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Natural Products

Revision of the Absolute Configurations of Chelocardin and Amidochelocardin

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Dedicated to the memory of Professor Heinz G. Floss (1934–2022), a pioneer in natural products biosynthesis.

Abstract: Even with the aid of the available methods, the configurational assignment of natural products can be a challenging task that is prone to errors, and it sometimes needs to be corrected after total synthesis or single-crystal X-ray diffraction (XRD) analysis. Herein, the absolute configuration of amidochelocardin is revised using a combination of XRD, NMR spectroscopy, experimental ECD spectra, and time-dependent density-functional theory (TDDFT)-ECD calculations. As amidochelocardin was obtained via biosynthetic engineering of chelocardin, we propose the same absolute configuration for chelocardin based on the similar biosynthetic origins of the two compounds and result of TDDFT-ECD calculations. The evaluation of spectral data of two closely related analogues, 6-desmethyl-chelocardin and its semisynthetic derivative **1**, also supports this conclusion.

Chelocardin (**CHD**) is a natural antibiotic that was first isolated in the early 1960s from the actinobacterium *Amycolatopsis sulphurea* and reported to exhibit broad-spectrum antibacterial activity against Gram-positive and -negative multidrug-resistant pathogens.[1] The structure of **CHD** was elucidated a decade later using a combination of NMR, UV/Visible, and electronic circular dichroism (ECD) spectroscopy.[2] It has a tetracyclic structure closely related to that of the tetracycline antibiotics. However, it differs from those by possessing distinct functionalities such as a naphthalene ring corresponding to rings C and D of tetracyclines and a unique C-9-methyl substituent (Figure 1). **CHD** was likewise found to be effective against tetracycline-resistant strains, except for *Pseudomonas aeruginosa*, and twelve patients who orally received **CHD** in a small Phase II clinical study in the late 1970s were cured of urinary-tract infections (pyelonephritis).[3,4] In 2013, Lukežič et al. reported the **CHD** biosynthetic gene cluster (*A. sulphurea*), revealing 18 putative open reading frames including a type-II polyketide synthase.[5] Compared to typical tetracyclines (Figure 1), this cluster contained several distinct features including an additional gene for a putative two-component cyclase/aromatase that could afford the unique aromatic scaffold, a gene for a putative aminotransferase for C-4 with the opposite stereochemistry to tetracyclines, and a gene for a putative C-9 methylase.[5] Owing to its atypical structure, superior biological activity, and potential to overcome the accelerating antibiotic resistance, its mode of action and

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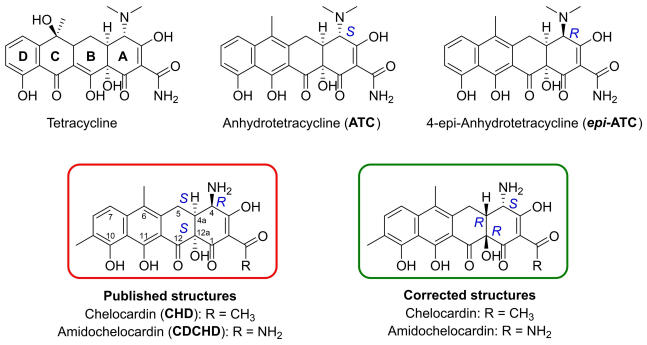


Figure 1. Structures of tetracyclines used as references, and reported and revised structures of chelocardin (**CHD**) and amidochelocardin (**CDCHD**).

