Biosynthesis

Isolation, Crystal and Solution Structure Determination, and Biosynthesis of Tubulysins—Powerful Inhibitors of Tubulin Polymerization from Myxobacteria**

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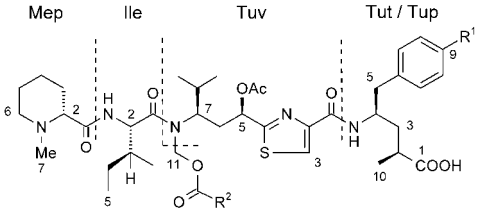
The antifungal and cytotoxic myxobacterial metabolite epothilone[1] was known for many years before Bollag et al.[2] discovered in 1995 that its potent cytotoxicity is based on the induction of tubulin polymerization. In fact, epothilone was the first natural product after taxol to be found to have this mode of action, and, even more surprisingly, it was able to displace taxol from its binding site on microtubules. This and the fact that it retained its activity for taxol- and multidrug-resistant tumor cells initiated extensive worldwide chemical and biological research activity[3,4] and clinical development.[5] However, it also triggered the reinvestigation of other toxic natural products for which no mode of action was known. Very soon, this led to the discovery that discodermolide,[6] eleutherobin/sarcodictyin,[7] laulimalide,[8] peloruside,[9] dictyostatin-1,[10] jatrophane,[11] and hemiasterlin[12] were also tubulin-polymerization inducers or inhibitors.

In our hands, the macrodiolide disorazol[13] and a novel group of tetrapeptides named tubulysins[14] from myxobacteria turned out to be, contrary to epothilone, inhibitors of tubulin polymerization, thereby mimicking the activity of the vinca alkaloids. Both disorazol and the tubulysins surpass epothilones, vinblastine, and taxol by a factor of 20–1000 with respect to growth inhibition potential; however, their therapeutic efficacy as anticancer drugs has still to be evaluated. Here we report on the isolation, structure elucidation, biosynthesis, and biological properties of tubulysins A–I (**1**–**9**; Mep = *N*-methyl pipecolic acid, Ile = isoleucine, Tuv = tubuvaline, Tut/Tup = tubutyrosine/tubuphenylalanine).

Two different species of myxobacteria, *Archangium gephyra* and *Angiococcus disciformis*, were identified to produce tubulysins, and the compounds were isolated by multistep chromatography from culture extracts. Whereas *A. gephyra* produces 2–4 mg L^{-1} of tubulysin A and as minor components tubulysins B, C, G, and I, all of which are characterized by a *p*-hydroxyphenyl residue, *A. disciformis* produces 0.5 mg L^{-1} of the phenyl analogues tubulysins D, E, F, and H.[15] Structure elucidation of tubulysins by NMR spectroscopy was seriously complicated by signal broadening and even lack of signals for certain carbon and hydrogen atoms. At this stage an important clue came from a biosynthetic labeling study. Feeding with the ^{13}C-enriched presumed polyketide precursors indicated incorporation of three acetate units and three methyl groups from methionine (see below). Thus, assignment of the ^{13}C NMR spectra was facilitated, and, most importantly, the signal for the C11 atom of the Tuv building block became visible as a broad peak at around $\delta = 70$ ppm. With this information, structure elucidation of tubulysin A (**1**; $C_{43}H_{65}N_5O_{10}S$) by 1D and 2D NMR spectroscopy was straightforward (Table 1).



		R^1	R^2
Tubulysin	A (**1**)	OH	$CH_2CH(CH_3)_2$
	B (**2**)	OH	$CH_2CH_2CH_3$
	C (**3**)	OH	CH_2CH_3
	D (**4**)	H	$CH_2CH(CH_3)_2$
	E (**5**)	H	$CH_2CH_2CH_3$
	F (**6**)	H	CH_2CH_3
	G (**7**)	OH	$CH{=}C(CH_3)_2$
	H (**8**)	H	CH_3
	I (**9**)	OH	CH_3

