Conformational Preferences of Natural and C3-Modified Epothilones in Aqueous Solution

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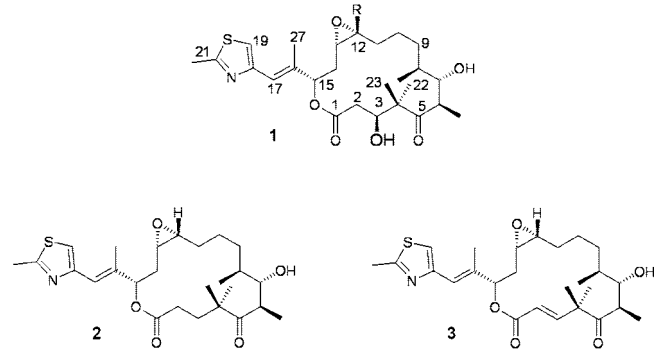
The conformational properties of the microtubule-stabilizing agent epothilone A (**1a**) and its 3-deoxy and 3-deoxy-2,3-didehydro derivatives **2** and **3** have been investigated in aqueous solution by a combination of NMR spectroscopic methods, Monte Carlo conformational searches, and NAMFIS calculations. The tubulin-bound conformation of epothilone A (**1a**), as previously proposed on the basis of solution NMR data, was found to represent a significant fraction of the ensemble of conformations present for the free ligands in aqueous solution.

Introduction

Epothilones are polyketide-based bacterial natural products, whose major variants epothilone A (Epo A, **1a**) and epothilone B (Epo B, **1b**; Chart 1) have been shown to exhibit potent in vitro and in vivo (**1b**) antitumor activity. In analogy to the important clinical anticancer drugs paclitaxel (taxol) and docetaxel, the inhibition of tumor cell growth by epothilones is based on the suppression of microtubule dynamics, which is associated with cell cycle arrest in mitosis and the induction of apoptosis.[1,2] However, in contrast to taxol, epothilones are not (or only very poor) substrates for the P-gp efflux pump and, therefore, exhibit virtually identical potency against drug-sensitive and many drug-resistant tumor cell lines (including taxol-resistant lines) in vitro.[3] In addition, epothilones are considerably more water-soluble than taxol,[4] thus enabling the use of less problematic formulation vehicles for clinical applications. Based on this favorable biological and physicochemical property profile, epothilones represent promising leads for the development of new anticancer drugs.[1,5]

As indicated by competitive binding studies, the microtubule binding sites of taxol and epothilones are identical or at least largely overlapping, thus implying that epothilones, like taxol, bind to β-tubulin.[3,6] Based on the premise of an identical taxol/epothilone binding site, several pharmacophore models have been proposed for epothilones[7] on the basis of the X-ray crystal structure of free Epo B (**1b**),[4] and/or a number of structure–activity relationship (SAR) studies.[1,5] However, overall, neither molecular modeling nor a whole set of SAR data so far have provided a coherent picture for the binding mode of epothilones to β-tubulin. Recent experimental studies on the bioactive, tubulin-bound conformation of epothilones by electron crystallography (EC)[8] and solution NMR methods[9,10] have led to significantly diverging conclusions.[13] Thus, according to the structure derived from solution NMR studies[10] the macrolactone ring of tubulin-bound Epo A (**1a**) possesses a conformation

Chart 1. Structures and Atom Numbering of Epo A (**1a**, R = H), Epo B (**1b**, R = Me), 3-Deoxy-Epo A (**2**), and 3-Deoxy-2,3-didehydro-Epo A (**3**)



largely similar to that determined by X-ray crystallography of free Epo B (**1b**, Figure 1).[4] A similar conformation has also been reported for Epo A (**1a**) free in solution for a variety of organic solvents and for Epo B (**1b**) in the solid state.[4,11,12] A distinctly different conformation of tubulin-bound Epo A (**1a**) has been derived by electron crystallography (EC) at intermediate resolution (2.9–4.2 Å) (Figure 1).[8] In this structure, the oxygen atom of the epoxide ring points inward; thus, the conformation of the macrocycle in this structure is different from all other structural proposals for the bioactive conformation of epothilones put forward so far.[10,12]

