Epothilones

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Design and Synthesis of 12-Aza-Epothilones (Azathilones)—"Non-Natural" Natural Products with Potent Anticancer Activity**

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Natural products provide an immeasurable pool of lead structures for drug discovery, and more than 50% of today's

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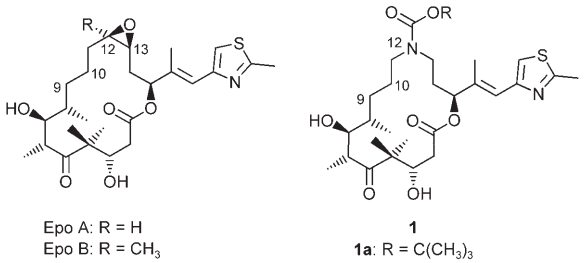
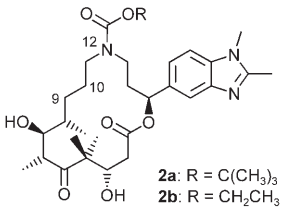
prescription drugs are ultimately derived from compounds first obtained from natural sources.[1] While the screening of large collections of natural products will continue to be an important strategy for the identification of new drug leads or compounds of biochemical and pharmacological interest,[2] various approaches have been developed in the more recent past that aim at expanding the structural scope of natural-products-based drug discovery. In particular, this includes the de novo construction of libraries of "natural-products-like" compounds through diversity-oriented synthesis (DOS),[3] as pioneered by Schreiber et al., and the design of natural-products-based libraries, a concept that was introduced by Waldmann et al. in 2002[4] and has been continuously refined over the last few years.[5]

Our own research in the area of natural-products-based drug discovery has been directed towards the development of new biologically active scaffolds (or chemotypes) through the extensive structural modification (rather than simple peripheral derivatization) of existing natural product leads.[6] In this context we have also investigated the biological activity of 12-aza-epothilones, which are characterized by the replacement of a backbone carbon atom by nitrogen in the epothilone macrocycle.[6c,d,7] The resulting analogues may best be described as "non-natural" natural products,[8] as they still retain most of the (two-dimensional) structural features of the natural product lead; at the same time they are structurally unique, as they are outside of the general scope of nature's biosynthetic machinery for polyketide biosynthesis, which is not programmed for the incorporation of single nitrogen atoms in a regular polyketide backbone.[9] As a result of these studies we discovered that 12-aza-epothilones **1** (termed





"azathilones") can retain much of the antiproliferative activity of natural epothilones, depending on the nature of the acyl substituent attached to the backbone nitrogen atom.[6d,10]

Unfortunately, our first-generation synthesis of these compounds proved to be relatively inefficient and tedious.[6d] At the same time, the activity of azathilones **1** even in the best case, i.e. with R = *tert*-butyl (**1a**), was ca. 15–50 times lower than that of Epo A. These shortcomings led us to investigate alternative synthetic routes to this class of compounds, with the concurrent incorporation of additional structural modifications designed to enhance biological activity. Apart from available data on the structure–activity relationships (SARs) for azathilones **1** with regard to the nature of the acyl substituent attached to N12 (vide supra),[6d] the design of these analogues was additionally guided by the results of previous studies in our laboratory, which had revealed a general activity-enhancing effect of a dimethylbenzimidazole side chain in combination with the natural epothilone macrocycle.[6a,b,11] These considerations resulted in compounds **2a** and **2b** as initial targets for total synthesis and biological investigation, both of which eventually proved to be highly potent inhibitors of human tumor growth in vitro. In this communication we now wish to report on the total synthesis of azamacrolides **2a** and **2b**,[10] either through ring-closing olefin metathesis (RCM) or by a macrolactonization approach, and the preliminary biological characterization of these compounds.

Macrocycle formation through RCM featured as a particularly attractive approach to the target azathilones, as it offered simultaneous access to specific unsaturated analogues (as the immediate cyclization products), which could be interesting new antiproliferative agents in their own right.[12] Our RCM-based synthesis of target structure **2a** and its 9,10-didehydro derivative **9** (Scheme 1) involved three key strategic steps, namely, 1) the stereoselective aldol reaction between aldehyde **3**[13] and ketone **4**[14] (d.r. 8:1), 2) esterification of carboxylic acid **7** with the unsaturated alcohol **10**,[15] and 3) RCM of bisolefin **8**.

