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ORIGINAL ARTICLE

Engineered production and evaluation of 6′-deoxy-tallysomycin H-1 revealing new insights into the structure–activity relationship of the anticancer drug bleomycin

Dong Yang[1,4], Hindra[1,4], Liao-Bin Dong[1], Ivana Crnovcic[1] and Ben Shen[1,2,3]

The bleomycins (BLMs), a family of glycopeptide antibiotics, are currently used clinically in combination with a number of other agents for the treatment of malignant tumors. Other members of the BLM family include tallysomycins (TLMs), phleomycins and zorbamycin (ZBM). We previously cloned and characterized the biosynthetic gene clusters for BLMs, TLMs and ZBM. Applications of combinatorial biosynthesis strategies to the three biosynthetic machineries enabled the engineered production of several BLM analogs with unique structural characteristics and varying DNA cleavage activities, thereby providing an outstanding opportunity to study the structure–activity relationship (SAR) for the BLM family of anticancer drugs. We now report the engineered production of a new BLM–TLM–ZBM hybrid metabolite, named 6′-deoxy-TLM H-1, which consists of the 22-desmethyl-BLM aglycone, the TLM A C-terminal amine and the ZBM disaccharide, by heterologous expression of the *zbmGL* genes from the ZBM biosynthetic gene cluster in the *Streptoalloteichus hindustanus* Δ*tlmH* mutant strain SB8005. Evaluation of the DNA cleavage activities of 6′-deoxy-TLM H-1 as a measurement for its potential anticancer activity, in comparison with TLM H-1 and BLM A2, reveals new insight into the SAR of BLM family of anticancer drugs.
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INTRODUCTION

The bleomycins (BLMs) are currently used clinically, under the trade name Blenoxane, in combination with a number of other agents to treat various cancers.[1–3] The BLMs are thought to cause metal-dependent oxidative cleavage of DNA in the presence of molecular oxygen. Early development of drug resistance and cumulative dose-dependent pulmonary toxicity are the major limitations of BLMs in chemotherapy.[1,3,4] In the past four decades, the development of active and particularly less toxic analogs has been intensively pursued through organic synthesis and microbial fermentation.[3,5,6]

Other members of the BLM family of natural products include tallysomycins (TLMs), phleomycins and zorbamycin (ZBM) (Figure 1a).[7–9] As a family, they feature (i) a hybrid peptide–polyketide agylcone containing a bithiazole or thiazonylthiazole moiety, (ii) a varying C-terminal amine and (iii) a disaccharide or its 6′-deoxy variant. TLMs differ from the other BLM family members by the presence of an additional talose moiety attached to the (aminoethyl) bithiazole moiety and the absence of the CH_3 group at C-22 in the valerate moiety (Figure 1a). TLM S10b, a TLM analog obtained by fermenting the producing strain in a medium supplemented with 1,4-diaminobutane, has been shown to exhibit antitumor activity similar to that of the BLMs but with less severe toxicity in preclinical studies.[10,11] However, TLM S10b failed to yield the desired response in phase II clinical trials due to poor cell penetration.[12,13]

In our continued efforts to study biosynthesis of the BLM family of anticancer drugs as a model system for hybrid peptide–polyketide natural products,[14] we have cloned the TLM biosynthetic gene cluster from *Streptoalloteichus hindustanus* E465-94,[15] in addition to the BLM cluster from *Streptomyces verticillus* ATCC15003[16] and *Streptomyces mobaraensis* DSM40847[17] and the ZBM cluster from *Streptomyces flavoviridis* ATCC21892.[18] Manipulations of the TLM biosynthetic machinery in *S. hindustanus* generated the Δ*tlmK* and Δ*tlmH* mutant strains SB8003[19] and SB8005[20] that produce TLM K-1, and TLM H-1 and TLM H-2, respectively (Figure 1b). TLM H-1, featuring the 22-desmethyl-BLM aglycone with a TLM C-terminal amine, cleaves plasmid DNA nearly as efficient as TLM A and BLMs, providing an additional scaffold for structural modifications and structure–activity relationship (SAR) studies for the BLM family of anticancer drugs.[20]