As it is generally accepted that the protein-bound conformation of natural products (or any other low-molecular-weight ligand) is also represented to a measurable extent in the ensemble of conformations for the compound free in aqueous solution,[14] conformational studies in water should provide additional guidance for a plausibility assessment of the existing structural models for the tubulin-bound state of Epo A (**1a**). Building on previous work related to other tubulin-interacting agents,[15] we have thus undertaken the elucidation of the feasible conformational space for Epo A (**1a**) in aqueous solution, using a combination of computational and spectroscopic methods. Somewhat surprisingly, this problem has not been previously addressed in the literature, perhaps due to the compound's limited solubility (at least for the purpose of NMR studies); rather, the aqueous conformation of Epo A (**1a**) has been assumed to be similar to that observed in DMSO solution.[12]

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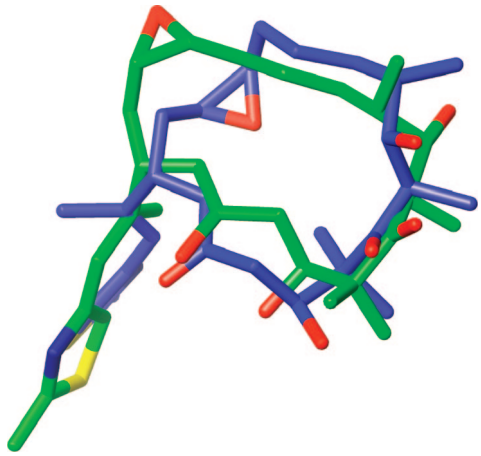

Figure 1. Superimposed structures of tubulin-bound Epo A (**1a**), as determined by solution NMR spectroscopy (green) or electron crystallography (EC; blue).

To address the effect of the 3-OH group on the conformational preferences of Epo A (**1a**) in water, we have also prepared the 3-deoxy derivatives **2** and **3** and investigated their conformational behavior. According to the EC structure of the tubulin/Epo A (**1a**) complex, this hydroxyl group should be involved in a H-bond with the protein; on the other hand, the Epo B (**1b**) derivatives corresponding to **2**[16] and **3**[17] have previously been shown to retain significant antiproliferative activity.

Results and Discussion

Small flexible molecules are present in solution as rapidly interchanging mixtures of conformers, which due to the short time scale of the interconversion cannot be distinguished by NMR methods. However, the relative populations of conformers in such mixtures can be estimated by fitting the experimental data (*J* couplings, NOE) to the data of an a priori computed set of theoretical structures.[18] In this study, a conformational ensemble was generated by a restraint-free Monte Carlo conformational search, and the pool of structures to be fitted was extended with the published solution conformations of epothilones.[4,8,10,12] The NAMFIS protocol[18] was then utilized to evaluate the population distribution of the conformational ensemble on the basis of NOE-derived distance and $^3J_{HH}$ experimental constraints. Out of 805 possible conformations 15 were identified by the procedure as feasible and the molar fractions of these conformations were estimated based on the NOE and *J* data. Subsequently, the mapped feasible conformational space was compared with the dihedral angles of the bound structures derived from EC[8] and NMR[10] studies (Figure 2).

This analysis revealed that the dihedral angles present in the NMR-derived tubulin-bound structure are also probable in aqueous solution, whereas this is not the case for many of the angles of the EC-derived structure. This implies that significant (and unfavorable) conformational changes would be required for Epo A (**1a**), upon translocation from solution to its binding pocket, to bind to tubulin in the EC-derived conformation. No such conformational alterations are required for binding in the NMR-derived conformation[10] because the latter was found to be a feasible component of the conformational ensemble in aqueous solution. Similarly, the solid state and solution structures described by Höfle[4] and Taylor,[12] respectively, were observed as components of the aqueous ensemble.

Many of the most significant differences between the EC- and NMR-derived tubulin-bound conformations of **1a** are found in the C7−C9 and the C10−C14 regions (Chart 1). For these