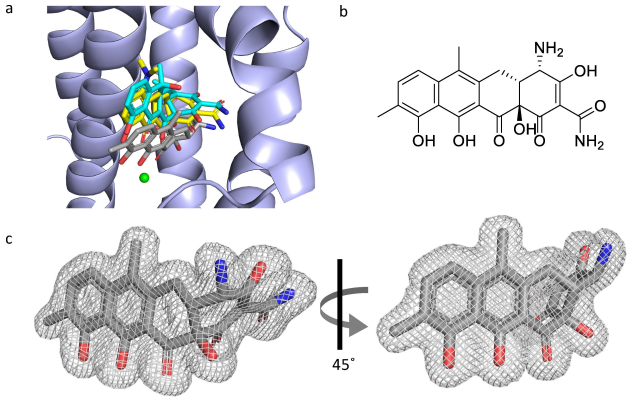


Figure 2. a) Cartoon representation of the ChdA-**CDCHD** structure (ChdA: slate; **CDCHD**: grey). TetR bound tetracycline (cyan; pdb: 2trt) and minocycline (yellow; pdb: 6rxb) were superposed to the ChdA-**CDCHD** structure. For clarity only tetracyclines are shown as stick (blue, nitrogen; and red, oxygen). b) **CDCHD** as observed in the complex crystal structure and c) Polder map of **CDCHD** contoured at 3.5 σ.

biosynthesis were extensively investigated.[6] Our prior research efforts resulted in the identification of a new lead compound, amidochelocardin (2-carboxamido-2-deacetyl-chelocardin, **CDCHD**), via biosynthetic engineering of **CHD**.[7,8] The carboxamide moiety—an important structural feature responsible for the biological activities of the tetracyclines—was introduced into **CHD** to afford **CDCHD**, with an improved broad-spectrum activity against all Gram-negative pathogens of the ESKAPE panel including *P. aeruginosa*.[7,8] Similarly, 6-desmethyl-**CHD** (**6-DM-CHD**) was generated by biosynthetic engineering (unpublished).

Considering that both **CDCHD** and **CHD** are produced by the same biosynthetic gene cluster and their similar NMR data, the relative and absolute stereochemistry of **CDCHD** were assigned to be identical to those of **CHD**.[8] However, the structure of **CDCHD** in complex with ChdA (a member of TetR-family transcriptional regulators) revealed that although the relative configuration of the stereocenters at C-4, C-4a, and C-12a was in agreement with the reported one, surprisingly the absolute configuration of protein-bound **CDCHD** was opposite to that reported in the literature. Therefore, we revisited both the relative and absolute configurations of **CDCHD** and **CHD** using a combination of experimental and calculated NMR and ECD data, as well as single-crystal X-ray diffraction (XRD) of a semisynthetic derivative of **6-DM-CHD**.

As part of our ongoing efforts to better understand the self-resistance of the native producer to **CDCHD**, we determined the crystal structure of this compound in complex with ChdA—a TetR family protein responsible for the regulation of efflux resistance protein, ChdR.[9] As expected, **CDCHD** bound to ChdA in manner similar to the tetracyclines, stabilized primarily by a number of H-bonding and hydrophobic interactions (Figures 2a and S1). The Mg^{2+} is coordinated in an octahedral fashion by the keto-enolate group of **CDCHD**, His100, and three water molecules (Figure S1b)—a feature highly conserved in all TetR-tetracycline structures.[10] Therefore, it appears that Mg^{2+} chelation is the key determinant in orienting the core cyclic scaffold of both **CDCHD** and tetracyclines in a manner that leads to a highly similar mode of binding (Figure 2a). However, unlike tetracyclines, the kink between ring A and B is such that the carboxyamide moiety is pointing in the opposite direction in **CDCHD** (Figure S1c), stabilized by both intra- and intermolecular interactions (Figure S1a). Fortuitously, the high resolution of the complex structure (1.85 Å; Table S1) along with unambiguous ligand density revealed that the absolute configuration of **CDCHD** disagreed with that proposed previously (Figures 2b-c and S1).[8,11] These findings prompted us to reinvestigate the absolute configurations of **CDCHD** and **CHD**.

The absolute configuration of **CHD** had originally been determined by comparison of its ECD data to those of 4-epianhydrotetracycline (***epi*-ATC**, Figure 1), showing similar negative signs for the 290 nm Cotton effect (CE).[2] The ECD spectra of **CHD** and ***epi*-ATC**, however, are not completely superimposable and according to our measurements, these have oppositely signed CEs above 350 nm (Figure S27). In fact, the ring-A chromophore of ***epi*-ATC** is different from that of **CHD** but rather similar to that of **CDCHD**. While H-4 and H-4a were determined to be *syn*-oriented based on their small coupling constants, the orientation of OH-12a with respect to H-4a was not determined experimentally.[2,8] Subsequent publications assumed that OH-12a in **CHD** and **CDCHD** was *syn*-oriented to H-4a based on their structural similarity. Owing to these discrepancies, we reexamined the relative and absolute configurations of these compounds via ECD analysis, semisynthetic studies, and evaluation of $^{2,3}J_{H,C}$ values.