[1]Department of Chemistry, The Scripps Research Institute, Jupiter, FL, USA; [2]Department of Molecular Medicine, The Scripps Research Institute, Jupiter, FL, USA and [3]Natural Products Library Initiative at The Scripps Research Institute, The Scripps Research Institute, Jupiter, FL, USA
[4]These authors contributed equally to this work.
Correspondence: Professor B Shen, Department of Chemistry, The Scripps Research Institute, 130 Scripps Way, #3A1, Jupiter, FL 33458, USA.
E-mail: shenb@scripps.edu
Dedicated to Professor Hamao Umezawa, Institute of Microbial Chemistry, on the occasion of the 60th anniversary of worldwide marketing of kanamycin, which was first discovered by Professor Umezawa in 1957.
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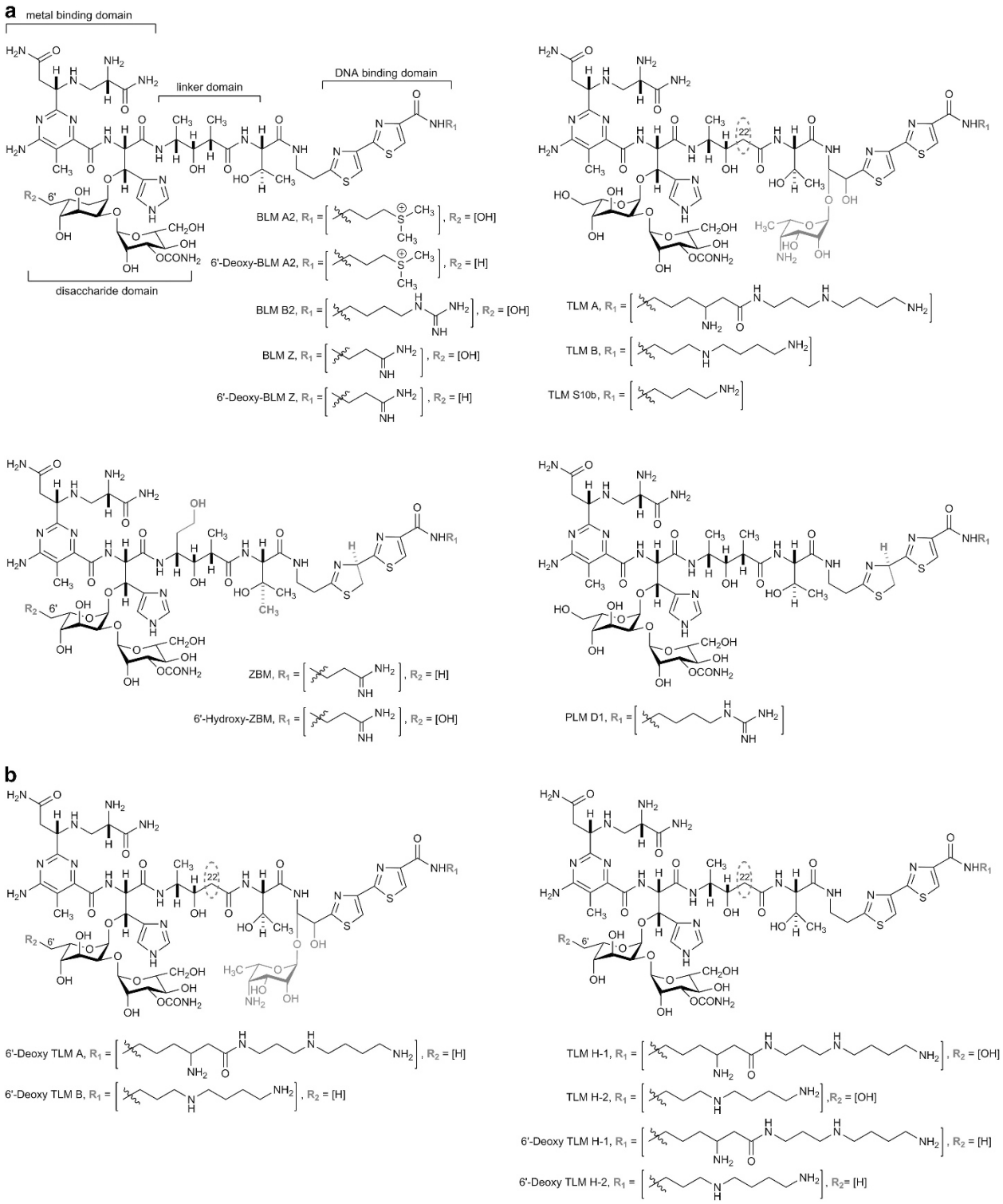

Figure 1 (**a**) Structures of selected members of the bleomycin (BLM) family of antitumor antibiotics: BLM A2, BLM B2, tallysomycin (TLM) A, TLM B, TLM S10b, zorbamycin (ZBM) and phleomycin (PLM) D1, as well as the previously engineered BLM analogs 6′-deoxy-BLM A2, BLM Z, 6′-deoxy-BLM Z and 6′-hydroxy-ZBM. (**b**) Structures of engineered BLM analogs produced by *S. hindustanus* recombinant strains: TLM H-1 and TLM H-2 by SB8005, 6′-deoxy-TLM A and 6′-deoxy-TLM B by SB8006, and 6′-deoxy-TLM H-1 and 6′-deoxy-TLM H-2 by SB8007. The structural variation sites are shown in red. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