Tubulysin A turned out to be a linear tetrapeptide of *N*-methyl pipecolic acid (Mep), isoleucine (Ile), a novel amino acid named tubuvaline (Tuv), and a novel chain-extended tyrosine analogue named tubutyrosine (Tut). In tubulysins D, E, F, and H the latter is replaced by tubuphenylalanine (Tup). Whereas the 5-acetoxy residue in Tuv is common to all tubulysins, the *N*-acyloxymethyl substituent varies in size from 3-methylbutyrate in tubulysins A (**1**) and D (**4**) to acetate in tubulysins H (**8**) and I (**9**). The *N*-acyloxymethyl substituent may also be regarded as a formaldehyde *N*,*O*-acetal, and formaldehyde was indeed liberated upon acidic hydrolysis. Remarkably, *N*,*O*-acetals of this type have been found only twice in nature, as the methyl ether[16] or *O*-glycoside.[17]

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Table 1: ^{13}C and ^{1}H NMR (150 and 600 MHz) spectroscopic data of tubulysin A (**1**).[a]

C atoms	δ [ppm] in [D$_6$]DMSO	δ [ppm] in CD_3OD	H atoms	δ [ppm]	Multiplicity in [D$_6$]DMSO	J [Hz]	δ [ppm]	Multiplicity in CD_3OD	J [Hz]
Mep:									
C1	172.8	173.3	2-H	2.46	dd	10.4, 3.1	3.05	brs	
C2	68.1	69.5	3a-H	1.37	m		1.66	m	
C3	24.8	31.1	3b-H	1.57	m		1.92	m	
C4	22.8	23.6	4a-H	1.16	qt	12.3, 3.6	1.41	qt	12.8, 3.7
C5	29.6	25.5	4b-H	1.63	m		1.83	m	
C6	54.7	56.4	5a-H	1.42	qt	12.5, 3.3	1.67	m	
C7	43.8	44.1	5b-H	1.55	m		1.76	m	
			6a-H	1.93	m		2.45	brs	
			6b-H	2.82	dt	11.5, 3.5	3.15	brd	11.8, 3.5
			7-H$_3$	2.04	s		2.41	s	
Ile:									
C1	174.2	176.3	2-H	4.42	t	9.1	4.68	d	9.0
C2	52.6	55.3	2-NH	7.92	d	8.8	–		
C3	35.1	37.4	3-H	1.93	m		2.05	m	
C4	24.1	25.4	4a-H	1.09	m		1.25	ddq	13.7, 9.0, 7.4
C5	10.1	10.8	4b-H	1.48	ddd	13.4, 7.5, 2.8	1.67	m	
C6	15.3	16.4	5-H$_3$	0.80	t	7.7	0.95	t	7.4
			6-H$_3$	0.83	d	6.1	1.02	d	6.8
Tuv:									
C1	159.7	162.7	3-H	8.18	s		8.15	s	
C2	149.8	150.9	5-H	5.75	dd	11.3, 2.2	5.91	dd	11.1, 2.1
C3	124.2	125.4	6a-H	2.15	brs		2.38	brs	
C4	168.5	170.9	6b-H	2.40	m		2.51	brt	12.0
C5	68.9	70.9	7-H	4.38	brs		4.42	brs	
C6	34.3	35.9	8-H	1.83	brs		1.95	brs	
C7	55.8	58.7	9-H$_3$	0.68	d	6.7	0.86	d	6.7
C8	30.0	32.2	10-H$_3$	0.98	d	6.5	1.10	d	6.5
C9	19.3	20.4	11a-H	5.26	d	12.1	5.50	d	12.1
C10	20.2	20.7	11b-H	6.20	brd	12.1	6.12	brd	12.1
C11	68.9	70.5	5-OAc	2.11	s		2.20	s	
5-OAc	169.8	172.0	2′a-H	2.08	dd	14.9, 7.1	2.12	dd	15.0, 7.0
5-OAc	20.5	20.8	2′b-H	2.14	dd	14.9, 7.2	2.18	dd	15.0, 7.4
C1′	171.3	173.3	3′-H	1.90	m		2.04	m	
C2′	42.7	44.4	4′-H$_3$	0.82	d	6.7	0.93	d	6.6
C3′	25.0	26.8	5′-H$_3$	0.81	d	6.7	0.90	d	6.6
C4′	22.1	22.8							
C5′	22.0	22.8							
Tut:									
C1	177.1	180.7	2-H	2.38	m		2.58	ddq	9.3, 4.6, 7.0
C2	36.2	38.4	3a-H	1.52	ddd	13.6, 10.6, 4.9	1.65	m	
C3	37.6	39.3	3b-H	1.84	ddd	13.5, 9.1, 4.3	2.06	m	
C4	49.0	51.1	4-H	4.11	m		4.33	ddt	9.6, 5.4, 6.6
C5	39.5	41.1	4-NH	7.79	d	8.2	–		
C6	128.5	130.1	5a-H	2.67	dd	13.8, 6.3	2.85	dd	13.9, 7.0
C7	129.9	131.5	5b-H	2.73	dd	13.8, 7.3	2.87	dd	13.9, 6.3
C8	114.9	116.2	7-H	6.97	AA′	8.4	7.08	AA′	8.4
C9	155.5	157.0	8-H	6.62	BB′	8.4	6.70	BB′	8.4
C10	18.0	18.7	9-OH	9.1	brs		–		
			10-H$_3$	1.06	d	7.0	1.21	d	7.0

