Combinatorial Biosynthesis

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Construction of a New Class of Tetracycline Lead Structures with Potent Antibacterial Activity through Biosynthetic Engineering

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Abstract: *Antimicrobial resistance and the shortage of novel antibiotics have led to an urgent need for new antibacterial drug leads. Several existing natural product scaffolds (including chelocardins) have not been developed because their suboptimal pharmacological properties could not be addressed at the time. It is demonstrated here that reviving such compounds through the application of biosynthetic engineering can deliver novel drug candidates. Through a rational approach, the carboxamido moiety of tetracyclines (an important structural feature for their bioactivity) was introduced into the chelocardins, which are atypical tetracyclines with an unknown mode of action. A broad-spectrum antibiotic lead was generated with significantly improved activity, including against all Gram-negative pathogens of the ESKAPE panel. Since the lead structure is also amenable to further chemical modification, it is a platform for further development through medicinal chemistry and genetic engineering.*

The evolution of multidrug-resistant pathogens presents one of the greatest challenges to modern health care. There is a critical shortage of effective antibiotics, particularly against Gram-negative pathogens belonging to the ESKAPE group (see Table 1), including *Pseudomonas aeruginosa*, which is an important pathogen that causes hospital-acquired infections.[1] Consequently, new antibiotics from novel antibiotic classes that bypass current resistance mechanisms, and preferably with a new mode of action,[2] need to be translated rapidly into the clinic. Approaches such as screening natural product libraries and developing chemically synthesised antibiotics based on novel targets have shown that the identification of promising anti-infective lead structures is a rare event.[3]

Based on more than 70 years of antibacterial drug development, an attractive alternative is to reassess unexploited and underutilised structural scaffolds of proven antibacterial potency and resistance-breaking properties, and to develop these further by using cutting-edge synthetic biology.

Antibiotics such as tetracyclines (TCs), which were formerly highly-effective against both Gram-positive and Gram-negative pathogens, are now ineffective owing to widespread antibiotic resistance.[4] TCs are bacteriostatic; the first major class of therapeutics to be termed broad-spectrum antibiotic.[5] Clinically important TCs, such as doxycycline, minocycline, and glycylcyclines (a new class of TCs), are termed typical TCs. Their mode of action involves binding to the ribosome during polypeptide elongation to inhibit translation.[6] Chelocardin (CHD, **3**; Figure 1b), which is produced by *Amycolatopsis sulphurea*,[7] is regarded as a structurally atypical TC and shows bactericidal activity.[8] Initial data on the mode of action of CHD exist, but the exact mechanism of action has yet to be elucidated.[8,9] CHD is effective against many multidrug-resistant pathogens, including some difficult-to-treat Gram-negative bacteria. Importantly, it is also effective against TC-resistant strains, except for *Pseudomonas aeruginosa* (Table 1).[10] No toxicity from

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Table 1: CHD and CDCHD activities against clinical isolates.

Species	N	Drug	Number of isolates inhibited by the indicated concentration [μg mL^{-1}]											MIC_{50}[a]	MIC_{90}[a]
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	>64		
E. faecium	10	CHD					2	5	3					4	8
		CDCHD					4	6						2	2
S. aureus MR	20	CHD			1	3	5	10	1					4	4
		CDCHD			1	1	10	8						2	4
K. pneumoniae	25	CHD			2	5	8	4	3	3				2	16
		CDCHD	1	2	4	7	7	4						1	4
A. baumannii	20	CHD					2	5	8	4	1			8	16
		CDCHD						2	12	5	1			8	16
P. aeruginosa	15	CHD									1	8	6	64	>64
		CDCHD							8	5	2			8	32
E. cloacae	20	CHD				1	12	5	2					2	4
		CDCHD			2	11	7							1	2
E. coli	15	CHD			3	4	4	2	1	1				2	8
		CDCHD		2	6	3	3	1						0.5	2
All organisms	125	CHD			**6**	**13**	**33**	**31**	**18**	**8**	**2**	**8**	**6**	**4**	**16**
		CDCHD	**1**	**4**	**13**	**22**	**31**	**21**	**20**	**10**	**3**			**2**	**8**

[a] MIC = minimal inhibitory concentration.

