8a*); 140.8 (C-4a*); 141.2 (C-11*); 152.9 (C-12*); 154.0 (C-10*); 166.8 (CO). ^{13}C NMR (125.8 MHz, DCOOD): δ 29.0 (CH_2, C-8); 53.5

(O*C*H$_3$); 56.3 (O*C*H$_3$); 61.5 (O*C*H$_3$); 61.5 (O*C*H$_3$); 108.3 (CH, C-9); 122.6 (C-12a*); 124.8; (C-12b*); 125.1 (C-7a); 129.4 (C-3*); 129.6 (CH, C-2*); 130.5 (CH, C-4*); 131.1 (CH, C-7*); 134.4 (CH, C-1*); 139.4 (C-8a*); 140.4 (C-4a*); 141.0 (C-11*); 143.9 (C-4b); 152.8 (C-12*); 154.2 (C-10*); 168.2 (CO). *Assignments not confirmed. MS (70 eV): *m/z* (%) 380 (100, M$^+$), 365 (6), 349 (11). HRMS: calcd for $C_{21}H_{20}N_2O_5$ (380.40 g/mol): 380.1372; found: 380.1359.

5.2.11. 10,11,12-Trimethoxy-8*H*-5-oxa-6-aza-dibenzo-[*e,g*]azulen-3-carboxylic acid methyl ester (34). The enamino ketone **32** (prepared from 513 mg (1.44 mmol) of ketone **31**) was dissolved in 10 mL of dry methanol. Sodium carbonate (99 mg, 0.93 mmol), 994 mg (14.3 mmol) of hydroxylamine hydrochloride and 15 drops of glacial acetic acid were added. The mixture was heated in a sealed tube at 100 °C for 2 h. After addition of water the aqueous phase was extracted once with chloroform. The organic phase was dried with Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash chromatography (silica gel, dichloromethane/methanol 9.9:0.1) and contained 121 mg (0.32 mmol) of isoxazole **34**. Recrystallization was accomplished with ethyl acetate/*n*-hexane. Light yellow crystals. Ratio of atropoisomers: 5:4. Yield: 22%; mp: 138 °C. UV (CF_3CH_2OH): λ_{max} (log ε) 297 nm (4.22). IR (KBr): ν (cm^{-1}) 2936 (CH), 1718 (CO), 1592 (C=C), 1141, 1092 (COC). ^{1}H NMR (400 MHz, $CDCl_3$): major conformer: δ 3.39 (s, 3H, OC*H*$_3$); 3.40 (d, 1H, 8-H, ^2J = 14.5 Hz); 3.49 (d, 1H, 8-H, ^2J = 14.6 Hz); 3.86 (s, 3H, OC*H*$_3$); 3.89 (s, 3H, OC*H*$_3$); 3.94 (s, 3H, OC*H*$_3$); 6.59 (s, 1H, 9-H); 7.81 (d, 1H, 1-H, ^3J = 8.2 Hz); 8.12 (dd, 1H, 2-H, ^3J = 8.3 Hz, ^4J = 1.9 Hz); 8.25 (s, 1H, 7-H); 8.55 (d, 1H, 4-H, ^4J = 2.0 Hz). Minor conformer: δ 3.36 (s, 3H, OC*H*$_3$); 3.45 (d, 1H, 8-H, ^2J = 14.5 Hz); 3.61 (d, 1H, 8-H, ^2J = 14.3 Hz); 3.86 (s, 3H, OC*H*$_3$); 3.90 (s, 3H, OC*H*$_3$); 3.96 (s, 3H, OC*H*$_3$); 6.60 (s, 1H, 9-H); 7.91 (d, 1H, 1-H, ^3J = 8.4 Hz); 8.07 (dd, 1H, 2-H, ^3J = 8.3 Hz, ^4J = 1.9 Hz); 8.21 (s, 1H, 7-H); 8.51 (d, 1H, 4-H, ^4J = 2.0 Hz). ^{13}C NMR (100.5 MHz, $CDCl_3$): major conformer: δ 28.3 (CH_2, C-8); 52.2 (O*C*H$_3$); 56.0 (O*C*H$_3$); 61.0 (O*C*H$_3$); 61.1 (O*C*H$_3$); 107.0 (CH, C-9); 121.3 (C-7a*); 123.3 (C-12a*); 127.5 (C-12b*); 129.1 (C-3*); 129.2 (CH, C-2); 129.8 (CH, C-4); 133.4 (CH, C-1); 136.4 (C-8a*); 138.9 (C-4a*); 141.6 (C-11); 149.0 (CH, C-7); 152.9 (C-12*); 153.5 (C-10*); 160.7 (C-4b*); 166.4 (CO). Minor conformer: δ 28.9 (CH_2, C-8); 52.3 (O*C*H$_3$); 56.0 (O*C*H$_3$); 60.9 (O*C*H$_3$); 61.1 (O*C*H$_3$); 107.1 (CH, C-9); 118.4 (C-7a*); 123.1 (C-12a*); 126.2 (C-12b*); 127.8 (CH, C-4); 128.2 (CH, C-2); 128.9 (C-3*); 133.5 (CH, C-1); 137.6 (C-8a*); 138.6 (C-4a*); 141.4 (C-11); 152.0 (CH, C-7); 153.0 (C-12*); 153.7 (C-10*); 163.8 (C-4b*); 166.3 (CO). *Assignments not confirmed. MS (70 eV): *m/z* (%) 381 (100, M$^+$), 366 (3), 354 (3), 352 (4), 350 (6). HRMS calcd for $C_{21}H_{19}N_1O_6$ (381.38 g/mol): 381.1212; found: 381.1197.