[a] DMSO = dimethyl sulfoxide.

The absolute configuration of the seven stereocenters of the tubulysins, as depicted for compounds **1**–**9**, was first determined by acidic hydrolysis, degradation, and partial synthesis, coupled with GC analysis.[18] However, some doubts remained with the assignment of the Tuv C5 and Tup C2 atoms, whose configuration may have been inverted during functional-group manipulation. Fortunately, tubulysin A crystallized spontaneously from a methanol/water solution, and an X-ray crystal structure analysis could be performed to confirm the structure and the proposed absolute configuration (Figure 1).[19]

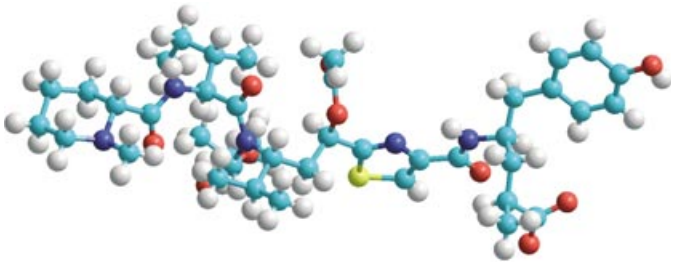

Figure 1. Stereoview of the crystal structure of tubulysin A (**1**) (ORTEP plot, hydrogen atoms added).

In the unit cell we find one tubulysin A and five interstitial water molecules, but no methanol. Due to the limited resolution (≤ 1.01 Å) of the X-ray experiment, hydrogen atoms could not be located directly. Nevertheless, short interatomic distances and the geometry of the carboxyl group indicate that tubulysin A crystallizes as a zwitterion. From the exterior angles the carboxyl group appears to be ionized (C7-C9-O2 118(1)° and C7-C9-O3 120(1)°; crystal structure numbering differs from that shown in the structures above).[20] On the other hand, the piperidine nitrogen atom shows one short intermolecular contact to the carbonyl oxygen atom (contact distance N5–O1 2.85 Å). This is compatible only with a protonated piperidine nitrogen atom.

The protonation state of tubulysin A in methanol was investigated by observation of the chemical shifts of the Mep *N*-methyl and Tut 2H signals. After addition of ammonia and acetic acid the free and signals of the protonated *N*-methyl groups were observed at $\delta = 2.19$ and 2.72 ppm, respectively, and the Tut 2H signals were observed at $\delta = 2.42$ and 2.60 ppm. Based on these data, in free tubulysin A, with *N*-methyl and Tut 2H siganls observed at $\delta = 2.41$ and 2.58 ppm, respectively, the carboxyl group is only sparingly deprotonated, whereas the extent of protonation of the piperidine nitrogen atom is about 40%.