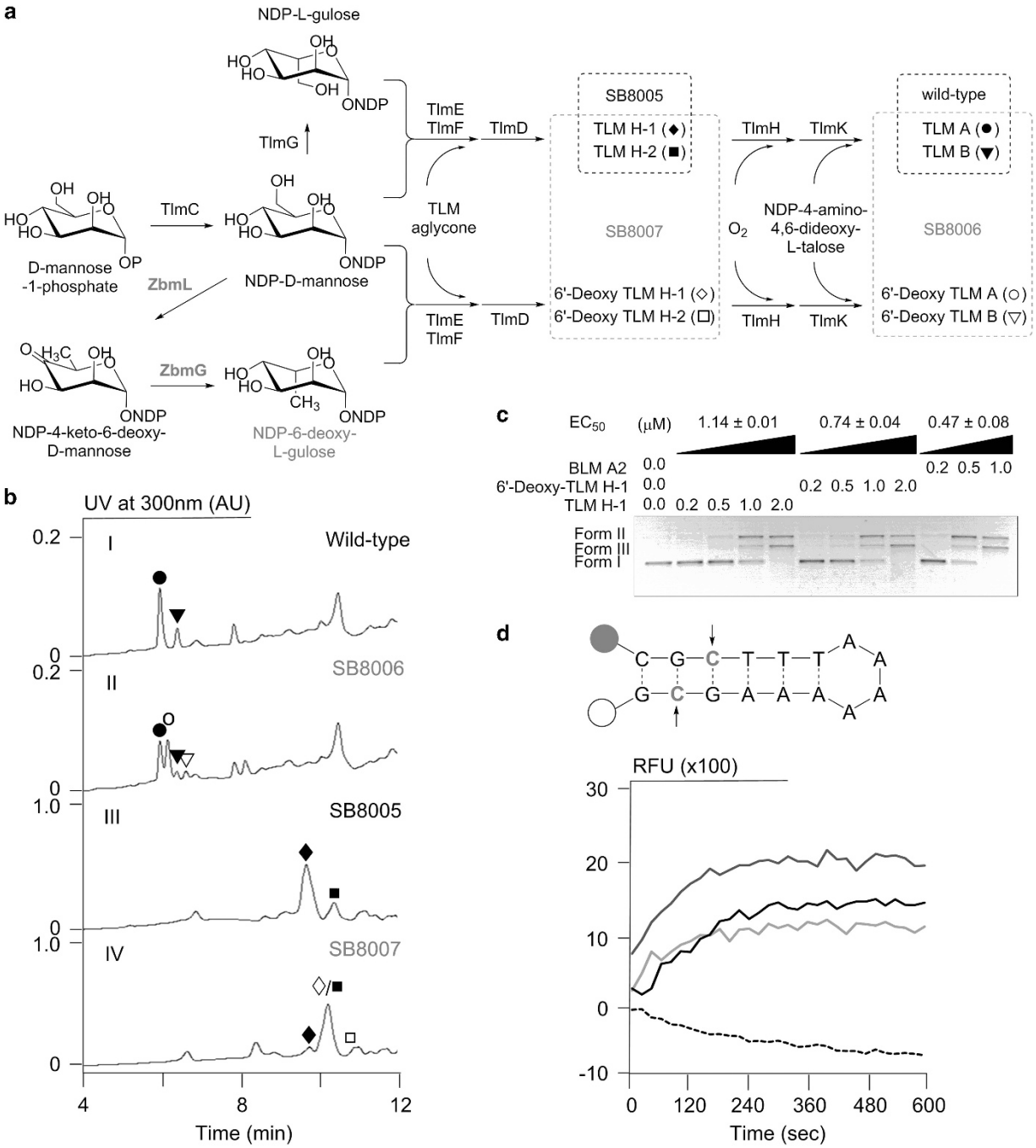

Figure 2 Engineered production of 6′-deoxy-TLM analogs and evaluation of the DNA cleavage activity 6′-deoxy-TLM H-1 in comparison with BLM A2 and TLM H-1. (**a**) Proposed pathways for TLM and ZBM sugar biosynthesis in *S. hindustanus* and *S. flavoviridis* and heterologous expression of *zbmGL* in the *S. hindustanus* wild type and Δ*tlmH* mutant SB8005 (highlighted by the black dotted line rectangles) for engineered production of 6′-deoxy-TLM analogs, as exemplified by SB8006 for the production of 6′-deoxy-TLM A and 6′-deoxy-TLM B and SB8007 for the production of 6′-deoxy-TLM H-1 and 6′-deoxy-TLM H-2, respectively (highlighted by red dotted line rectangles). (**b**) HPLC chromatograms of metabolite profiles of SB8006 (panel II) and SB8007 (panel IV), in comparison with the *S. hindustanus* wild type (panel I) and the Δ*tlmH* mutant (panel III), highlighting the engineered production of the 6′-deoxy-TLM analogs. TLM A (●); TLM B (▼); 6′-deoxy-TLM A (o); 6′-deoxy-TLM B (▽); TLM H-1 (♦); TLM H-2 (■); 6′-deoxy-TLM H-1 (◇); 6′-deoxy-TLM H-2 (□). (**c**) DNA cleavage efficiency of 6′-deoxy-TLM H-1 as observed in plasmid relaxation assays with pBluescript II SK(+) in comparison with BLM A2 and TLM H-1. Form I, supercoiled plasmid DNA; form II, open circular plasmid DNA; and form III, linearized plasmid DNA. (**d**) DNA cleavage kinetics as observed by time-dependent fluorescence emission intensity of molecular beacon (arrows indicating cleavage sites), treated with 6′-deoxy-TLM H-1 (blue) in comparison with BLM A2 (black) and TLM H-1 (red). Dash lines represent the negative control without adding BLM or engineered analogs. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Combinatorial biosynthesis has emerged as a powerful strategy to generate novel analogs of complex natural products by genetic engineering of biosynthetic pathways.[21,22] Cloning and comparative genomics analysis of BLM, TLM and ZBM biosynthetic gene clusters (Supplementary Figure S1) have provided the molecular basis for the formulation of hypotheses to engineer novel analogs for the BLM family of anticancer drugs.[14–20,23,24] We have shown previously that BLM, TLM and ZBM share similar sugar biosynthetic pathways (Supplementary Figure S2).[14] The disaccharide moiety of BLMs and TLMs is biosynthesized and attached to the BLM aglycones by Blm/TlmCDEFG, whereas that of ZBM is afforded by ZbmCDEFGL.[14,18,24] It has been further revealed from the engineered production of 6'-deoxy-BLM Z in *S. flavoviridis* SB9026[24] and 6'-deoxy-BLM A2 in *S. mobaraensis* SB11[17] that while BlmCDEF and ZbmCDEF are functionally equivalent, BlmG and ZbmGL specifically catalyze the biosynthesis of NDP-L-gulose and NDP-6′-deoxy-gulose, respectively, differentiating the disaccharide moieties between BLM and ZBM (Figures 1 and 2a). Evaluation of DNA cleavage activities of the 6′-deoxy-BLM analogs suggested the role of the disaccharide moiety in fine-tuning BLM activities.[17,24] These findings inspired us to alter the disaccharide moiety of TLM H-1 in the current study.

