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The isolation, total synthesis and structure elucidation of chlorofusin, a natural product inhibitor of the p53-MDM2 protein-protein interaction

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Abstract

Inhibitors of key protein-protein interactions are emerging as exciting therapeutic targets for the treatment of cancer. One such interaction between MDM2 (HDM2) and p53, that silences the tumour suppression activities of p53, was found to be inhibited by the recently isolated natural product chlorofusin. Synthetic studies on this complex natural product summarized herein have served to reassign its chromophore relative stereochemistry, assign its absolute stereochemistry, and provided access to a series of key analogues and partial structures for biological evaluation.

1 Introduction

1.1 p53-MDM2

The tumor suppressor p53 is an important part of an innate protection mechanism where it acts as a transcription factor that initiates cell cycle arrest and apoptosis in response to DNA damage.^{1–4} The activity of p53 is modulated by MDM2 (HDM2), which binds p53 preventing it from acting as a regulator of cell division^{5–7} and targeting it for nuclear export and degradation.^{8,9} Overexpression of MDM2 has been implicated in many cancers,^{10–16} defining the p53-MDM2 interaction as an attractive target for therapeutic intervention.^{4,17} An X-ray crystal structure of the N-terminal domain of MDM2 bound to the 15-residue transactivation domain of p53 revealed that the formation of the complex is mediated by the interaction of three hydrophobic residues of a p53 α -helix with a hydrophobic cleft of MDM2.¹⁸ Molecules that bind this hydrophobic cleft of MDM2 disrupt the protein-protein interaction with p53, restoring the regulatory function of p53 and inhibiting tumor growth.^{4,19–22}

1.2 Isolation, initial structural assignment, and activity of chlorofusin

In 2001, chlorofusin (**1**, Fig. 1) was reported by Williams as the most abundant MDM2-p53 inhibitor isolated from the fermentation broth of a *Fusarium* species of microfungus (corrected as *Microdochium caespitosum*) in the course of an activity-guided screening program involving the examination of over 53,000 microbial extracts.²³ Chlorofusin was found to disrupt the MDM2-p53 interaction in a DELFIA-modified ELISA assay ($IC_{50} = 4.6 \mu M$), but was inactive against the TNF α :TNF α protein-protein interaction and showed no cytotoxicity against Hep G2 cells at $4 \mu M$. Through the use of surface plasmon resonance (SPR) spectroscopy, chlorofusin was later shown to directly bind the N-terminal domain of MDM2 ($K_D = 4.7 \mu M$)

and fitting of a biphasic model to the data has been interpreted as an initial fast binding event followed by a second slow step.²⁴ Thus, chlorofusin represents an exciting lead for antineoplastic intervention that acts by a rare disruption of a protein-protein²⁵ interaction although the structural details of its interaction with MDM2 have yet to be established.

On the basis of thorough spectroscopic and chemical degradation studies, the chlorofusin structure was proposed to consist of a densely functionalized, azaphilone-derived chromophore linked through the terminal amine of ornithine to a cyclic peptide composed of nine amino acid residues.²³ Two of the cyclic peptide amino acids possess a nonstandard or modified side chain, and four possess the *D*-configuration. These studies provided the cyclic peptide structure and connectivity, although the stereochemical assignment of the two asparagine residues (Asn3 and Asn4) could not be made and were simply established to have opposite stereochemistries (L and D).

Similarly, the structure and relative stereochemistry of the unusual azaphilone-derived chromophore was also proposed, although an assignment of its absolute stereochemistry was not possible (Fig. 2). The relative stereochemistry was assigned using gradient 1D NOE studies in which irradiation of the proton attached to C8 with a mixing time of 50 ms resulted in NOEs to the protons attached to C10 as well as C12, whereas a shorter mixing time of 25 ms resulted in NOEs to only the protons attached to C10. In addition, irradiation of the C4-Me protons provided a long range NOE to the proton attached to C8, but only if conducted with very extended mixing times (500 ms). These results were interpreted to indicate that the protons attached to C8, C10 and C4-Me all lie on the same face of the chromophore and led to the initial relative stereochemical assignment represented by **2** and **3** in Fig. 2.