Initial attempts to cyclize **8** employing the first-generation Grubbs catalyst[16] met with complete failure and no conversion was observed. In contrast, the use of the dihydroimidazol-2-ylidene-based second-generation catalyst[16] produced the cyclic olefin in excellent yield (85%) and with exclusive *E* selectivity. No trace of the corresponding *Z* product could be isolated, and similar observations have been made for the RCM-based cyclization of the analogue of **8** containing the natural epothilone side chain.[24]

Unfortunately, the efficiency of the cyclization reaction was thwarted by serious difficulties encountered in the subsequent reduction of the C9–C10 double bond, which proved to be extremely sluggish under all experimental conditions investigated (thus leading to low yields and also side reactions such as reductive ester cleavage with H_2/Pd-C without reduction of the double bond). The only viable approach for the transformation of **9** into **2a** involved the use of in situ generated diimide, which had been successfully employed in the transformation of 9,10- and 10,11-didehydroEpo D, respectively, into Epo D (= 12,13-deoxyEpoB),[17] and which produced **2a** in 31% yield from **9** after purification by preparative HPLC.

While this approach provided sufficient material for initial biological testing, it was clear that an alternative strategy would have to be developed for more extensive profiling and eventual in vivo studies, should those appear to be warranted. In light of the highly promising biological data obtained for

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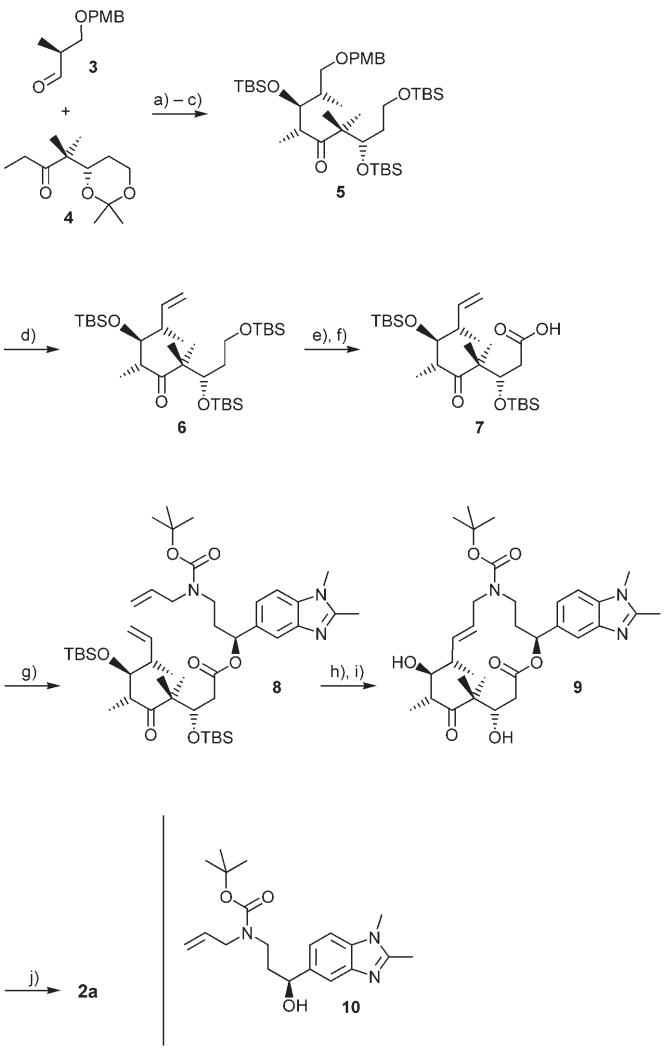
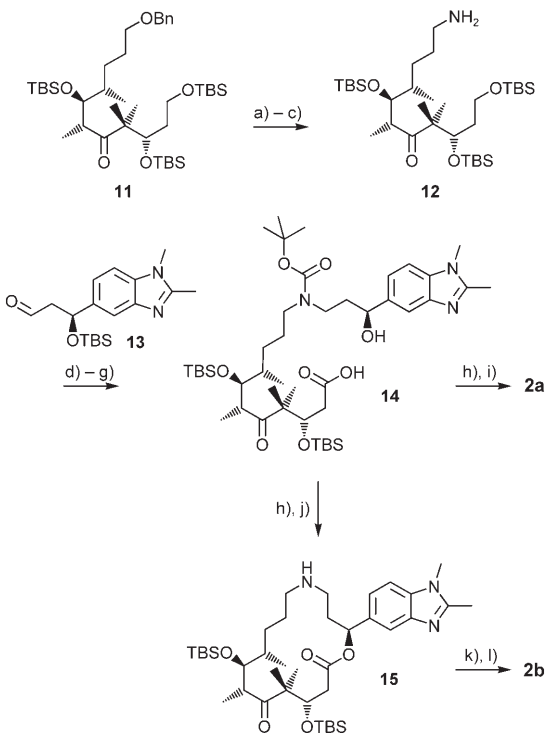