Considering the similar chromophore functionalities of **CDCHD** and ***epi*-ATC**, as well as their identical relative configurations, the latter served as a reference to compare their ECD data. As observed in Figure 3, the CEs of these two compounds are mirror images of each other, indicating that the absolute configuration of **CDCHD** is opposite to that of ***epi*-ATC** and consistent with that determined by the XRD data. The calculated ECD spectra of (4*S*,4a*R*,12a*R*)-**CDCHD** also show good agreement to the experimental ECD spectrum at various levels of theory for the low-energy

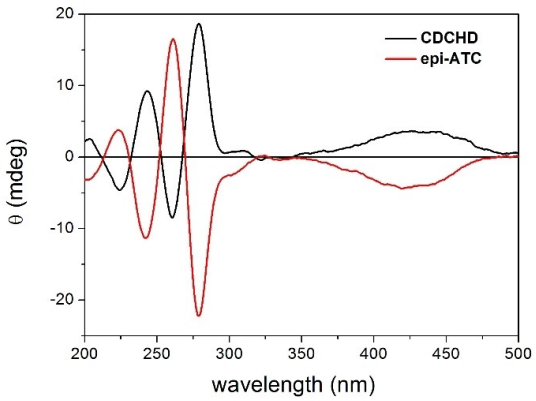


Figure 3. Experimental ECD spectra of **CDCHD** and ***epi*-ATC** in MeOH.

conformers obtained by density-functional theory (DFT) optimization at two different levels (Figures 4 and S29).

We then attempted to verify the relative configuration of **CHD**. A clear ROESY correlation between H-4 and H-4a in the NMR data of **CHD** indicated their *syn*-orientation (Figure S7). Despite the observation of a broad signal at 10.1 ppm in the ^{1}H NMR spectrum, we could not conclusively assign it to a hydroxyl proton, due to the absence of any HMBC and ROESY correlations from it. To resolve the ambiguity in the orientation of OH-12a, DFT NMR and time-dependent density-functional theory (TDDFT)-ECD calculations were performed for two diastereomers of **CHD** [(4*S*,4a*R*,12a*R*)-**CHD** and **12a-*epi*-CHD** ((4*S*,4a*R*,12a*S*)-**CHD**)] with variation in the stereochemistry at position C-12a.[12] The calculated NMR data were compared with the experimental chemical shift values obtained in DMSO-d_6, MeCN-d_3 and CD_3OD (Tables S12–19 and S21–28). In the case of DMSO-d_6, the results were evaluated against both the literature and the current data set. The calculated ^{1}H NMR data supported the (4*S**,4a*R**,12a*R**) relative configuration, while the ^{13}C NMR data gave either ambiguous results or preferred the other diastereomer. The overall DP4+ statistical analysis resulted in over 99 % sum probability for the (4*S**,4a*R**,12a*R**) configuration in DMSO-d_6 (Tables S20 and S29). In contrast to DFT NMR calculations, TDDFT-ECD calculations gave more clear-cut results clearly favoring the (4*S*,4a*R*,12a*R*) configuration. While the Boltzmann-averaged calculated ECD spectra of (4*S*,4a*R*,12a*R*)-**CHD** reproduced well the major transitions of the experimental spectrum of **CHD** at various levels of theory, those of (4*S*,4a*R*,12a*S*)-**CHD** gave a mismatch, reproducing the low-wavelength transitions while giving mirror-image agreement for the high-wavelength transition (Figures 5 and S28). Thus, the absolute configuration of **CHD** can be elucidated as (4*S*,4a*R*,12a*R*), implying that the absolute configuration of **CHD** described in literature needs revision. The H-4—H-4a distances for the lowest-energy computed ωB97XD/def2-TZVP PCM/MeOH conformers of (4*S*,4a*R*,12a*R*)-**CHD** and (4*S*,4a*R*,12a*S*)-**CHD** were 2.37 and 2.36 Å, respectively, which were in line with the observed ROESY correlation. Furthermore, some relevant $J_{H,C}$ values (HSQMBC NMR analysis) determined for **CHD** were comparable to those of **CDCHD** (Table S4; Figures S21–22), suggesting that the relative and absolute configurations of **CHD** should be analogous to those of **CDCHD**.[13]