1.3 Biosynthesis of chlorofusin

To date, a single investigation aimed at defining the biosynthesis of chlorofusin has been conducted by growing *Microdochium caespitosum* in a ¹³C-labeled acetate (CH₃¹³CO₂Na) growth medium and examining the isolated natural product using ¹³C NMR spectroscopy.²⁶ Strong ¹³C NMR signal enhancement was observed for carbons 1, 3, 5, 7, 9, 11, 15, 17, alternating carbons along the alkyl chain of the 2-aminodecanoic acid residue, and the carbonyls of Leu5, Leu7 and ADA8 (Fig. 3). The backbone and side chain carbonyls of the two asparagine residues were also enriched to a lesser degree with ¹³C. These results are consistent with the biosynthetic origin of common amino acids²⁷ and serve to establish the acetogenic origin of the 2-aminodecanoic acid residue. The alternating incorporation of ¹³C into the chromophore establishes the acetogenic origin of this structure and is in agreement with previous studies on the fungal biosynthesis of the azaphilones.²⁸

2 Cyclic peptide syntheses

2.1 Boger cyclic peptide synthesis

In 2003, the Boger group reported the synthesis of two chlorofusin cyclic peptide diastereomers bearing either the *L*-Asn3/*D*-Asn4 or *D*-Asn3/*L*-Asn4 stereochemistry and correlated the former with the spectroscopic properties (¹H and ¹³C NMR) of the natural product.^{29a} In this work, three key subunits were assembled, sequentially coupled, and cyclized to provide the 27-membered nine residue cyclic peptide (Scheme 1). The coupling and macrocyclization sites were chosen to minimize the use of protecting groups and maximize the convergency of the synthesis, and a deliberate late stage incorporation of the subunit bearing the two asparagine residues allowed convenient access to both diastereomers required to assign the absolute stereochemistry.

The data obtained from COSY, HMBC, and HMQC NMR experiments indicated that the ¹H and ¹³C NMR chemical shifts of peptide **17** closely matched those reported for the natural

product as compared to those of peptide **15**. Characteristic of this similarity in ^1H NMR chemical shifts, the nine α -CH chemical shifts of **17** exhibited ≤ 0.02 δ differences from those reported for chlorofusin (av $\Delta\delta = 0.016$), whereas those of **15** exhibited much larger differences of 0.1–0.6 δ (av $\Delta\delta = 0.34$). Analogous comparisons of the ^{13}C NMR shifts of the α -carbons provide similar distinctions (**15**, $\Delta\delta = 0.5$ –3.0, av $\Delta\delta = 1.91$ vs **17**, $\Delta\delta = 0.0$ –0.8, av $\Delta\delta = 0.47$). In addition, Williams reported a series of diagnostic long range NOEs for the cyclic peptide, including a key NOE between both β -methylene hydrogens of Asn3 and the two methines of Thr6 including the α -H ($\text{C}_2^6\text{-H}$). While not as extensively defined as those in the Williams study, the long range NOEs observed in the NOESY and ROESY NMR experiments for **15** and **17** were sufficient to indicate that peptide **17** satisfies the previously reported NOEs while **15** does not.

Removal of the Orn9 side chain SES protecting group of **15** and **17** afforded the corresponding free amines and permitted the synthesis of a series of Orn9 derivatives (e.g., NHCbz, NHFmoc, NHBoc, N-phthalimide, and NH-naphthalene acetamide). Cyclic peptides **15** and **17**, and their corresponding free amines, along with their Orn9 derivatives were assayed for inhibition of MDM2/p53 binding in an ELISA assay enlisting p53 immobilized via a biotin linker and full length MDM2, however no activity was observed ($\text{IC}_{50} > 250$ μM).

More recently, Boger and Lee^{29b} have reported an alternative and improved convergent synthesis of **17** enlisting the same three peptide subunits assembled in an alternative order with the final macrocyclization conducted at the more favorable D-ADA/L-Orn site.

2.2 Searcey cyclic peptide synthesis

A disclosure by Searcey and coworkers concurrent with that of Boger described the synthesis of the L-Asn3/D-Asn4 diastereomers of the chlorofusin cyclic peptide incorporating either a D-ADA8 (**18**) or L-ADA8 (**19**) residue and served to verify the assigned D-stereochemistry of the 2-aminodecanoic acid residue.³⁰ In this work, the linear peptides were efficiently constructed and cyclized on solid phase utilizing an L-Asp residue immobilized on a Rink amide resin (Scheme 2). Initially the ADA8 was installed as a racemic mixture and the final compounds were separated by HPLC, and a subsequent resynthesis utilizing enantiomerically pure D-Fmoc-ADA-OH provided **19** and allowed the stereochemical assignment of each product. Highlights of this strategy include concurrent side chain deprotection/product release from the resin and the requirement for only one late stage HPLC purification step.