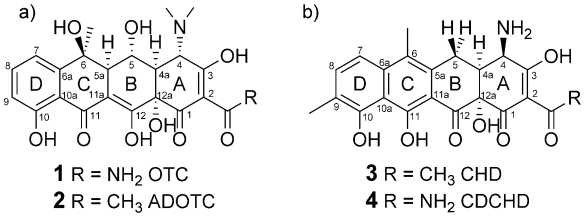

Figure 1. Structures of oxytetracycline (OTC, **1**), 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC, **2**), chelocardin (CHD, **3**), and 2-carboxamido-2-deacetyl-chelocardin (CDCHD, **4**).

CHD was observed following oral administration in rats or dogs.[11] In a small Phase II clinical study in the late 1970s, twelve patients receiving CHD orally were cured of urinary-tract infections (pyelonephritis).[11] Only one minor gastrointestinal adverse event was reported, while three patients were cleared of tetracycline-resistant infections, thus showing the potential of CHD to combat difficult infections. Nevertheless, CHD was not developed further as an antibiotic.

We re-examined CHD activity with a panel of multidrug-resistant clinical isolates (Table 1) and the results confirm its reported potency.[10] Although CHD has the typical four-ring TC backbone, it also has several unique structural features that are believed to contribute to a mode of action most likely different to that of other TCs. Compared to clinically used TCs, these features include: an alternative aromatization pattern at rings D and C; functionalization of CHD at C9; and opposite sterochemistry of the non-methylated amino group at C4 (Figure 1). Furthermore, whereas typical TCs bind to the ribosome in a cleft into which TCs fit owing to a kink between rings A and B,[12] CHD is a more planar molecule and consequently may not bind to the ribosome. No cross-resistance is known. One important feature of all medically-important TCs is the carboxamido moiety at C2 (**1**, Figure 1). In contrast, CHD carries an acetyl group at C2 (**3**, Figure 1). Biosynthetically, this difference is due to priming by acetate instead of malonamate during initiation (Figure 2b).[13] Although the exact mechanism of initiation in oxytetracycline (OTC) biosynthesis is still not well understood, it is carried out by a minimal polyketide synthase (PKS) OxyABC, consisting of two ketosynthase units KSα and KSβ, and an acyl-carrier protein (ACP), an amidotransferase (AMT) OxyD, which catalyzes the transamination reaction of malonate to malonamate (Figure 2), and an acyltransferases (AT) homologue, OxyP.[13] The function of the AT domain in OTC biosynthesis is not yet clearly defined. Interestingly, inactivation of *oxyP* did not abolish the biosynthesis of OTC but did increase the proportion of 2-acetyl-2-decarboxamido-oxytetracycline (**2**, ADOTC) compared to OTC (**1**; Figure 1).[13] Moreover, the carboxamido moiety in OTC is crucial for the antibacterial activity of typical TCs.[14] Recently, we reported the cloning of the gene cluster for CHD biosynthesis, which enabled the production of CHD analogues by using biosynthetic engineering.[15] Since CHD is exclusively acetate-primed (Figure 2b), the CHD biosynthetic cluster, as expected, contained no AT or AMT homologue.[15] We thus attempted to prime CHD biosynthesis with the carboxamido moiety. Aiming to produce an amidated analogue of CHD, specifically 2-carboxamido-2-deacetyl-chelocardin (CDCHD, **4**; Figure 1b), *oxyD* and *oxyP* from the *S. rimosus otc* gene cluster[16] were introduced into *A. sulphurea* individually and in combination by using three integrative plasmids: pAB03oxyD, pAB03oxyP and pAB03oxyDP (Figure S1 in the Supporting Information). The resulting *A. sulphurea* transformants were analyzed for production of CHD and CDCHD by HPLC (Figure S2a) and LC–MS (Figure S3). Remarkably, in the genetically engineered PKS system of *chd* in *A. sulphurea*, efficient malonamate priming is indeed possible but, interestingly, it is dependent on both *oxyP* and *oxyD*. This is in contrast to OTC biosynthesis in *S. rimosus*, where *oxyP* is practically dispensable. Biosynthesis of the malonamate-primed CDCHD was efficient, reaching a yield of around 80% of the total CHD produced by this strain (Figure S2). The structure of CDCHD, particularly the