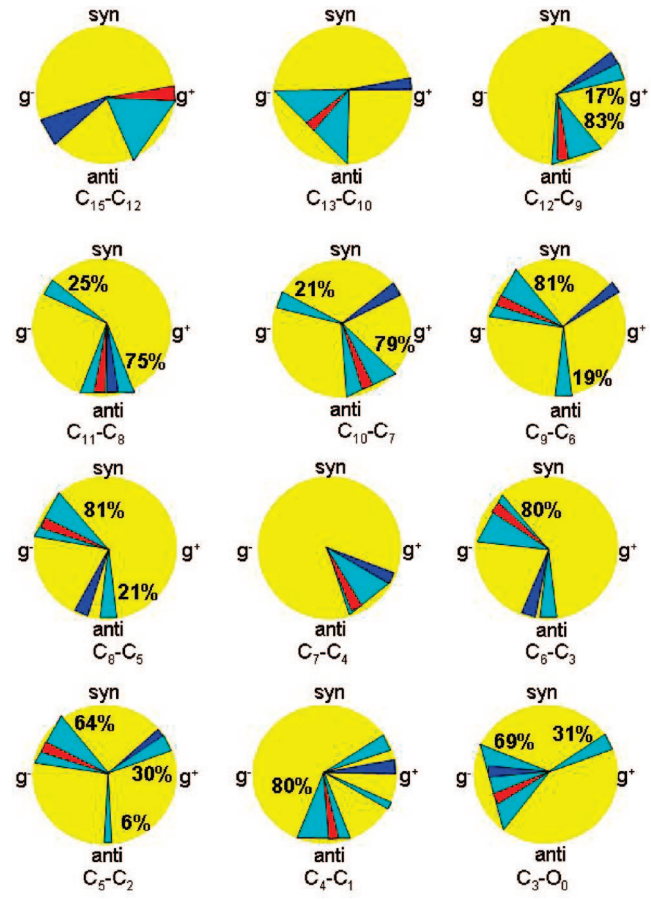

Figure 2. Feasible torsional angles for **1a**. Dihedral angles are indicated as pseudo-Newman projections of the individual C−C bonds. Hence, the structure appears in the subfigures as viewed along the bond between the participating carbon atoms, their identity being indicated below each subfigure. The bonds from the carbon atom closer to the observer to the preceding carbon in the macrolide are presumed to be at 0 degree and are omitted. Those from the more remote carbon atom to the constitutionally next carbon are drawn as projections in the plane of the paper. The available conformational angle/space for various experimental conditions is indicated. Cyan: Dihedral angles accessible for the solution ensemble, according to NAMFIS analysis of NMR and computational data. Red: NMR-derived tubulin-bound structure.[9,10] Blue: EC-derived tubulin-bound structure.[8]

regions, four torsion angles in the EC structure are not part of the feasible conformational ensemble of Epo A (**1a**) in aqueous solution (Figure 2). In addition, the C10−C11 torsion angle of about 90° that is observed in the EC-derived structure, although accessible in aqueous solution, would appear to be in conflict with the potent biological activity of the *trans*-10,11-didehydro derivatives of Epo D.[19] In contrast, the *anti*-conformation about the C10−C11 bond as observed in the NMR-derived structure is in agreement with the biological activity data. Similarly, a 90° torsion angle about the C2−C3 bond, as suggested by the EC structure, cannot be readily reconciled with the high biological activity of the *trans*-3-deoxy-2,3-didehydro derivatives of Epo A (**1a**) and B (**1b**),[17] although this conformation is moderately populated in aqueous solution. On the other hand, the 150° C2−C3 torsion angle present in the NMR-derived structure is included in a major family of conformers in the solution conformational ensemble of Epo A (**1a**) and it is able to explain the available biological activity data.

As for the conformation of the unsaturated thiazole-containing side chain on C15, both the solution NMR as well as the EC structure of tubulin-bound Epo A (**1a**) are characterized by a

syn-periplanar C16−C17−C18−C19 dihedral angle, while previous NMR studies with unbound Epo A (**1a**) in DMSO solution had indicated no preference for the thiazole ring orientation.[12] In contrast, the X-ray structure of Epo A (**1a**), with crystals obtained from an apolar organic solvent, shows an antiperiplanar C16−C17−C18−C19 torsion angle.[4] For unbound Epo A (**1a**) in aqueous solution NAMFIS analysis indicated the presence of both *syn*- and *anti*-periplanar conformations for the C16−C17−C18−C19 (74% *syn*, 26% *anti*) as well as the H15−C15−C16−C17 dihedral angle (65% *syn*, 35% *anti*). At the same time, we also observed an increase in the integral ratio of the NOE cross peaks H19↔H27 and H19↔H17 upon changing the solvent from DMSO to water, which is indicative of a higher preference for the *syn*-periplanar conformation of the C16−C17−C18−C19 dihedral angle in water as compared to DMSO solution. Nevertheless, the conformation about the C17−C18 bond remains averaged for unbound Epo A (**1a**) even in aqueous solution at ambient temperature. The presence of conformational flexibility around the C17−C18 bond was confirmed by variable temperature measurements performed in CD_3OD solution, which gave temperature coefficients of −1.64 and +0.43 ppbK^{-1} for H19 and H17, respectively, upon raising the temperature from 208 to 298 K. The $\Delta\delta/\Delta T$ values of these protons were significantly higher than those of all other protons in the molecule (typically 0–0.3 ppbK^{-1}), implying a higher mobility around the C17−C18 bond, as compared to the conformationally more restrained C−C bonds of the macrolactone part.