Scheme 1. Reaction conditions: a) **4**, LDA, −78 °C, 5 h, then addition of **3**, −90 °C, 75 min, 76%, d.r. 8:1; b) PPTS, MeOH, RT, 20 h, 86%; c) 1. TBSOTf, 2,6-lutidine, −78 °C → RT, 1.5 h; 2. flash chromatography; 76%; d) 1. H_2/Pd-C, MeOH, RT, 20 h; 2. TPAP, NMO, 4-Å MS, CH_2Cl_2, RT, 1 h; 3. $MePPh_3Br$, LiHMDS, THF, 0 °C, 1.5 h, 79% (three steps); e) CSA (1.0 equiv), CH_2Cl_2/MeOH 1:1, 0 °C, 1 h, 87%; f) PDC (11 equiv), DMF, RT, 64 h, 85%; g) **10**, DCC (1.2 equiv), DMAP (0.3 equiv), CH_2Cl_2, 0 °C, 15 min, RT, 15 h, 60%; h) 2nd-generation Grubbs catalyst (0.15 equiv, incremental addition), CH_2Cl_2, reflux, 8 h, 85%; i) HF·pyridine, pyridine, THF, RT, 4 h, 70%; j) KO_2C-N=N-CO_2K (excess), AcOH, CH_2Cl_2, 31%, pure **1** obtained through purification by preparative HPLC. CSA = (+)-camphorsulfonic acid, DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, LDA = lithium diisopropylamide, LiHMDS = lithium 1,1,1,3,3,3-hexamethyldisilazide, NMO = 4-methylmorpholine-*N*-oxide, PDC = pyridinium dichromate, PMB = *para*-methoxybenzyl, PPTS = pyridinium *p*-toluenesulfonate, TBS = *tert*-butyldimethylsilyl, TPAP = tetrapropylammonium perruthenate.



Scheme 2. Reaction conditions: a) H_2/Pd-C, EtOAc, RT, 62 h, 86%; b) HN_3, DEAD, PPh_3, THF, 0 °C, 25 min, RT, 30 min, 96%; c) H_2/Pd-C, MeOH, RT, 3 h, 92%; d) 1. **13** (1.1 equiv), $NaBH(OAc)_3$ (1.6 equiv), AcOH (2.0 equiv), 4-Å MS, RT, 2.5 h; 2. Boc_2O, Et_3N, THF, 0 °C, 45 min, 60% (two steps); e) CSA (1.1 equiv), CH_2Cl_2/MeOH 1:1, 0 °C, 3 h, 80%; f) PDC (15 equiv), DMF, RT, 24 h, 50%; g) TBAF (6 equiv), THF, RT, 24 h; h) 2,4,6-$Cl_3C_6H_2C(O)Cl$, Et_3N, THF, 0 °C, 20 min, then diluted with toluene and added to a solution of DMAP in toluene, 75 °C, 1 h, 44% (two steps); i) HF·pyridine, pyridine, THF, RT, 2.5 h, then preparative HPLC, 40%. j) $ZnBr_2$ (4.0 equiv), CH_2Cl_2, RT, 2.5 h, quant.; k) $CH_3CH_2OC(O)Cl$, Et_3N, THF, 0 °C, 30 min; l) HF·pyridine, pyridine, THF, RT, 3.5 h, then preparative HPLC, 32% (two steps). Bn = benzyl, Boc = *tert*-butyloxycarbonyl, DEAD = diethylazodicarboxylate, TBAF = tetrabutylammonium fluoride.

2a (vide infra), we thus embarked on the elaboration of an alternative route to **2a** that would be based on macrolactonization rather than RCM.