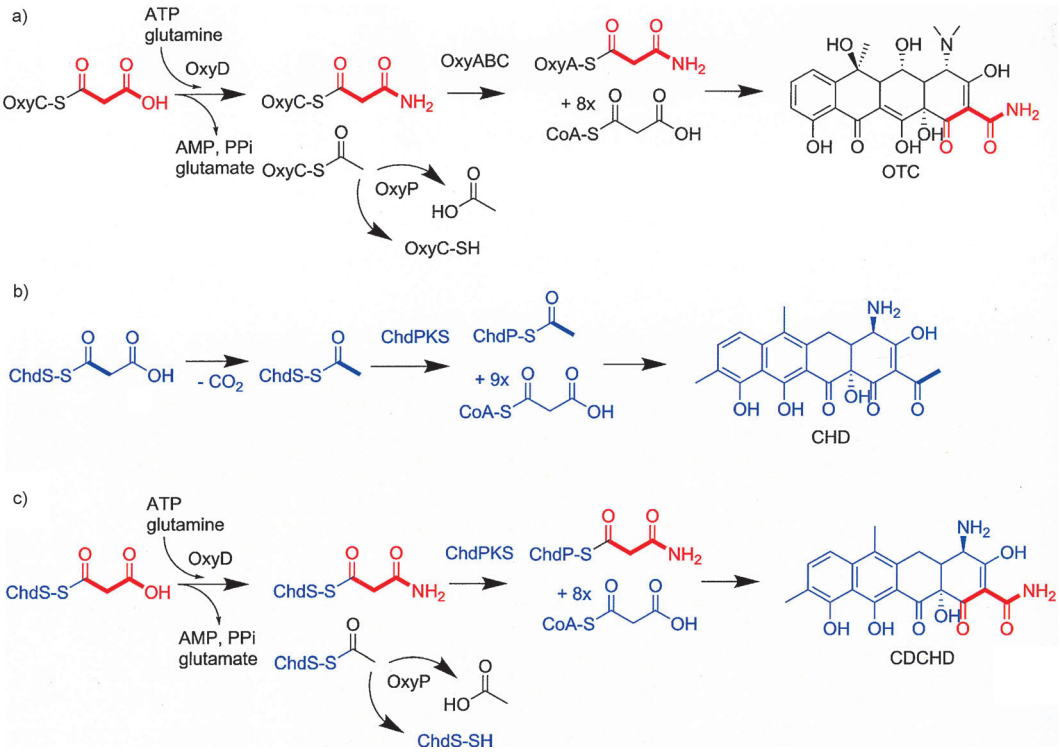

Figure 2. Priming steps in OTC biosynthesis (a)[13] and CHD biosynthesis (b),[15] and proposed malonamate priming in CDCHD biosythesis (c). The starter unit in OTC biosynthesis is indicated in red, the CHD structure in blue, and OTC in black.

presence of the carboxamido moiety, was confirmed by LC–MS, HRMS, and 2D NMR spectroscopy (Figure 3 for the most important NMR correlations, also see the Supporting information). Incomplete conversion of CHD into CDCHD could be due to insufficient supply/processing of the putative

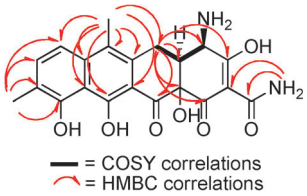

Figure 3. Significant HMBC and COSY correlations of CDCHD.

starter unit, malonamyl-CoA, or competition by acetate starter units through direct transfer to the KSα. Engineered enzymes often produce the desired product at significantly lower yield, thus seriously hampering downstream development.[17] Remarkably, however, the highest yield of CDCHD in the recombinant strain containing pAB03oxyDP was around 400 mg L^{-1} (Figure S2b), compared to a yield of 900 mg L^{-1} CHD achieved when using the wild-type *A. sulphurea* strain containing an empty plasmid.