To understand whether the temperature-induced chemical shift changes of H19 and H17 in CD_3OD may reflect an increasing shift of the side chain conformational equilibrium toward an *anti*-periplanar conformation about the C17−C18 bond, the theoretical shift changes upon reorientation of the epothilone side chain from the C16−C17−C18−C19 *syn*-periplanar to an *anti*-periplanar conformation were determined by ab initio (b3lyp/6–311 g(2df,2pd)) calculations. These calculations predicted changes of −0.44 ppm and +0.32 ppm for δ(H19) and δ(H17), respectively, for a *syn*- to *anti*-periplanar transition of the C16−C17−C18−C19 dihedral angle. The computational data thus support the idea that the *anti*-periplanar conformation becomes more accessible at higher temperatures in *polar* solvents, while the *syn*-periplanar conformation is energetically more favorable. This finding is in agreement with the results of the NAMFIS analysis of **1a**, which points to a clear preference for the *syn*-periplanar conformation of the C16−C17−C18−C19 dihedral angle in water at room temperature. In contrast, in crystals of Epo A (**1a**) obtained from an *apolar* organic solvent (CH_2Cl_2), the *anti*-periplanar conformation for the C16−C17−C18−C19 dihedral angle was found to be preferred.[4] Overall, the above data clearly suggest that the proposed bioactive conformation of the side chain of Epo A (**1a**; which is similar for the EC- and NMR-derived structures) is considerably populated in aqueous solution, which facilitates binding to the target.

Investigation of the aqueous conformations of 3-deoxy-Epo A (**2**) and the corresponding unsaturated derivative 3-deoxy-2,3-didehydro-Epo A (**3**; Chart 1) allowed the assessment of the impact of the 3-OH group on the conformational preferences in the C1−C5 region. According to the EC-derived structure, this OH group is involved in protein recognition by the formation of a hydrogen bond with T274 of β-tubulin.[8] According to this structural proposal, the bioactivity of 3-deoxy derivatives would be expected to be significantly reduced compared with the parent natural products. However, both **2** and **3** showed intriguingly potent tubulin polymerization activity, with EC_{50} values of 5.6 μM for **2** and 4.8 μM for **3** (compared to 4.6 μM for Epo A (**1a**)). Likewise, **2** and **3** exhibit potent antiproliferative activity, with IC_{50} values in the sub-100 nM range (Table 1).

Table 1. Antiproliferative Activity of Epo A (**1a**), **2**, and **3** against Human Cancer Cell Lines[a]

	IC_{50} [nM] (±SD)		
cmpd	MCF-7 (breast)	PC-3 M (prostate)	HCT-116 (colon)
1a	2.9 ± 0.3	6.4 ± 1.5	2.8 ± 0.4
2	58.4 ± 6.8	79.0 ± 21.3	84.4 ± 11.6
3	8.7 ± 2.4	24.8 ± 4.1	16.2 ± 1.8

[a] For experimental details, see Experimental Methods.

These data are in line with the results of previous studies on the bioactivity of the corresponding Epo B (**1b**) derivatives, which had revealed very similar trends.[16,17] The similar activity of **1a**, **2**, and **3** in tubulin polymerization assays points to a limited role of the 3-OH group in the direct interaction of epothilones with tubulin, and this finding is in agreement with the proposed binding mode of Epo A (**1a**) to β-tubulin, as derived by the INPHARMA solution NMR method.[20] According to this model, the 3-OH group does not engage in a direct interaction with any amino acid side chain, but is oriented toward a polar, solvent accessible pocket. In addition, the very similar activity of Epo A (**1a**), Epo B (**1b**), and their respective 3-deoxy-2,3-didehydro derivatives clearly points to an *anti*-periplanar conformation of the C2−C3 bond in the bioactive conformation of epothilones, which is again in line with the NMR-derived structural proposal for the tubulin-bound state of Epo A (**1a**).