Here we report (i) heterologous expression of the *zbmGL* genes in the *S. hindustanus* wild type (WT) and Δ*tlmH* mutant strains, (ii) isolation and structure elucidation of a new BLM analog, named 6′-deoxy-TLM H-1, consisting of the 22-desmethyl-BLM aglycone, the TLM A C-terminal amine, and the ZBM disaccharide, and (iii) evaluation of the DNA cleavage activities of 6′-deoxy-TLM H-1, in comparison with TLM H-1 and BLM A2, revealing new insight into the SAR of BLM family of anticancer drugs.

RESULTS AND DISCUSSION

Heterologous expression of *zbmGL* in *S. hindustanus* WT and mutant strains

We previously constructed the *S. hindustanus* Δ*tlmH* mutant strain SB8005, which produced two new TLM analogs TLM H-1 and TLM H-2; TLM H-1 displayed DNA cleavage activity similar to its parent compound TLM A.[20] To alter the disaccharide moiety of TLMs to the ZBM disaccharide, we first cloned the *zbmGL* genes, under control of the *ErmE** promoter, into the integrative plasmid pSET152[25] to construct the expression plasmid pBS9081. Introduction of pBS9081 into either the *S. hindustanus* WT[15] or SB8005 (Δ*tlmH* mutant)[20] strain afforded the recombinant strains SB8006 (WT/pBS9081) or SB8007 (SB8005/pBS9081), respectively, both of which were fermented under conditions described previously for TLM production,[15,19,20] using the *S. hindustanus* WT and SB8005 strains as controls.

The *S. hindustanus* WT strain produced TLM A and TLM B with TLM A as the predominant metabolite[15] (Figure 2b, panel I). HPLC analysis of the SB8006 fermentation revealed two new metabolites, in addition to TLM A and TLM B, with distinct retention times in comparison to TLM A and TLM B (Figure 2b, panel II). Upon HPLC-HR-ESI-MS analysis, one metabolite (Figure 2b, panel II, o) afforded a $[M+Cu]^{2+}$ ion at *m/z* 888.8344 (Supplementary Figure S3), indicative of an MW of 1777.6, which differs from TLM A by mass of 16. The other metabolite (Figure 2b, panel II, ∇) afforded a $[M+Cu]^{2+}$ ion at *m/z* 824.7860 (Supplementary Figure S3), indicative of an MW of 1649.6, which differs from TLM B by mass of 16. The MS analysis of the new metabolites supported the assignment of the new metabolites as 6′-deoxy-TLM A and 6′-deoxy-TLM B (Figure 2b, panel II and Figure 1b), respectively. SB8006 produced TLMs and the deoxy-TLM analogs in approximately a 1:1 ratio, with a combined titer for all TLMs similar to that by the WT strain on the basis of HPLC analysis.

The *S. hindustanus* SB8005 strain produced TLM H-1 and TLM H-2 with TLM H-1 as the predominant metabolite (Figure 2b, panel III). HPLC analysis of the SB8007 fermentation revealed one dominant new metabolite, whose retention time overlapped with TLM H-2 (Figure 2b, panel IV, ◊), and although TLM H-1 could still be detected, it was produced at a much lower titer than that of SB8005. Upon HPLC-HR-ESI-MS analysis, this new metabolite (Figure 2b, panel IV, ◊) afforded a molecular ion at *m/z* 800.3029 (Supplementary Figure S3), indicative of an MW of 1600.6. The new metabolite with 16 mass unit less than TLM H-1 supported the assignment of the new metabolite as 6′-deoxy-TLM H-1. While 6′-deoxy-TLM H-2 was not detected by HPLC analysis with UV detection presumably due to its extremely low titer, its $[M+Cu]^{2+}$ ion at *m/z* 736.2548 was apparent upon HPLC-HR-ESI-MS analysis (Figure 2b, panel IV, □ and Supplementary Figure S3). SB8007 produced 6′-deoxy-TLM H-1 in a titer similar to that of TLM H-1 by SB8005 on the basis of HPLC analysis.

