MEDI#87

Design and Synthesis of *N*-alkylated Tubulysin Analogs and their Folate Conjugates.

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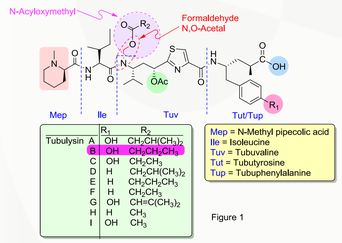
254th American Chemical Society National Meeting

Introduction

Tubulysins are natural products isolated from myxobacterial species. They are potent mitotic poisons as they inhibit the polymerization of tubulin into microtubules. Majority of natural isolated tubulysins possess an acid, base, and enzyme-sensitive *N*-acyloxymethyl substituent, as well as an enzyme-labile acetate group; both of these functional groups are essential for their potent cytotoxicity. Herein, we present the design and synthesis of more stable tubulysin analogs based on our reported synthesis of tubulysin B. These synthetic tubulysin analogs are less prone to degradation under acidic/basic conditions and enzymatic hydrolysis. Folate conjugates of these highly potent tubulysin analogs were also synthesized.

Structural Variety of Tubulysins

Natural Tubulysins:



	R_3	R_4
Tubulysin A	OH	$CH_2CH(CH_3)_2$
B	OH	$CH_2CH_2CH_3$
C	OH	CH_2CH_3
D	H	$CH_2CH(CH_3)_2$
E	H	$CH_2CH_2CH_3$
F	H	CH_2CH_3
G	OH	$CH=C(CH_3)_2$
H	H	CH_3
I	OH	CH_3

Mep = N-Methyl pipecolic acid
Ile = Isoleucine
Tuv = Tubuvaline
Tut = Tubutyrosine
Tup = Tubuphenylalanine

Figure 1

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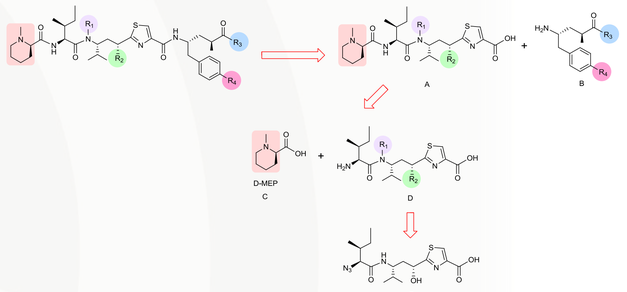
Retrosynthetic Analysis of Tubulysins and Analogs

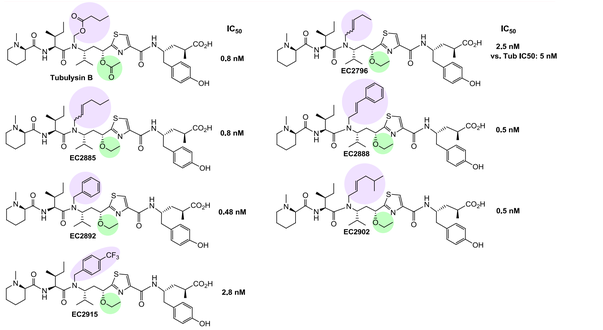


Based on our synthetic method, there are several potential sites on Tubulysin B which can be conveniently modified. Our focus was on the modification of highlighted sites, i.e. MEP, *N,O*-acetal side chain, acetate, phenolic hydroxyl and acid groups of Tut. Intermediate E is the common starting point for all syntheses.

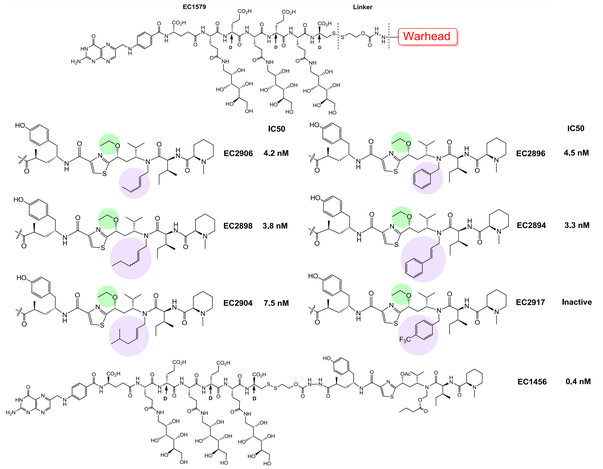
In vitro Activities of Selected Tubulysin Analogs Synthesized previously