This approach employed the reductive amination of aldehyde **13** with amine **12** (obtained in three steps from the known protected tetrol **11**[14a]) to assemble the heteroaliphatic skeleton of **2a** (Scheme 2). The reaction was best conducted with a slight excess of aldehyde **13** (1.1 equiv) and $NaBH(OAc)_3$ as the reducing agent in the presence of AcOH (2 equiv) and 4-Å molecular sieves. Owing to its pronounced polarity (arising from the presence of the secondary amino group as well as the benzimidazole moiety) the reductive-amination product was not purified but directly converted into the corresponding *N*-*tert*-butyloxycarbonyl derivative, which was obtained in 60% yield (based on amine **12**). Selective cleavage of the primary *tert*-butyldimethylsilyl (TBS) ether with CSA followed by oxidation of the resulting free alcohol with PDC and removal of the TBS protecting group from C15–O with TBAF then led to seco acid **14**, which was cyclized under Yamaguchi conditions[18] to produce fully protected **2a** (44% based on C15-O-TBS-protected **14**). Subsequent selective removal of the TBS protecting groups with HF·pyridine gave target structure **2a** in 40% yield (after HPLC purification).

In a preliminary attempt to assess the importance of the *tert*-butyl moiety of **2a** for biological potency (vide infra), we also prepared the closely related azathilone **2b**, which

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incorporates a N12-ethoxycarbonyl substituent in place of the *tert*-butyloxycarbonyl moiety present in **2a**.[19] Azathilone **2b** was obtained from bis-TBS-protected **2a** through highly selective cleavage of the *tert*-butyloxycarbonyl group from N12 ($ZnBr_2$, CH_2Cl_2, RT)[20] followed by acylation of the resulting free amine **15** with ethyl chloroformate and subsequent removal of the TBS protecting groups with HF·pyridine (Scheme 2).

The data summarized in Table 1 indicate that azathilone **2a** is a highly potent antiproliferative agent, which inhibits the growth of different types of drug-sensitive human cancer cell lines (A549, HCT-116, PC-3M, KB-31) with IC_{50} values in the low nanomolar range.

Table 1: αβ-Tubulin-polymerizing and antiproliferative activity of azathilones **2a**, **2b**, and **9**.

Cmpd	EC_{50} (Tubulin polym.) [μM][a]	IC_{50} [nM][b]				
		A549	HCT-116	PC-3M	KB-31	KB-8511
2a	3.9 ± 0.6	1.9 ± 0.4	1.6 ± 0.5	2.3 ± 0.6	0.34 ± 0.15	222 ± 48
2b	5.4 ± 0.4	18.0 ± 3.1	23.9 ± 2.9	12.3 ± 1.1	12.6 ± 1.0	> 1000
9	9.1 ± 0.7	920 ± 85	1009 ± 71	973 ± 64	n.d.[e]	n.d.[e]
1a	5.6 ± 0.4	130 ± 24	110 ± 19	126 ± 22	31[c]	105[c]
Epo A	4.6 ± 0.5	3.2 ± 0.5	2.2 ± 0.3	3.4 ± 0.4	2.15[d]	1.91[d]

[a] Concentration required to induce 50% of the maximum αβ-tubulin polymerization achievable with the respective compound (10 μM of porcine brain tubulin). Tubulin polymerization was determined by turbidity measurements at 340 nm (A_{340}).[22] 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 compounds investigated in this study. [b] IC_{50} values for inhibition of human cancer cell growth. KB-31, KB-8511: cervix; A549: lung; HCT-116: colon; PC-3M: prostate. KB-8511 is a P-glycoprotein 170 (P-gp170)-overexpressing multidrug-resistant subline of the KB-31 parental line. Cells were exposed to compounds for 72 h. Cell numbers were determined by quantification of protein content of fixed cells by staining with methylene blue.[23a] For further experimental details see reference [23b]. Values represent the means of at least three independent experiments (± standard deviation). [c] Data from reference [6d]. [d] Data from reference [6a]. [e] Not determined.

The antiproliferative activity of **2a** is thus comparable to that of Epo A, with the exception of the multidrug-resistant KB-8511 line, where **2a** is significantly less potent (vide infra). Likewise, **2a** induces tubulin polymerization in vitro with potency similar to that of Epo A (Table 1, Figure 1A), which strongly suggests that inhibition of human cancer cell proliferation by **2a**, as for natural epothilones, is a consequence of interference with microtubule functionality. This view is further corroborated by the fact that treatment of cancer cells with **2a** results in cell cycle arrest at G_2/M, which mirrors the effects on the cell cycle observed upon treatment with Epo A or B[7] (Figure 1B).