NAMFIS conformational analysis based on NOE and *J* measurements as well as Monte Carlo conformational searches indicated largely unaltered conformational preferences of the 16-membered ring in **2** or **3** relative to the conformation of Epo A (**1a**). For this analysis, the stereospecific assignments of the CH_2-2 and CH_2-3 protons were derived by estimation of the long-range $^3J_{C1/5/22/23,H3}$ and $^3J_{C4H2}$ together with $^3J_{H2H3}$ coupling constants and their comparison with back-calculated values.[21,22] The conformational analysis of solution ensembles generally indicated similar dihedral angles for all three compounds investigated, with a single major difference being observed for the C1−C2 torsion, as shown in Figure 3.

For compound **3** the alteration of this dihedral angle is related to the preference of the ester group for an in-plane orientation with the conjugated double bond between C2 and C3, which is only possible for the O−C1−C2−C3 dihedral angle in a *syn*- or *anti*-conformation (Figure 3). As a consequence, the orientation of the C1 carbonyl is altered from that of natural Epo A (**1a**), however, apparently without any significant effect on biological activity. This indicates that the C1 carbonyl group may not be involved in any energetically important interactions with tubulin.

For 3-deoxy-Epo A (**2**), a moderate increase in flexibility was observed for the C1−C2 and the C2−C3 bonds (as compared to Epo A (**1a**)), but the C1−C2−C3−C4 *anti*-periplanar conformation remains largely preferred. Thus, removal of the 3-OH group does not dramatically alter the conformational preferences of the macrolide ring, either in the presence (compound **3**) or the absence (compound **2**) of a geometric constraint about the C2−C3 bond. On the other hand, the slight increase in conformational flexibility observed in the C1−C4 region of derivative **2** for entropic reasons may lead to somewhat reduced binding affinity and this may ultimately be reflected in the lower cellular activity of **2** as compared to Epo A (**1a**) or **3**.

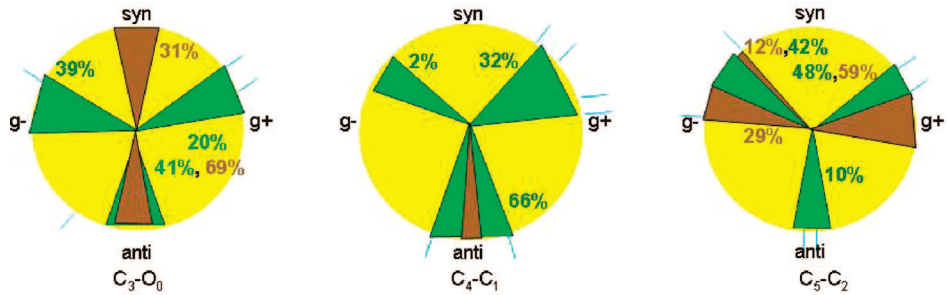

Figure 3. Feasible torsional angles (C1−C2, C2−C3, and C3−C4) of the solution ensembles of **2** (green) and **3** (brown) are shown. Comparable angles of Epo A (**1a**) are shown with cyan lines and in Figure 2. For further explanations, see Figure 2.

It should be emphasized, however, that the differences between all compounds are small at the level of tubulin polymerization in vitro and it is not clear how these effects quantitatively translate into antiproliferative activity in cells.

Conclusions

Based on the investigation of the conformational preferences of unbound Epo A (**1a**) in aqueous solution, the NMR-derived tubulin-bound conformation of this natural product is a probable component of the aqueous conformational ensemble. In contrast, the EC-derived tubulin-bound structure of Epo A (**1a**) was not found to be significantly populated by free Epo A (**1a**) in water. Similar conformational preferences as for Epo A (**1a**) were observed for its 3-deoxy derivatives **2** and **3**. Thus, the removal of the 3-OH group, either with or without concomitant incorporation of a *trans* double bond between C2 and C3, does not alter the preferred overall conformation of the macrolide ring, although some minor changes were observed in the C1−C5 region. However, these changes are well tolerated at the level of tubulin interactions and also in terms of cellular activity. In summary, the conformational analysis presented in this work clearly indicates that the NMR-derived tubulin-bound conformation of Epo A (**1a**) corresponds to a low energy conformation of the free ligand in aqueous solution, and access to this conformation is not critically dependent on the presence of the 3-OH group. Together with the bioactivity data, these findings also suggest that the 3-hydroxyl group may not be directly involved in the fundamental network of interactions between epothilones and β-tubulin.