In contrast to **CDCHD**, the C-4 position of **CHD** tends to slowly epimerize in solution.[2,14] In an attempt to minimize the possibility of epimerization of **CHD**, we synthesized an *N*-substituted derivative of **6-DM-CHD**, a novel natural product obtained via biosynthetic engineering (unpublished). Briefly, a gene knockout of *chdMI*, a putative C-6 methyltransferase from **CHD** pathway, was performed in *A. sulphurea*, resulting in the accumulation of **6-DM-CHD**. The choice of using **6-DM-CHD** lies in its improved stability during fermentation, isolation, and purification processes compared to the parent **CHD**. Reaction between **6-DM-CHD** and acryloyl chloride in the presence of *N*,*N*-diisopropylethylamine (DIPEA) in *N*-methyl-2-pyrrolidone

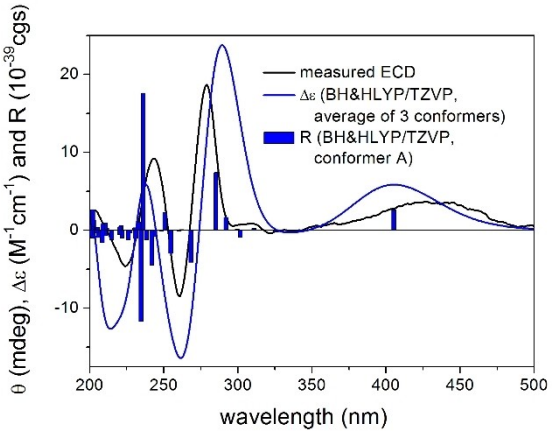


Figure 4. Experimental ECD spectrum of **CDCHD** in MeOH compared with the Boltzmann-weighted BH&HLYP/def2-TZVP PCM/MeOH ECD spectrum of the three lowest-energy ωB97XD/def2-TZVP PCM/MeOH conformers of (4*S*,4a*R*,12a*R*)-**CDCHD**. Bars represent the computed rotational strength values for the lowest-energy conformer.

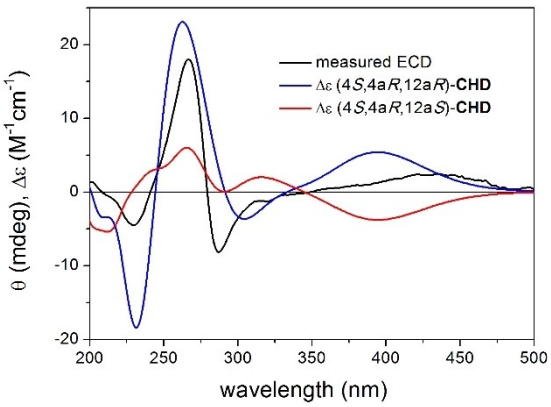


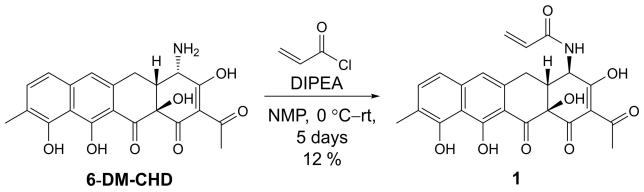
Figure 5. Experimental ECD spectrum (black) of **CHD** in MeOH compared with the Boltzmann-weighted CAM-B3LYP/def2-TZVP PCM/MeOH ECD spectra of the low-energy ωB97XD/def2-TZVP PCM/MeOH conformers of (4*S*,4a*R*,12a*R*)-**CHD** (blue) and (4*S*,4a*R*,12a*S*)-**CHD** (red).

(NMP) at 0 °C-rt for 5 days afforded compound **1** in 12 % yield (Scheme 1).