In tubulysins the Tuv *N*-acyloxymethyl substituent causes considerable crowding around the Ile–Tuv amide bond, and slow rotation in this tertiary amide is obviously responsible for the extensive line broadening of the Tuv C6 and C11 methylene signals and the C7 and C8 methine signals. Additionally, slow proton exchange at pH 7 causes line broadening in the vicinity of the Mep nitrogen atom and for the Tut 2H atom. Both ^{1}H and ^{13}C NMR signals sharpen up at elevated temperatures; however, even at 80 °C the linewidth of these signals is still greater than that of other signals. As expected, this and the overlap of ^{1}H NMR signals complicated the solution-conformation analysis. Whereas ROESY spectra of tubulysin A in methanol showed a surplus of H–H contacts, NOE contacts in DMSO indicate the presence of one predominant conformer (Figure 2), which is very similar to that in the crystal structure. While no NOE contacts were observed across the thiazole ring, a strong NOE interaction between the Tuv 5H and Tut 4NH atoms indicates rotation of the thiazole ring by 180°, which brings these hydrogen atoms into close proximity. From vicinal coupling constants (Table 1) and NOE interactions, it is determined that the Tut side chain, including the C10 atom, is in an extended staggered conformation with some rotational freedom for the *p*-hydroxybenzyl group. The tripeptide part Mep–Ile–Tuv exists in a well-defined conformation, as indicated by a tight network of NOE contacts. In addition, vicinal coupling constants, for example, in Ile (2H, 3H, 4NH: 9.1 Hz) and Tuv (5H, 6Ha, 6Hb: 11.3, 2.2 Hz) are in full accord with this conformation.

Biosynthetic considerations suggested that the tubulysin backbone is formed by condensation of the common amino

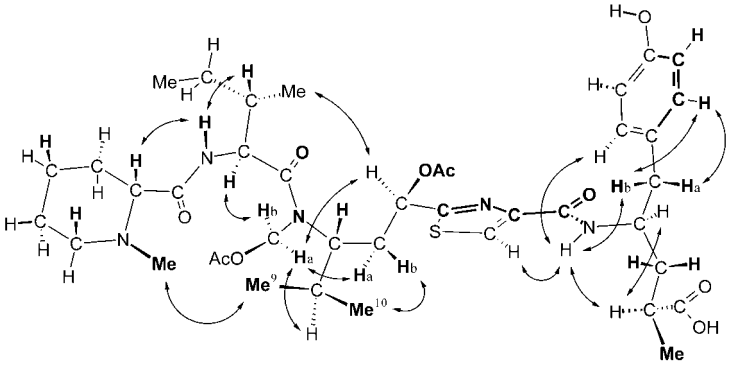

Figure 2. Conformation of tubulysin A (**1**) in [D$_6$]DMSO, based on the crystal structure, NOE correlations, and vicinal proton coupling constants. Ac = acyl.

acids pipecolic acid (lysine), isoleucine, valine, cysteine, and tyrosine or phenylalanine with two acetate units, followed by *C*- and *N*-methylation. Feeding with ^{13}C-labeled acetate and methionine indeed confirmed incorporation of these building blocks with high efficiency in the expected positions (Figure 3,

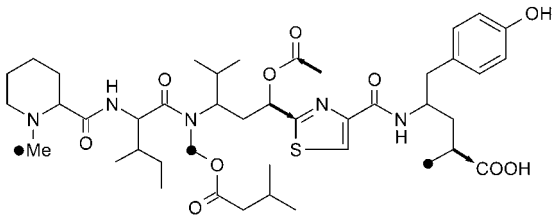

Figure 3. Biosynthesis of tubulysin A (**1**) from [$^{13}C_2$]acetate (→) and [$^{13}CH_3$]methionine (●).