Most of the NOEs observed for chlorofusin²³ were detected in the NOESY spectrum of **19**, including the cross peaks between Leu5 and ADA8 and between Asn3 and Thr6, although the presence of additional cross peaks suggested conformational differences from the chromophore-bearing natural product. In contrast, the NOESY spectrum of **18** had notable differences when compared with that for chlorofusin, including the absence of a long range cross peak between Asn3 and Thr6. The diastereomer **19** incorporating the D-ADA residue was found to be a close match to the data reported for chlorofusin, **18** exhibited substantial spectroscopic differences. Screening of these two compounds in an osteosarcoma cell line which contains wild type p53 and overexpresses HDM2, the human homologue of MDM2, provided no discernable evidence of p53 activation.³⁰

In an extension of their initial work and demonstrating the utility of their solid phase synthesis, several analogues of the chlorofusin cyclic peptide were prepared in a subsequent study by Searcey³¹ (Fig. 4). Based on an examination of cyclodecapeptide β -hairpin constructs with high binding affinities for MDM2,³² they proposed that Ala2, Orn9, and D-ADA8 of the chlorofusin peptide may be responsible for its interaction with the p53 binding site on MDM2. Thus, the peptide analogues explored contained modifications at the Ala2 and Orn9 positions. A key element of this approach is the on-resin cyclisation that allows analogue synthesis while

still immobilized on the solid phase. Orthogonal use of an Mtt (methyltrityl-) protecting group on the Orn9 side chain amine led to the selective on-resin deprotection (using 1% TFA) of Orn9- δ NH₂ that was then reacted with various acid chlorides. The resultant substituted benzamides were chosen to mimic possible interactions involving the 4-, 5-, 6-, and 7-positions of the chromophore with MDM2. As in their previous work, this strategy required only one final global deprotection/cleavage step and only one HPLC purification step for each analogue constructed.

Evaluation of these eight cyclic peptide analogues was conducted in an ELISA assay adapted from the work of Kahne³³ and measured disruption of the interaction between HDM2 (17-125) and immobilized biotinylated SGSG-p53 peptide (17-27). Although the control p53 peptide (SQETFSDLWKLLPEN) produced an IC₅₀ value of 4.2 μ M in this assay, comparing well with reported values, the chlorofusin analogues displayed no p53/MDM2 inhibitory activity with IC₅₀ values outside their solubility range in the assay.

2.3 Nakata cyclic peptide syntheses

Recently, Nakata³⁴ has reported an additional solution phase synthesis of the chlorofusin cyclic peptide employing a Boc/TMSE strategy and key macrocyclization at the D-ADA/L-Orn site, which features the late stage incorporation of the L-Orn9 residue in order to expedite and anticipate the synthesis of chlorofusin and its chromophore analogues.

3 Boger total synthesis, structural reassignment, and absolute configuration of chlorofusin

3.1 Retrosynthetic analysis

The chlorofusin chromophore is derived from a class of compounds referred to as azaphilones, so named for their ability to condense with ammonia or primary amines.³⁵ Given what is known about the electrophilic nature of the azaphilones, at least two potential routes to chlorofusin could be envisioned: (1) condensation of an appropriate azaphilone with the intact cyclic peptide followed by chromophore oxidative spirocyclization, or (2) construction of the fully functionalized chromophore appended to a smaller peptide fragment followed by peptide coupling and macrocyclization to afford chlorofusin. The former requires the conduct of an azaphilone chromophore oxidative spirocyclization on a complex, late stage synthetic intermediate with control of the resulting relative and absolute stereochemistry or effective separation and characterization of the resulting diastereomers, whereas the latter requires that the chromophore *N,O*-spiroketal in the smaller peptide fragment not unravel during the late stages of the ensuing cyclic peptide synthesis.

Since the absolute configuration of the chromophore was unknown and the relative stereochemical assignment came under question as the work progressed, the route pursued in the Boger groups first generation synthesis^{36,37} permitted access to both enantiomeric series and to all possible diastereomers, albeit optimized to access the original diastereomer assigned by Williams. As discussed herein, confidence in a requisite reassignment of the relative stereochemistry emerged with the observation of diagnostic spectroscopic distinctions (¹H and ¹³C NMR) made possible by access to all diastereomers. Due to concerns for an unambiguous stereochemical assignment as the studies progressed, the strategy of early stage chromophore oxidative spirocyclization with clear stereochemical assignments for all diastereomers became the focus of these efforts and was effectively conducted on the azaphilone adduct with an ornithine-threonine dipeptide. Ultimately, even an assignment of the chromophore absolute configuration emerged from diagnostic distinctions observed in the spectroscopic properties of the series of all eight diastereomers derived from the chromophore adduct (four diastereomers in each of the two enantiomeric series) that was ultimately

confirmed by the total synthesis of the natural product. In retrospect, a late stage chromophore elaboration may have failed to produce sufficient quantities of the natural product for detection and may not have allowed an unambiguous stereochemical assignment given that the natural product was found to possess a contrathermodynamic *N,O*-spiroketal stereochemistry.