Yellow needle-shaped crystals of **1** suitable for XRD were obtained by dissolving the compound in a mixture of DME and cyclohexane (1:7), followed by slow evaporation of solvent at 25 °C over five days. The crystal structure of **1** revealed (4*R*,4a*R*,12a*R*) absolute configuration (absolute structure (Flack) parameter = 0.03(8); Figure 6). ECD spectra of (4*R*,4a*R*,12a*R*)-**1** computed at various levels of theory reproduced well all the major transitions of the experimental spectrum indicating the same absolute configuration as that obtained by XRD analysis (Figures S36–37).

The crystal structure of the semi-synthetic derivative **1** shows that both C4a and C12a have (*R*)-configuration, and thus have the same absolute stereochemistry at positions C-4a and C-12a as observed for **CDCHD** in the co-crystal structure, and not (*S*) as previously reported. Interestingly, C-4 possesses (*R*)-configuration, which is opposite to that of the corresponding position in the corrected structure of **CDCHD**. This change in relative stereochemistry is likely due to epimerization of the parent **6-DM-CHD** at this position under the reaction conditions. To verify this, we measured the ECD spectra of **6-DM-CHD** in MeOH and the solvent mixture used in the synthesis reaction. In MeOH, **6-DM-CHD** shows a gradual decrease of the Cotton effect signal intensity over 135 days, indicating a slow epimerization (Figure S33). On the other hand, we observed a swift distortion of the Cotton effect signal in an NMP/DIPEA (39:1) mixture within the first hour and a nearly complete loss of Cotton effect after five days, indicating the rapid epimerization of **6-DM-CHD** during the *N*-acylation reaction (Figure S34). Together, these data suggest that the relative and absolute configuration of **CHD** and **6-DM-CHD** are most probably the same as for **CDCHD** (4*S*,4a*R*,12a*R*), with an inverted configuration at position C4 likely due to the epimerization of **CHD** and related compounds. Notably, ECD analysis of the C4-NH_2 functionalized product (**1**) shows marked stability with no epimerization in MeOH over 24 h (Figure S35).

In conclusion, we have revised the absolute configuration of **CDCHD** to (4*S*,4a*R*,12a*R*) using a combination of NMR, XRD, ECD, and computational studies, which is opposite to that previously reported for **CDCHD** and **CHD**. The two compounds are proposed to have identical absolute configurations, considering their similar biosynthetic origins. This supposition is supported by the absolute configuration assigned via XRD analysis for a closely related semi-synthetic analogue of **6-DM-CHD** (**1**). Although the absolute configuration of C4-NH_2 in **1** (4*R*) is opposite to that in **CDCHD** (4*S*), a likely result of ready epimerization at this position, the relative and absolute configurations of the other stereocenters are identical to those of **CDCHD** (4a*R* and 12a*R*). These results underscore the importance of using a combination of appropriate techniques and reference compounds for comparison during stereochemical assignments of natural products. The corrected structures of (amido)chelocardins are important for establishing the total synthesis of the chelocardin family and the design of optimized derivatives.



Scheme 1. C-4 amide derivatization of 6-desmethyl-chelocardin (**6-DM-CHD**).

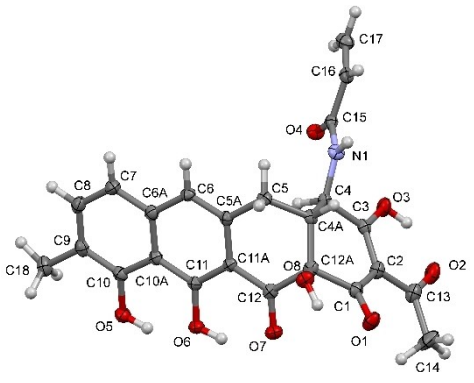


Figure 6. Molecular structure of **1** with atomic numbering scheme, showing that all three stereogenic atoms C4, C4a, and C12a have (*R*)-configuration. Displacement ellipsoids are drawn at the 50 % probability level.

Supporting Information

Instrumentation details, experimental methodology, and relevant NMR, MS, ECD, and XRD data are provided in the Supporting Information. The crystallography data that support the findings of this study are openly available in Protein Data Bank at https://www.rcsb.org/ and Cambridge Crystallographic Data Centre at https://www.ccdc.cam.ac.uk/ .[15] The authors have cited additional references within the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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