5.2.12. 11,12,13-Trimethoxy-9*H*-5,7-diazatribenzo-[*a,c,e*]cyclohepten-3-carboxylic acid methyl ester (35). To a freshly prepared solution of sodium methanolate (50 mg sodium in 5 mL of dry methanol) was added 114 mg (1.1 mmol) of formamidine acetate under an argon atmosphere. After stirring for 15 min at rt the enamino ketone **32** (prepared from 356 mg (1.0 mmol) of ketone **31**) dissolved in 10 mL of dry methanol was added. After refluxing the reaction mixture under argon for 19 h, 10 mL water was added. The mixture was extracted with chloroform and the organic phase was dried with Na_2SO_4, excess of solvent was removed under vacuo. Flash chromatography (dichloromethane/methanol 9.8:0.2) yielded 157 mg (0.40 mmol) of pyrimidine **35**, which could be recrystallized from dichloromethane/*n*-hexane. Light yellow crystals, yield: 40%; mp 193 °C. UV (CF_3CH_2OH): λ_{max} (log ε) 273 nm (4.30). IR (KBr): ν (cm^{-1}) 2929 (CH), 1721 (CO), 1593, 1571 (C=C), 1095, 1058 (COC). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.42 (s, 3H, OC*H*$_3$); 3.55 (br s, 2H, 9-H); 3.85 (s, 3H, OC*H*$_3$); 3.90 (s, 3H, OC*H*$_3$); 3.94 (s, 3H, OC*H*$_3$); 6.67 (s, 1H, 10-H); 7.87 (d, 1H, 1-H, ^3J = 8.2 Hz); 8.15 (dd, 1H, 2-H, ^3J = 8.2 Hz, ^4J = 2.1 Hz); 8.61 (s, 1H, 8-H); 8.69 (d, 1H, 4-H, ^4J = 2.1 Hz); 9.17 (s, 1H, 6-H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 36.1 (CH_2, C-9); 52.3 (O*C*H$_3$); 56.1 (O*C*H$_3$); 61.0 (O*C*H$_3$); 61.1 (O*C*H$_3$); 106.0 (CH, C-10); 122.3 (C-13a*); 128.9 (C-13b*); 129.3 (CH, C-2); 131.3 (CH, C-4); 132.2 (CH, C-1); 134.0 (C-9a*); 135.9 (C-3*); 137.4 (C-8a*); 139.5 (C-4a*); 141.7 (C-12); 152.7 (C-13*); 154.0 (C-11*); 154.1 (CH, C-8); 157.5 (CH, C-6); 161.4 (C-4b*); 166.6 (CO). *Assignments not confirmed. MS (70 eV): *m/z* (%) 392 (100, M$^+$), 383 (7), 377 (10), 361 (4). HRMS calcd for $C_{22}H_{20}N_2O_5$ (392.42 g/mol): 392.1372; found: 392.1356.

5.2.13. 6-Amino-11,12,13-trimethoxy-9*H*-5,7-diaza-tribenzo[*a,c,e*]cycylohepten-3-carboxylic acid methyl ester (36). To a freshly prepared solution of sodium methanolate (50 mg sodium in 5 mL of dry methanol) was added 119 mg (1.25 mmol) of guanidinium hydrochloride under an argon atmosphere. After stirring for 15 min at rt the enamino ketone **32** (prepared from 356 mg (1.0 mmol) of ketone **31**) dissolved in 10 mL of dry methanol was added. After refluxing the reaction mixture under argon for 5.5 h, the resulting light yellow precipitate was isolated by filtration. Recrystallization from isopropanol furnished 272 mg (0.67 mmol) of the aminopyrimidine **36** as light yellow crystals containing 0.24 equiv of isopropanol. Yield: 67%; mp: 238 °C. UV (CF_3CH_2OH): λ_{max} (log ε) 224 nm (4.61), 277 nm (4.41). IR (KBr): ν (cm^{-1}) 3325 (NH), 3171 (NH), 2939 (CH), 1726 (CO), 1653 (C=N), 1594 (C=C), 1110, 1089 (COC). ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.16 (d, 1H, 9-H, ^2J = 13.5 Hz); 3.44 (s, 3H, OC*H*$_3$); 3.59 (d, 1H, 9-H, ^2J = 13.5 Hz); 3.72 (s, 3H, OC*H*$_3$); 3.86 (s, 3H, OC*H*$_3$); 3.91 (s, 3H, OC*H*$_3$); 6.68 (s, 2H, N*H*$_2$); 6.94 (s, 1H, 10-H); 7.76 (d, 1H, 1-H, ^3J = 8.2 Hz); 8.05 (dd, 1H, 2-H, ^3J = 8.2 Hz, ^4J = 1.8 Hz); 8.23 (s, 1H, 8-H); 8.49 (d, 1H, 4-H, ^4J = 1.8 Hz). ^{13}C NMR (125.8 MHz, CF_3COOD): δ 34.6 (CH_2, C-9); 54.0 (O*C*H$_3$); 56.3 (O*C*H$_3$); 62.1 (O*C*H$_3$); 62.2 (O*C*H$_3$); 107.9 (CH, C-10); 122.6 (C-13a*); 127.0 (C-13b*); 129.8 (C-8a*); 132.6 (CH, C-2*); 133.5 (C-3*); 133.7 (CH, C-4*); 133.8 (CH, C-1*); 138.3 (C-9a*); 141.4 (C-12*); 142.4 (C-4a*); 145.7 (CH, C-8); 152.7 (C-13*); 155.4 (C-11*); 155.6