3.2 Chlorofusin chromophore synthesis

The azaphilone intermediate **29** was initially prepared by a route based on the work of Whalley,³⁸ and subsequently accomplished using a second, more concise route based on the work of Porco,³⁹ the latter of which is shown in Scheme 3. Regioselective bromination of **20**, via *o*-lithiation of **21**, was followed by demethylation under standard conditions (BBr₃, 88%) to generate **23**. Sonogashira coupling of aryl bromide **23** and alkyne **24** provided the azaphilone precursor **25** (Pd(Cl)₂(PPh₃)₂, CuI, Et₃N, 73%). Oxidative cyclization of **25** using Porco's methodology³⁹ generated the azaphilone **26** in good yield (Au(OAc)₃, CF₃COOH, dichloroethane, 72%). Acylation of the tertiary alcohol of **26** to give **27** (butyric anhydride, 91%), regioselective C6-chlorination yielding **28** (NCS, 89%) and removal of the TBDPS protecting group (HF-pyridine, 72%) proceeded smoothly to provide azaphilone **29**. The racemic azaphilones **26-29** could be chromatographically separated into their constituent enantiomers, and the most effective chiral phase resolution was achieved with **28** ($\alpha = 1.33$). The absolute stereochemistry of the enantiomerically pure azaphilones was assigned based on the sign of their optical rotation (α_D) and the sign of the longest wavelength Cotton effect (350-370 nm) in their CD spectra (Fig. 5).⁴⁰⁻⁴² This assignment was further confirmed by application of Porco's asymmetric oxidative cyclization⁴³ of the OTIPS variant of **25** conducted using the catalyst generated from Cu(I) and (-)-sparteine to provide the corresponding azaphilone (94% ee), matching the assignment made based on CD. As fate would dictate and since (+)-sparteine is not yet readily accessible, this asymmetric route currently only provides access to the chromophore unnatural enantiomer series.

3.3 Chromophore model studies

Following extensive exploration of direct and indirect oxidative spirocyclization methods,³⁹ oxidative spirocyclization with I₂ assisted by AgNO₃ in the presence of DMSO-H₂O (I₂, AgNO₃, H₂O, DMSO, 48 h) allowed access to all four diastereomers (**33A-33D**) of a simplified chlorofusin chromophore (Fig. 6).

This reaction appears to be initiated by reversible iodonium ion formation and subsequent iodoetherification with *N,O*-spiroketal formation followed by slow Ag(I)-assisted iodide displacement by H₂O providing the desired products directly. Notably, the lone pair of the endocyclic amine is tied up in the π -system of the chromophore as a vinylogous amide, and accounts for the relative stability of the iodonium intermediate, and the predominance of C8/C9 syn products arising from S_N2-like trans intramolecular opening of the iodonium ion. The major products (54%), in which the C8 and C9 oxygen substituents are syn possessing the C8/C9 stereochemistry found in the original Williams assignment, represent those that formally arise from a trans iodoetherification reaction followed by water S_N2 displacement of the iodide. The structures of **33A** and **33B**, the major products of the oxidative spirocyclization reaction, were established by X-ray crystallography and the C8 and C9 oxygen substituents of both compounds were found to be oriented syn with respect to one another with the C8-OH occupying an axial orientation. *N,O*-Ketal equilibration studies unambiguously established the structure of the remaining two minor anti diastereomers. A detailed comparison of the spectroscopic properties of **33A-33D** to chlorofusin provided the first indication that the C8 and C9 oxygen substituents of chlorofusin may be oriented anti with respect to one another, and not syn as originally assigned. For the purposes herein, these may be summarized simply as observed chemical shift differences averaged over the entire chromophore or restricted to the more diagnostic comparisons as presented in Fig. 6, both of which indicate that the anti

diastereomer **33D** provides the best match with the chlorofusin chromophore. However, the chromophore NMR signals could be impacted by the appended benzyl group and an additional, more comparable system **37** was examined, incorporating an *N*-butyl substituent as well as a C4 butyrate versus acetate. Significantly, the observations with **33A-33D** initiated a switch in the strategy from targeting the total synthesis of chlorofusin by this group to one that could also unambiguously establish the chromophore stereochemistry. As such, the efforts were refocused on preparing and spectroscopically characterizing all possible chromophore diastereomers rather than exclusively targeting the Williams syn isomer.