Site 1	Site 2	Site 3	Site 4	Site 5	KB	KB-DR	MDA-MB -231
MEP	$OCOC_3H_7$	(*R*)-OAc	OH	OH	0.5 nM	4 nM	0.7 nM
MEP	$OCOC_3H_7$	(*R*)-OAc	$NHNH_2$	OH	2 nM	10 nM	0.9 nM
MEP	OC_3H_7	(*R*)-OAc	OH	OH	0.89 nM	3 nM	0.7 nM
MEP	OC_5H_{11}	(*R*)-OAc	OH	H	.3nM	.7nM	.01nM
MEP	OC_5H_{11}	OMe	OH	OH	0.68 nM	0.94 nM	0.26 nM
MEP	OC_5H_{11}	OMe	OH	H	0.45 nM	0.98 nM	0.3 nM
	OC_5H_{11}	OMe	OH	H	2 nM	7.3 nM	0.5 nM
MEP	OC_5H_{11}	(*R*)-$OCONC_2H_5$	OH	OH	2.8 nM		
MEP	OC_5H_{11}	(*R*)-NHAc	OH	OH	171 nM		
MEP	OC_5H_{11}	(*R*)-NHAc	OH	H	95.5 nM		
MEP	$OCOC_3H_7$	(*S*)-NHAc	OH	OH	inactive		
MEP	$S(CH_2)_2CH_3$	(*R*)-OAc	OH	OH	1.4 nM		
MEP	$S(CH_2)_2OH$	(*R*)-OAc	OH	OH	26 nM		

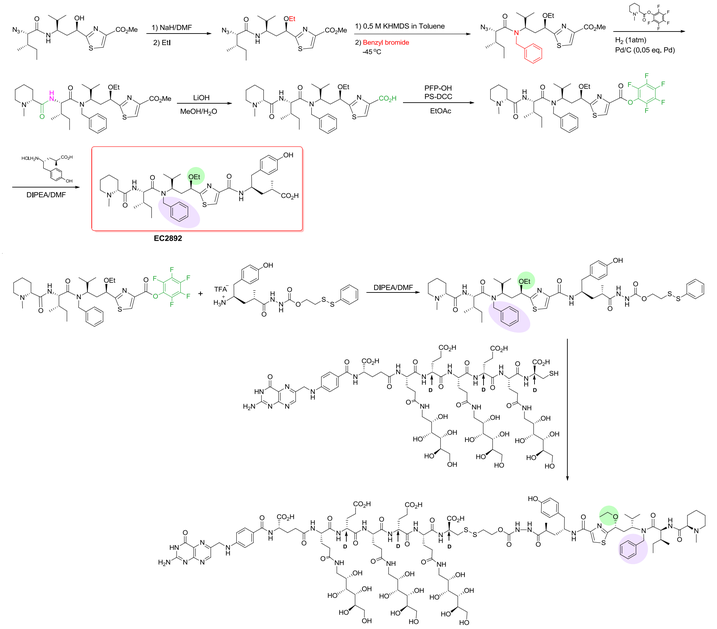
In vitro Activities of *N*-Alkylated Tubulysin Analogs



In vitro Activities of *N*-Alkylated Tubulysin Analog Conjugates



Typical Synthesis of Tubulysin Analogs and their Conjugates



Results and Conclusions

Structural features, critical for the tubulysin's cytotoxicity, were investigated. We found that the hydroxyl group on Tuv is very important to maintain the high activity of tubulysin analogs. Exchange of Tuv's (*R*)-OAc group with (*R*)-OMe or (*R*)-OEt had minimum influence on activity. Replacement *N*-acyloxymethyl substituent (dotted circle) with an alkyl or benzyl group resulted in *N*-alkyl tubulysin analogs possessing similar or higher activity. The folate conjugates of *N*-alkyl tubulysin analogs showed good activity against KB cells.