Isolation and structure elucidation of 6′-deoxy-TLM H-1

We scaled up the fermentation of SB8007 to produce and isolate sufficient quantities of 6′-deoxy-TLM H-1 for structural elucidation and evaluation of its DNA cleavage activities. Thus, the supernatant (4 l) obtained from 5 l of SB8007 cultures was collected and adjusted to pH 7.0 using 1.0 N HCl. After sequential column chromatography on Amberlite FPC3500(H), Diaion HP-20 resins and Sephadex LH-20, followed by semi-preparative HPLC on a C-18 column, the new 6′-deoxy-TLM H-1 metabolite was purified as a blue Cu^{2+} complex (15.2 mg). Further treatment of the 6′-deoxy-TLM H-1•Cu^{2+} complex (10.0 mg) with EDTA to remove Cu^{2+}, followed by HPLC on a C-18 column, finally afforded Cu^{2+}-free 6′-deoxy-TLM H-1 as a white powder (3.8 mg). The structural elucidation was carried out by a combination of HR-ESI-MS and 1D and 2D NMR spectroscopic analyses, as well as by comparison of the resultant data with TLM H-1,[20] 6′-deoxy-BLM A2,[17] 6′-deoxy-BLM Z[24] and ZBM.[9]

HR-ESI-MS analysis, in positive mode, of Cu^{2+}-free 6′-deoxy-TLM H-1 afforded an $[M+H]^+$ ion at *m/z* 1538.6832 (Supplementary Figure S6), consistent with the predicted molecular formula of $C_{62}H_{99}N_{21}O_{21}S_2$ (calculated for $[M+H]^+$ ion at *m/z* 1538.6839), and suggesting that it contained the ZBM disaccharide 2-*O*-(3-*O*-carbamoyl-α-D-mannosyl)-6-deoxy-L-gulose.[9,17,24] The structure of 6′-deoxy-TLM H-1 (Figure 1b) was finally established on the basis of comprehensive 1D and 2D NMR analysis (Supplementary Figures S4 and S7–S11). The ^1H and ^{13}C NMR spectra of 6′-deoxy-TLM H-1 are very similar to those of TLM H-1 except for the disaccharide moiety (Table 1).[20] The signals for the hydroxymethylene group (δ_C 62.8, C-6′; δ_H 3.40, mb; 3.51 mb) disappeared in 6′-deoxy-TLM H-1 and were replaced by methyl group signals (δ_C 14.4, C-6′; δ_H 0.88 (d, $J = 6.3$ Hz)) (Table 1 and Supplementary Figures S7 and S8). The signals for the disaccharide moiety of 6′-deoxy-TLM H-1 are almost identical to those of ZBM,[9] 6′-deoxy-BLM A2[17] and 6′-deoxy-BLM Z.[24] These analyses also enabled the full ^1H and ^{13}C NMR spectroscopic assignments of 6′-deoxy-TLM H-1 as summarized in Table 1.

Evaluating the DNA cleavage activity of 6′-deoxy-TLM H-1

DNA cleavage efficiency of 6′-deoxy-TLM H-1 was first determined by using the plasmid relaxation assay.[17,19,23,24] 6′-Deoxy-TLM H-1 was compared with TLM H-1 and BLM A2 for their ability to cleave pBluescript II SK(+) supercoiled plasmid DNA in the presence of Fe^{2+} as described previously. BLMs are known to mediate single- and double-strand cleavages, resulting in the conversion of supercoiled plasmid DNA (form I) to open circular (form II) or linearized

Table 1 ^{13}C (175 MHz) and ^{1}H (700 MHz) NMR data for 6′-deoxy-TLM H-1 in D_2O[a]