Upon analysis of the data presented in Table 1 in more detail, it also becomes apparent that **2a** is > 60 times more potent against drug-sensitive human cancer cells than the corresponding parent (natural-side-chain-containing) azathilone **1a**. Although the molecular origin of this activity difference is unclear, it should be noted that the potency increase observed for **2a** over **1a** dramatically exceeds the potency-enhancing effects previously observed for the dimethylbenzimidazole side chain in combination with polyketide-based macrocycles (2–15-fold).[6a,b,11] Equally intriguing is the observation that compound **9**, which incorporates a *trans* double bond between C9 and C10, is significantly less potent than the fully saturated azathilone **2a** (both at the levels of tubulin polymerization as well as cellular activity). A similar potency difference also exists between **1a** and its *trans* 9,10-didehydro derivative.[25] These findings are in marked contrast to the effects observed for Epo B and D, where the introduction of a *trans* double bond between C9 and C10 results in enhanced cellular potency,[12b,17a] and they may be indicative of differences in the bioactive conformation between azathilone-type analogues and natural epothilones.

Compared to azathilone **2a**, analogue **2b** exhibits somewhat reduced cellular activity (Table 1), but the compound still remains a very potent antiproliferative agent against all drug-sensitive cancer cell lines investigated. In addition, as **2b** can safely be assumed to be more acid-stable than **2a**, the former could in fact offer some advantage over the latter in vivo (for oral administration). Based on the tubulin-polymerization data shown in Table 1, **2a** is a more potent inducer of tubulin polymerization than **2b**, which indicates that hydrophobic interactions between the protein and the N12-carbamate substituent play an inportant role in the binding of these compounds to α/β-tubulin.[19] Whether the difference in tubulin-polymerizing activity between **2a** and **2b** can fully account for the difference in cellular potency is unknown at this

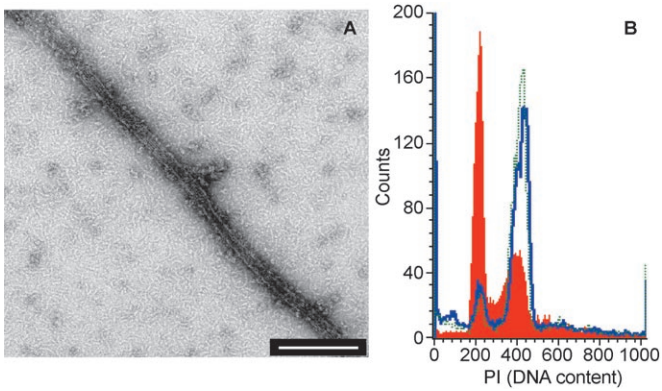

Figure 1. A: Compound **2a** induces the formation of microtubules in vitro. [20 μM of purified porcine αβ-tubulin was incubated with 20 μM of **2a** for 30 min in BRB80 buffer at RT]. Electron micrograph shows part of a single long microtubule. Scale bar: 100 nm. B: Cell-cycle effects of **2a** in PC-3M cells (G_1 vs. G_2/M). **2a** (250 nM) was incubated for 1 h with 2×10^5 cells and then removed. Cells were subsequently grown for 24 h prior to analysis of DNA content with propidium iodide. Vehicle control (red) shows cells in the G_1 phase of the cell cycle. In the presence of compound **2a** (blue line) or Epo A (250 nM; dotted green line) cells accumulate in G_2/M. PI = propidium iodide.

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point; the latter may also be affected by differences in non-target-related parameters such as cellular uptake or intracellular distribution.

Compared to their effects on drug-sensitive cancer cell lines, both **2a** and **2b** exhibit reduced antiproliferative activity against the P-gp-overexpressing human cervix carcinoma line KB-8511, which indicates that both compounds are substrates for the P-gp efflux pump. However, we have recently shown that the susceptibiliy of polyketide-based epothilone analogues to P-gp-mediated drug efflux can be modulated through adjustments in compound lipophilicity; this strategy will also be explored for lead structures **2a** and **2b**.[21]

In summary, we have achieved the total synthesis of two representative examples of a new class of highly potent microtubule-stabilizing agents, which are based on an azamacrolide backbone and which we have termed azathilones. While the conception of these compounds is closely connected to the structure of natural epothilones (hence the name "azathilones"), given the degree of structural divergence from the natural epothilone template, they may be considered as members of a distinct group of "non-natural" natural products with unique structural features and, as indicated by some preliminary SAR data, a unique SAR profile. Both compounds investigated in this study are potent growth inhibitors of drug-sensitive human cancer cells in vitro and, thus, should be attractive new lead structures for anticancer-drug discovery.

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