(C-4b*); 170.0 (CO*); 171.4 (C-6*). *Assignments not confirmed. MS (70 eV): *m/z* (%) 407 (100, M^+), 392 (7), 376 (5). HRMS calcd for: $C_{22}H_{21}N_3O_5$ (407.43 g/mol): 407.1481; found: 407.1468.

5.2.14. Tubulin binding assay. Calf brain tubulin was purified according to the method of Shelanski et al.,[36] by three cycles of assembly–disassembly and then dissolved in the assembly buffer containing 0.1 M MES, 0.5 mM $MgCl_2$, 2 mM EGTA and 1 mM GTP, pH 6.6 (the concentration of tubulin was about 2–3 mg/mL). Tubulin assembly was monitored and recorded continuously by turbidimetry at 400 nm in a UV spectrophotometer, equipped with a thermostated cell at 37 °C.[37] We determined for all newly synthesized drugs the IC_{50} values of their concentrations, which decreased by 50% the maximum assembly rate of tubulin without drug. The IC_{50} for all compounds were compared to the IC_{50} of colchicine, measured the same day under the same conditions.

5.2.15. In vitro characterization of inhibitor-induced effects with respect to the human MCF-7 breast cancer cell line growth. The human MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD; USA). Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay.[42] The MCF-7 cells were maintained in *L*-glutamine containing Eagle's MEM (Sigma München, Germany) supplemented with $NaHCO_3$ (2.2 g/L) sodium pyruvate (110 mg/L), gentamycin (50 mg/L; Sebio Walchsing, Germany) and 10% fetal calf serum (FCS; Gibco Eggenheim, Germany) using 75 cm^2 culture flasks (Falcon Plastics 3023) in a water-saturated atmosphere (95% air/5% CO_2) at 37 °C. The cell line was weekly passaged after treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (0.02%; EDTA; Boehringer Mannheim, Germany). Mycoplasma contamination was routinely monitored and only mycoplasma-free cultures were used.

In vitro testing of (−)-(*aR*,7*S*)-colchicine (**1**) and the newly synthesized allocolchicinoids for antitumour activity was carried out on exponentially dividing human breast cancer cell according to a previously published microtitre assay.[40,43] Briefly, in 96-well microtitre plates (Costar), 100 μL of a cell suspension in 500 cells/mL culture medium was plated into each well and incubated at 37 °C for 2–3 days in a humidified atmosphere (5% CO_2). By addition of an adequate volume of a stock solution of the respective compound (solvent: DMF) to the medium, the desired test concentration was obtained (max. content of DMF in the medium: 1 ppm). For each test concentration and for the control, which contained the corresponding amount of DMF, 16 wells were used. After the proper incubation time the medium was removed, the cells were fixed with a glutardialdehyde solution and stored at 4 °C. Cell biomass was determined by a crystal violet staining technique as described in Refs. 42 and 44. The effectiveness of the complexes is expressed as corrected T/C values according to the following equations: Cytostatic effect: $T/C_{corr}[\%] = [(T - C_o)/(C - C_o)] \times 100$, where T (test) and C (control) are the optical densities at 578 nm of the crystal violet extract of the cell lawn in the wells (i.e., the chromatin-bound crystal violet extracted with 70% ethanol), and C_o is the density of the cell extract immediately before treatment. Cytocidal effect: $T[\%] = [(T - C_o)/C_o] \times 100$. For automatic estimation of the optical density of the crystal violet extract in the wells a Microplate EL 309 Autoreader was used.

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Supplementary data

Supplementary data associated with this article can be found, in the online version at, doi:10.1016/j.bmc.2005.02.059.

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