No	δ_C *(in p.p.m.)*	δ_H *(J in Hz)*	*No*	δ_C *(in p.p.m.)*	δ_H *(J in Hz)*
1	170.7, s		32	146.9, s	
2	52.2, d	4.09, m[b]	33	162.8, s	
3	46.7, t	2.97, 2H, m[b]	34	124.9, d	8.23, s
4	176.0, s		35	149.0, s	
5a	40.0, t	2.62, m[b]	36	163.2, s	
5b		2.45, m[b]			
6	59.6, d	3.95, dd (8.4, 5.6)	37	38.8, t	3.48, 2H, m
7	165.4, s		38	24.6, t	1.78, 2H, m[b]
8	164.2, s		39	29.4, t	1.79, 2H, m[b]
9	111.0, s		40	48.6, d	3.70, m[b]
10	153.4, s		41	36.8, t	2.74, dd (15.4, 4.9)
					2.66, dd (15.4, 7.7)
11	167.6, s		42	172.1, s	
12	10.5, q	1.83, s	43	36.2, t	3.27, 2H, m
13	169.1, s		44	25.4, t	1.90, 2H, m
14	56.2, d	5.12, d (8.4)	45	45.1, t	3.05, 2H, m[b]
15	73.3, d	5.24, d (8.4)	46	46.9, t	3.08, 2H, m[b]
16	133.1, s		47	22.8, t	1.78, 2H, m[b]
17	118.3, d	7.46, s	48	23.9, t	1.78, 2H, m[b]
18	136.1, d	8.27, s	49	38.8, t	3.06, 2H, m[b]
19	14.3, q	1.22, d (7.0)	1′	99.0, d	5.24, d (2.8)
20	49.7, d	4.05, m	2′	70.5, d	3.97, m[b]
21	70.7, d	4.01, m[b]	3′	68.2, d	4.16, m[b]
22	39.6, t	2.61. m[b]	4′	71.5, d	3.70, m[b]
		2.48, m[b]			
23	174.0, s		5′	62.9, d	4.11, m[b]
24	172.1, s		6′	14.4, q	0.88, d (6.3)
25	59.2, d	4.26, d (4.2)	1″	98.8, d	5.05, d (0.7)
26	66.9, d	4.17, m	2″	68.3, d	4.15, m[b]
27	18.8, q	1.14, d (6.3)	3″	74.3, d	4.83, dd (9.8, 3.5)
28	39.0, t	3.72, 2H, m	4″	64.9, d	3.84, t (9.8)
29	31.9, t	3.29, 2H, m	5″	73.3, d	3.88, m
30	170.8, s		6″a	61.0, t	3.99, m[b]
			6″b		3.83, dd (11.9, 6.3)
31	119.1, d	8.08, s	7″	158.1, s	

[a]Assignments are based on 1D and 2D NMR experiments.
[b]Signals are overlapped.

(form III) plasmid DNA. At a concentration of 1.0 μM, 6′-deoxy-TLM H-1 was shown to nearly complete the cleavage of supercoiled DNA (into open circular and/or linear forms), whereas BLM A2 cleaved the plasmid DNA completely (Figure 2c). As the observed DNA cleavage activity was concentration dependent, the effective concentrations at 50% DNA cleavage (EC_{50}) were further evaluated (Supplementary Figure S5). The EC_{50} values were estimated to be 0.74 μM for 6′-deoxy-TLM H-1 and 1.14 μM for TLM H-1, in comparison with 0.47 μM for BLM A2, revealing that the modification on disaccharide of TLM H-1 improved on DNA cleavage activities.[17,24]

DNA cleavage kinetics of 6′-deoxy-TLM H-1 was next followed by using the break light assay.[26,27] In the presence of Fe^{2+}, BLM-mediated single-site cleavage of the molecular beacon could be followed by time-dependent fluorescence resonance energy transfer. In comparison with TLM H-1 and BLM A2, 6′-deoxy-TLM H-1 was shown to have a more rapid cleavage of the molecular beacon (Figure 2d). The anticancer activities of the BLM family of metabolites have been correlated with their *in vitro* DNA cleavage activities.[1,3–6] Evaluation of the *in vitro* DNA cleavage activity of 6′-deoxy-TLM H-1, compared with TLM H-1 and BLM A2 as controls, showed that a modification of the disaccharide moiety of TLM H-1 improved the DNA cleavage activity (Figures 2c and d, and Supplementary Figure S5), a finding that is consistent with the other 6′-deoxy-BLM analogs engineered previously.[17,24]

In summary, we took advantage of the genetic system developed for *S. hindustanus*[15,19,20] to investigate if novel BLM analogs could be produced by heterologous expression of the *zbmLG* genes[17,18,24] in the *S. hindustanus* WT and mutant strains. The recombinant strains SB8006 and SB8007 produced new 6′-deoxy-TLM analogs (Figures 2a and b and Supplementary Figure S3), one of which was subsequently isolated and characterized as the BLM–TLM–ZBM hybrid metabolite 6′-deoxy-TLM H-1 consisting of the 22-desmethyl-BLM aglycone, the TLM A terminal amine and the ZBM disaccharide (Figure 1b). This finding confirmed that ZbmLG can functionally constitute NDP-6-deoxy-L-gulose biosynthesis in *S. hindustanus* and that the TlmEF glycosyltransferase apparently can efficiently attach the designer sugar onto the 22-desmethyl-BLM aglycone (Figure 2a). This study has once again underscored the versatility of biosynthetic machineries of the BLM family of glycopeptide antibiotics for the generation of designer BLM analogs. Comparison of DNA cleavage efficiency and kinetics between BLMs and their engineered 6′-deoxy-BLM analogs supports the wisdom of altering the disaccharide moiety to fine-tune the activity for the BLM family of anticancer drugs (Figures 1,2c and d). The availability of 6′-deoxy-TLM H-1, which can be produced in sufficient quantities by scale-up microbial fermentation, should greatly facilitate future in depth SAR studies for the development of this promising BLM analog into a potential clinical drug.