Table 1 in the Supporting Information). The biosynthesis would be completed by P_{450}-mediated hydroxylation at the Tuv C5 atom and the Tuv *N*-methyl group, followed by acylation. Remarkably, the 5-hydroxy group is exclusively acetylated, whereas the *N*-hydroxymethyl group is acylated by a range of fatty acids. The fact that feeding the organism with specific fatty acids has no significant influence on the pattern of acyl groups indicates that these are specifically derived from a pool of fatty acid coenzyme A esters formed by amino acid degradation.[21] Similarly, feeding with an excess of phenylalanine or tyrosine had no influence on the ratio of tubulysins formed. Whereas the tubulysin synthase complex of *A. gephyra* accepts exclusively tyrosine, that of

A. disciformis is only partially selective for phenylalanine (Phe/Tyr = 8:1). With the recent cloning of the tubulysin megasynthase complex from *A. disciformis*,[22] the way is prepared for a detailed analysis and manipulation of the approximately 30 biosynthetic steps required to form tubulysin.

From a structural and biosynthetic point of view the tubulysins are related to dolastatin 10, which was isolated by Pettit et al. from the sea hare *Dolabella auricularia*.[23] Both compounds are polypeptide–polyketide hybrids of similar size and amino acid composition. Although the sequence of building blocks is significantly different, both compounds target the tubulin system by inhibiting polymerization. Taken together, these similarities are a strong indication for a common ancestor of the dolastatin 10 and tubulysin biosynthesis genes in bacteria,[24] an ancestor that during coevolution with eukaryotes has been optimized independently for the synthesis of two different molecules with high tubulin-binding affinity. It is a tempting idea to search now for "biosynthetic fossils" on the gene or product level.

The antiproliferative activity of tubulysins A–I correlates very well with their lipophilicity as indicated by the retention time on reversed-phase HPLC (Table 2). Regardless of the size of the Tuv 11-acyloxy residue, Tup-type tubulysins **4**–**6** and **8** are more lipophilic and more active than the Tut-type ones **1**–**3**, **7**, and **9**, which have a phenolic hydroxy group. Within the two groups tubulysins D and A with a 3-methylbutyryl residue are the most active, and tubulysins H and I with an acetyl residue are the least active. Overall, the activity varies by a factor of 60 from 0.011–0.68 ng mL^{-1}. Contrary to the antiproliferative activity, the target activity, that is, inhibition of tubulin polymerization, is more or less the same for all tubulysins and is lower than cell culture activity by several orders of magnitude. These results can be explained, in part, by the preferential uptake of the lipophilic tubulysins from the culture medium. Indeed, incubation of L929 cells with a mixture of tubulysins A and D (50 ng mL^{-1}) resulted in 25- and 100-fold enrichments, respectively, in the cells, according to HPLC/ESI-MS analysis. Whether this is the result of an active inward transport or of diffusion followed by binding to abundant tubulin in the cytoplasm is not known. Even with the multidrug-resistant cell line KB-V1, which has an active P-glycoprotein export system, the intracellular concentration of tubulysins remains high; this results in activity superior to that of dolastatin and vinblastine.

Table 2: Biological activity, inhibition of tubulin polymerization, and lipophilicity of naturally occurring tubulysins A–I (**1**–**9**), dolastatin 10, and vinblastine.

Tubulysin	Lipophilicity t_R[a] [min]	L929[b] IC_{50} [ng mL^{-1}]	KB-V1[c] IC_{50} [ng mL^{-1}]	Tubulin-polymerization inhibition [%][d]
D	17.5	0.011	0.25	47
E	16.4	0.013	0.25	48
F	15.3	0.015	0.35	50
H	13.9	0.031	1.3	36
A	13.6	0.070	1.4	46
G	13.0	0.093	2.9	39
B	12.6	0.091	2.3	57
C	11.6	0.30	4.0	57
I	10.4	0.68	6.8	34
dolastatin 10	17.4	0.10	1.5	–
vinblastine	17.7	23	93	20

[a] Retention time on RP-18 chromatography is used as a measure of the lipophilicity.[25] [b] Mouse fibroblasts (DSMZ ACC 2). [c] Human cervix carcinoma, multidrug-resistant cell line (DSMZ ACC 149). [d] Determined at 10 μM tubulin and 1 μM tubulysin and vinblastine, respectively.[26]

Ongoing work on the chemical modification of tubulysin A indicates that its inhibitory activity can be increased by a factor of 10. The tubulysins are therefore ideal candidates for immunoconjugation and tumor targeting. On the other hand, there is sufficient scope for reducing activity by derivatization while improving the therapeutic index. At first sight, total synthesis also seems an easy task; however, as we observed, clustering of space-demanding groups in the center of the structure poses an unexpected challenge.