Experimental Methods

Compounds. Epo A (**1a**) was a generous gift of Novartis Pharma AG, Basel, Switzerland. Compounds **2** and **3** were synthesized according to Scheme S1 (Supporting Information). The synthesis of **3** has been previously described in the literature.[17] Detailed procedures for the preparation of **2** and **3** are provided in the Supporting Information.

NMR Experiments and Computational Studies. NMR studies were carried out on Bruker Avance 900, 700, and 600 MHz spectrometers. The NOE build up studies were performed on 0.5 mmol/dm^3 solutions of epothilone derivatives at mixing times of 40, 80, 120, 160, and 200 ms. No water suppression was necessary. For **1a** and **2**, the measurements were run for a 5% deutero-ethyleneglycole 95% D_2O solvent mixture at 270 K, for **3** a 15% DMSO, 5% ethyleneglycole, and 80% D_2O mixture was studied at 288 K. The temperature and content of solvent mixtures were optimized to reach tumbling rates of the molecules that allow for the observation of a sufficient number of NOEs for structure calculation. Distances were calculated from NOEs with the reference distance of 1.78 Å for geminal protons. For the distance calculation, methyl signals were treated as follows: $d = \{[(d_1^6)^{-1} + (d_2^6)^{-1} + (d_3^6)^{-1}]/3\}^{-6}$. The J couplings were derived from E.COSY and high resolution ^{1}H spectra. Long range $^3J_{CH}$ coupling constants were estimated by integration of the corresponding cross peak intensities in a HMBC spectrum.[21] Stereochemical assignment was performed by comparison of the experimental $^3J_{CH}$s and $^3J_{HH}$s with back-calculated values.[22]

Conformational searches were performed using the OPLS-2005 all-atom force field as implemented in the program Macromodel 7.5.[23] The general born solvent accessible (GB/SA) surface area method developed by Still[24] was used in all calculations. The number of torsion angles allowed to vary during each Monte Carlo step ranged from n to $n - 1$, where n is the total number of rotatable bonds. Conformational searches were conducted by use of the torsional sampling (MCMM) search method implemented in the Batchmin program. First 100.000 Monte Carlo step runs were performed, and those conformations within 25 kJ mol^{-1} of the global minimum were kept. PR conjugate gradient (PRCG) minimization (5000 steps) was used in the conformational search. In the subsequent minimization to fully converged structures, PRCG and truncated Newton conjugate gradient minimization was applied. This procedure resulted in 953 conformations for **2**, 620 structures for **3**, and 3561 structures for **1**. The low energy structures (for **1** the first 800 conformations starting from the global minimum) were then included in the NAMFIS analysis.[18] As experimental restraints, these calculations included 12 distances and 8 Js for **3**, 11 distances and 12 Js for **2**, and 18 distances and 11 Js for **1**. A summary of experimental constraints and calculated distances and coupling constants of conformers selected by the NAMFIS analyses are available as part of the electronic Supporting Information.

Biological Assays. EC_{50} values for the induction of tubulin polymerization (i.e., the concentration required to induce 50% of the maximum $\alpha\beta$-tubulin polymerization achievable) were determined with 10 μM of porcine brain tubulin. Tubulin polymerization was assessed through turbidity measurements at 340 nm (A_{340}).[25] For a given compound concentration, the achievement of an equilibrium state between soluble and polymerized tubulin is indicated by a stable plateau in A_{340}. Maximum tubulin polymerization is reached when increases in compound concentration no longer result in an increase of the plateau value for A_{340}. Similar maximum values for A_{340} were observed for all three compounds investigated in this study.

IC_{50} values for human cancer cell growth inhibition were determined for a 72 h exposure period of cells to compounds by quantification of protein content of fixed cells by methylene blue staining.[26] For further experimental details, compare ref 27. The EC_{50} values given in the text and the IC_{50} values in Table 1 all represent the means of three independent experiments (±standard deviation).

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Supporting Information Available: Details of the NAMFIS calculation as well as synthetic procedures for the preparation of **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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