Next, we isolated CDCHD and undertook comparative in vitro testing of CHD and CDCHD against a collection of well-characterised multidrug-resistant clinical isolates (Table 1 and the Supporting Information). Remarkably, the in vitro activity of CDCHD against these isolates was usually 2–4 times higher than that of CHD, thus confirming the importance of the 2-carboxamido moiety for activity. For multidrug-resistant enterobacteria, methicillin-resistant *Staphylocccus aureus*, and glycopeptide-resistant *Enterococcus faecium*, CDCHD showed in vitro activity superior to that of CHD, with corresponding MIC_{90} and MIC_{50} values differing 2- to 4-fold. CDCHD showed a remarkable improvement compared to CHD against *Klebsiella pneumoniae*, which was inhibited at 16 μg mL^{-1} or less by CHD and gave MIC_{90} and MIC_{50} values for CDCHD below 4 μg mL^{-1}. CHD was only slightly less active compared to CDCHD against *A. baumannii* and almost inactive against *P. aeruginosa* (for which the MIC_{90} value was >64 μg mL^{-1}; Table 1). Importantly, CDCHD showed much higher in vitro activity against *P. aeruginosa* compared to CHD: 13 out of 15 multidrug-resistant isolates were inhibited at 16 μg mL^{-1} or lower. However, the MIC_{50} (8 μg mL^{-1}) and MIC_{90} (32 μg mL^{-1}) values for CDCHD against *P. aeruginosa* were still 8- to 16-times higher than with *S. aureus* or *E. faecium* (2–4 μg mL^{-1}). Relatively high MIC values against *A. baumannii* and *P. aeruginosa* and some multidrug-resistant *K. pneumoniae* species may indicate the involvement of intrinsic resistance mechanisms (e.g., efflux pumps). Additional studies to define the mechanisms of resistance to CHD and CDCHD are currently ongoing in our laboratories in order to understand the bacterial strategies used to escape their effects.

Although the enzymatic mechanisms leading to carboxamido moiety formation in TCs is of medicinal interest,[16,18] our study is, to the best of our knowledge, the first successful heterologous expression of the AT and AMT from the *otc* cluster to derive a novel malonamate-primed molecule. The addition of this carboxamido moiety significantly increases the biological activity of CHD. The acetate-primed impurity of OTC, ADOTC (**2**), also shows a tenfold reduction in antibacterial activity.[19]Our findings for CHD are thus in agreement with the structure–activity relationships (SAR) found in typical TC antibiotics.[14]

Our current understanding of the priming reactions in type II PKSs, which mostly involve minimal PKS ketosynthase complexes[13,20] and an additional auxiliary acyltransferase (AT), is still limited, particularly when considering applying combinatorial approaches to generate potentially useful compounds. Reconstitution of the OTC minimal PKS in a heterologous host resulted in the biosynthesis of a truncated polyketide initiated with acetate. The addition of *oxyD* alone to this artificial system was sufficient to produce the amidated derivative as the major product,[16] suggesting that rather than being an acyltransferase, OxyP is a thiolase that suppresses priming by acetate by removing the competing acetyl units, as similarly observed in other type II PKS systems.[21] However, in contrast to the priming steps in OTC biosynthesis,[13] where the AMT *oxyD* alone is sufficient for the efficient production of OTC, when only *oxyD* was expressed in *A. sulphurea*, amidated CDCHD was biosynthesised in very low yield (Figure S2). The involvement of OxyP thus has much greater importance in the priming of CDCHD.

Most of the semisynthetic TCs used in the clinic, or currently in clinical development, derive from 6-demethyltetracyclines (6-DMTCs).[22] New approaches for total synthesis routes were recently reported to further expand the chemical diversity of the TC scaffold.[23] Powerful biosynthetic engineering routes for the development of TC analogues have also been reported.[24] Our current report of the incorporation of the carboxamido moiety into chelocardins provides a unique diversification of the TC backbone and the resulting derivative shows potent antibacterial activity and could thus prove to be a promising drug lead.

Overall we have demonstrated efficient incorporation of the carboxamido starter moiety, which is known to be important for tetracycline activity, into CHD. A high yield of this novel compound (CDCHD) was obtained, and the process should be directly transferable to an industrial scale. Therefore, we not only demonstrated the utility of this biosynthetic engineering approach but also developed a unique tetracycline lead with potent antibacterial activity. This compound class can now be diversified further by semisynthesis or further biosynthetic engineering in the context of wider medicinal chemistry approaches to develop urgently needed novel broad-spectrum antibacterial drugs.

Keywords: antibiotics · biosynthesis · chelocardin · polyketides · tetracyclines

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