Experimental Section

Isolation of tubulysins from *Archangium gephyra*: A fermentation batch (270 L) of strain Ar 315 was harvested and extracted as described previously.[13a] The resulting crude extract (60 g) was partitioned between methanol and *n*-heptane to give a refined oily extract (52 g), which was separated by chromatography on Sephadex LH 20 with methanol as the eluent. The tubulysin-containing fraction (6.12 g) was further separated by MPLC (RP-18, methanol/50 mM ammonium acetate buffer (pH 6.5) 6:4) to give tubulysins A (856 mg), B (739 mg), and C (94 mg) as colorless amorphous solids. Tubulysins G (24 mg) and I (190 mg) were obtained from intermediate fractions by preparative HPLC (acetonitrile/50 mM ammonium acetate buffer (pH 6.5), 35:65).

Isolation of tubulysins from *Angiococcus disciformis*: A fermentation batch (300 L) of strain An d48 was harvested and extracted as described previously.[13a] The resulting crude extract (36 g) was passed through a Sephadex LH 20 column (methanol) to give a tubulysin-enriched fraction (3.3 g), which was further separated by MPLC (Merck Prepbar, RP-18, methanol/75 mM ammonium acetate buffer (pH 6.5) 6:4) to give a polar fraction (209 mg) containing tubulysins A, B, F, and H and a more lipophilic fraction (308 mg) containing tubulysins D and E. Repeat MPLC chromatography of these fractions on RP-18 and subsequently on Sephadex LH 20 (CH_2Cl_2/MeOH 8:2) yielded tubulysins D (74 mg), E (11 mg), F (5 mg), and H (1 mg).

Tubulysin A (**1**): Colorless crystals from methanol/water, m.p. 106–108 °C; colorless crystals from 10 mM sodium phosphate buffer (pH 7), m.p. 107–110 °C; $t_R = 13.6$ min (Nucleosil C18, 125 × 2 mm, 5 μm, acetonitrile/10 mM ammonium acetate buffer (pH 5.5), gradient from 30:70 to 95:5 over 20 min, 0.3 mL min^{-1}); $R_f = 0.42$ (silica gel 60 on aluminium sheets, dichloromethane/acetone/methanol 70:20:10); $[\alpha]_D^{22} = 15.3$ ($c = 5$, MeOH); UV (MeOH): λ_{max} (lgε) = 205 (4.44), 225 (4.20), 250 sh (3.86), 276 (3.25), 287 nm (3.08); IR (KBr): $\tilde{\nu}_{max} = 3390$, 2959, 2934, 2876, 1747, 1667, 1553, 1515, 1233 cm^{-1}; NMR: see Table 1; DCI MS: *m*/*z* (%): 844 (34) [M+H$^+$], 742 (22), 504 (6), 239 (30), 120 (17), 103 (100); HR DCI MS: *m*/*z* calcd. for $C_{43}H_{66}N_5O_{10}S$ [M+H$^+$]: 844.4530; found: 844.4543.

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[15] In addition to **1**–**9**, the major tubulysins were also isolated in varying amounts as their Mep-*N*-oxides, characterized by the *N*-methyl ^{1}H and ^{13}C NMR signals at $\delta = 3.10$ and 57.8 ppm, respectively. We found that these compounds were artefacts of the extraction with analytical grade ethyl acetate, which is commonly contaminated with peroxides. Unlike ether peroxides, the peroxide contaminants of ethyl acetate come from the (Tischtschenkow) production process. We found up to 3 mmol equiv L^{-1} of nonidentified peroxides in analytical grade ethyl acetate from several major distributors (analytical method: oxidation of triphenylphosphane). This observation should be a warning to all those who isolate *N*- or *S*-oxides from natural sources while using ethyl acetate as the solvent.

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[19] Experimental details of the crystal structure analysis of tubulysin A (**1**) can be found in the Supporting Information. CCDC-233087 (**1**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).

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