METHODS

General experimental procedures

Optical rotations were recorded on an AUTOPOL IV automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). HPLC-MS and HR-ESI-MS was conducted on an Agilent 1260 Infinity LC coupled to a 6230 TOF equipped with an Agilent Poroshell 120 EC-C18 column (50 mm × 4.6 mm, 2.7 μm) (Agilent Technologies, Santa Clara, CA, USA). NMR data were acquired using a Bruker Avance III Ultrashield 700 MHz spectrometer (Bruker, Billerica, MA, USA) at 700 MHz for ^{1}H NMR and 175 MHz for ^{13}C NMR, and the chemical shifts were given in δ (p.p.m.), and coupling constants (J) were reported in Hz. Semi-preparative HPLC was conducted on a Varian HPLC system (Varian, Inc., Woburn, MA, USA) with a Prostar 330 detector using an Alltima C18 column (250 × 10.0 mm, 5 μm) (Alltech Associates, Inc., Deerfield, IN, USA). Commercial kits (Omega Bio-Tek, Norcross, GA, USA) were used for gel extraction and plasmid preparation. All restriction endonucleases, Q5 high fidelity/OneTaq DNA polymerase and T4 DNA ligase were purchased from NEB (Ipswich, MA, USA), and the reactions were performed according to the manufacturer's procedures. DNA amplification was conducted using a thermocycler (Bio-Rad, Hercules, CA, USA). DNA concentration was measured using a NanoDrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Other common biochemicals and culture medium components were purchased from standard commercial sources.

Genetic manipulation of *S. hindustanus*

S. hindustanus WT[15] and SB8005 (Δ*tlmH* mutant)[20] strains were grown at 28 °C on ISP-4 medium supplemented with 28 mM $MgCl_2$, or cultured in liquid tryptic soy broth. *Escherichia coli* DH5α and ET12567/pUZ8002 were grown at 37 °C in lysogeny broth medium for general subcloning procedures and preparation of non-methylated plasmid DNA, respectively.[28,29] To construct the *zbmGL* expression plasmid pBS9081, the *zbmG* gene was PCR-amplified using *S. flavoviridis* SB9001 genomic DNA as a template and oligonucleotides zbmG-S2shSbfI/zbmG-ASEcoRI (Supplementary Table S2). The resultant *zbmG* DNA fragment was cloned into a *zbmL* and *ErmE** promoter-containing plasmid pBS9018,[17] at the *Sbf*I and *Eco*RI sites, to give pBS9079. An *Spe*I fragment harboring *zbmGL* under *ErmE** was then transferred into the *Xba*I site of pSET152[25] to yield an integrative vector pBS9080. To allow the selection of

transformants in *S. hindustanus*, which is resistant to apramycin but sensitive to thiostrepton, the apramycin-resistance gene of pSET152 backbone was replaced with a tandem ampicillin–thiostrepton resistance cassette.[29] The cassette was amplified using pBS9010[18] as a template and oligonucleotides replapra-S/replapra-AS (Supplementary Table S2) and used in the λRED-mediated PCR-targeting mutagenesis method to give pBS9081.[30] After passaging pBS9081 to the non-methylating *E. coli* ET12567/pUZ8002, this plasmid was introduced into *S. hindustanus* WT and SB8005 by electroporation.[15] Transformants SB8006 or SB8007 were selected by their thiostrepton-resistant phenotype and subsequently verified by PCR to harbor the *zbmGL* genes.

Fermentation, isolation and analysis

For the production of TLMs, *S. hindustanus* strains were cultured in 250-ml baffled flasks containing 50 ml of the seed medium composed of 1.5% glucose, 0.2% yeast extract, 0.5% peptone, 0.05% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.5% $CaCO_3$, adjusted to pH 7.2 with 1.0 N HCl. After growth at 28 °C and 250 r.p.m. for 2 days, 5 ml of the seed cultures were inoculated into 250-ml baffled flasks with 50 ml of production medium containing 2.5% sucrose, 0.5% glucose, 3% cotton-seed meal, 3% distiller's grains and solubles, 0.3% $(NH_4)_2SO_4$, 0.01% $CuSO_4{\cdot}5H_2O$. The resultant cultures were fermented at 28 °C and 250 r.p.m. for 7 days. The fermentation broths (50 ml) of *S. hindustanus* WT or recombinant strains were prepared and processed using the following steps. The culture was centrifuged, and the supernatant was adjusted to pH 7.0 with 1.0 N HCl, mixed with 5 g of Amberlite FPC3500(H) resin, and incubated at room temperature under gentle agitation for 30 min. After washing the resin with 50 ml of water, the resin was eluted with 25 ml of 20% NH_4OAc. The resulting eluate was mixed with 3 g of Diaion HP-20 resin and incubated at room temperature under gentle agitation for about 30 min. The Diaion HP-20 resin was then packed into a column, washed with 10 bed volumes of water and drained of excess water. The column was then eluted with five bed volumes of methanol, and the methanol eluate was combined and concentrated in vacuo to give the crude extract. The crude extracts were dissolved in 1.5 ml of methanol and subjected to HPLC-MS analyses for the production of TLMs and analogs. HPLC-MS was performed using a 15 min solvent gradient, from 100% solvent A (water with 0.1% formic acid) and 0% solvent B (100% methanol with 0.1% formic acid) to 30% A and 70% B; and a 5 min gradient, from 30% A and 70% B to 0% A and 100% B at a flow rate of 0.4 ml min^{-1} and with UV detection at 300 nm.

Large-scale fermentation and isolation

For large-scale fermentation, 50 ml of the seed cultures were inoculated into 2-l flasks containing 500 ml of the production medium, and incubated at 28 °C and 250 r.p.m. for 7 days. To isolate 6′-deoxy-TLM H-1, the SB8007 culture (5 l in total) was harvested. The supernatant (4 l) was collected, adjusted to pH 7.0 with 5.0 N HCl, mixed with 0.5 kg Amberlite FPC3500(H) resin, and incubated at room temperature under gentle agitation for 45 min. After washing the resin with 10 bed volumes of water, the resin was eluted with 2 l of 20% NH_4OAc. The resulting eluate was mixed with 100 g Diaion HP-20 resin and incubated at room temperature under gentle agitation for 45 min. The Diaion HP-20 resin was then packed into a column, washed with 10 bed volumes of water and drained of excess water. The column was then eluted with methanol, and the methanol eluates were combined and concentrated *in vacuo* to 3 ml. The crude extract was loaded onto a Sephadex LH-20 column and eluted with methanol to yield 15 fractions. Fractions containing 6′-deoxy-TLM H-1 were combined and evaporated to dryness. Final purification of 6′-deoxy-TLM H-1 was achieved by semi-preparative HPLC using a 20 min solvent gradient, from 100% solvent A (water with 0.1% HOAc) and 0% solvent B (methanol with 0.1% HOAc) to 40% solvent A and 60% solvent B, at a flow rate of 3.6 ml min^{-1} and with UV detection at 300 nm. After methanol evaporation and lyophilization, pure 6′-deoxy-TLM H-1 was obtained as a blue 6′-deoxy-TLM H-1•Cu^{2+} complex (15.2 mg). Cu^{2+}-free 6′-deoxy-TLM H-1 was obtained by treating the 6′-deoxy-TLM H-1•Cu^{2+} complex (10.0 mg) with 0.5 M EDTA-Na (pH 7.3). After the final HPLC purification, Cu^{2+}-free 6′-deoxy-TLM H-1 (3.8 mg) was obtained as a white powder.

6′-Deoxy-TLM H-1. White power; $[\alpha]^{D}_{23}$ +9.2 (c 0.002, H_2O); HR-ESI-MS for the $[M+H]^+$ ion at *m/z* 1538.6832 (calculated $[M+H]^+$ ion for $C_{62}H_{99}N_{21}O_{21}S_2$ at 1538.6839); ^1H and ^{13}C NMR data (in D_2O), see Table 1.

DNA cleavage assay

The DNA cleavage assays were performed using previously reported protocols.[17,19,23,24,26,27] Cu^{2+}-free BLM A2 was purified from BLM sulfate purchased from Cayman Chemical (Ann Arbor, MI, USA). Cu^{2+}-free TLM H-1 was isolated from *S. hindustanus* SB8005 fermentation.[20] In the plasmid relaxation assay, the pBluescript SK (+) plasmid DNA (75 ng), 10 μM Fe $(NH_4)_2(SO_4)_2$, 0.2 to 2 μM Cu^{2+}-free BLM A2/TLMs and 25 mM Tris-HCl pH 7.5 were mixed to give a reaction volume of 10 μl.[17,23,24] The reactions were incubated at 37 °C for 30 min and stopped by the addition of 6× loading dye containing 5 mM EDTA (pH 8.0). The resulting products were separated by electrophoresis on 1% agarose gels containing GelRed dye (0.5× strength), and gel electrophoresis was carried out in Tris-Acetate-EDTA buffer (Amresco, Solon, OH, USA) at 90 V for 1 h. To compare the DNA cleavage activities, the intensity of DNA bands (form I) were measured using the 'Analyze-Measure' menu in the ImageJ 1.49v software package.[31] Logarithmic values of TLMs/BLM concentrations and the ratio of cleaved supercoiled DNA were plotted using the Dr Fit software (sourceforge.net/projects/drfit/) to generate dose–response curves to estimate EC_{50} values.[32] In the break light assay (in 20 μl reactions), the assay mixtures contained 0.5 μM of the GC-BLM1 DNA beacon (5′-[6FAM]CGCTTTAAAAAAAGCG[BHQ1]-3′) and 0.2 μM Cu^{2+}-free BLM A2, TLM H-1, or 6′-deoxy-TLM H-1 in 10 mM Tris-HCl buffer (pH 7.5).[17,19,26,27] The assay reaction was initiated by addition of $(NH4)_2Fe(SO_4)_2$ (freshly prepared in water) to 10 μM final concentration. Fluorescence levels were measured every 20 s at 25 °C for 10 min, using a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA) with excitation and emission wavelengths of 485 and 520 nm, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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