Thus, chromophore derivatives **37A-37D** possessing the *N*-butyl substitution were synthesized (Fig. 6). As with **33A-33D**, the NMR data collected from the anti isomers **37C** and **37D** provided a better match with chlorofusin than the syn isomers **37A** or **37B**. An X-ray crystal structure of **37A**, representing the Williams-assigned diastereomer and the closest match by NMR to **33A**, confirmed that the C4, C8 and C9 oxygen substituents were all syn, whereas an X-ray of **37D**, the closest match with chlorofusin by NMR, confirmed that the relative orientation of the C8 and C9 oxygen substituents is anti and that the C4 methyl group is cis to the C8-OH and trans to the C9 oxygen of the spirotetrahydrofuran (Fig. 7). *N,O*-Ketal equilibration studies established the related syn/anti pairs and completed the unambiguous stereochemical assignments for the entire series. As the similarity of the model to the chlorofusin chromophore increased, the trends in the NMR data distinguishing the C8/C9 syn diastereomers from the anti diastereomers became more predominant. This, along with other more subtle differences, distinguished the two anti diastereomers with **37D**, not **37C**, being representative of the stereochemistry found in chlorofusin.

Although intuitively surprising but which now may be expected from the X-ray crystal structure of **37D** (Fig. 7) that shows an unobstructed path between C8-H and C4-Me (4.761 Å, C8-H-C13), the ROESY NMR for **37D** exhibited a weak cross-peak between C4-Me and C8-H as observed by Williams as a long range NOE in chlorofusin. Also observed in its X-ray structure is the diaxial orientation of the C8 and C9 oxygen substituents. In this conformation, the equatorial C8-H can exhibit NOEs to both C10-H and C12-H as observed by Williams for chlorofusin. Although these NOEs were not quantitated, it is notable that C8-H is closer to C10-H (2.484 Å) than C12-H (2.592 Å) in this X-ray consistent with such implications in the Williams work. In fact, the C8-OH is axial and the C8-H is equatorial in the X-ray structures for **33A**, **33B**, **37A** and **37D** indicating that the C8-H NOEs observed by Williams would be possible with all four diastereomers. This dominance of the C8-OH axial orientation arises from the A(1,3)-strain an equatorial C8-alcohol would experience with the C6-chloride that is further destabilized by the electronegative nature of the two interacting substituents (Cl/OH). With this understanding of the conformational properties of the chromophore diastereomers and with the characterization data from both the **33** and **37** model systems in hand, the stereochemistry of the chlorofusin chromophore was confidently reassigned as either the (4*S*, 8*R*, 9*S*) or (4*R*, 8*S*, 9*R*) anti diastereomer (**37D** enantiomers).

3.4 L-Orn-L-Thr azaphilone conjugation and elaboration to all eight chromophore diastereomers and stereochemical assignment of chlorofusin

At this stage, it was clear that a final stage chromophore coupling to the intact cyclic peptide followed by oxidative spirocyclization would be difficult to implement. Not only would this require the separation, characterization, and unambiguous stereochemical assignment of the four diastereomers generated in each enantiomeric series, but the reassigned anti isomer represented a minor, contra-thermodynamic product of the cyclization reaction in the model systems. As such, the less ambitious, more manageable step of linking and elaborating the chromophore on a smaller dipeptide constituting L-Orn9-L-Thr1 of chlorofusin was examined using both enantiomeric series and their four possible diastereomers. Not only did this provide

intermediates with more manageable physical and spectroscopic properties and a third model on which to carefully reassess the chlorofusin stereochemical assignments, but it provided an opportunity to probe the chromophore absolute stereochemical assignment.

To this end, dipeptide **38** was condensed with either (*R*)-**29** or (*S*)-**29** (84% for **39**, 71% for **41**) to provide **39** and **41**, respectively (Fig. 8). Oxidative spirocyclization of **39** (I₂, AgNO₃, H₂O, DMSO, 72 h) provided **40A-40D** and similar treatment of **41** provided **42A-42D**. The ¹H and ¹³C NMR spectra of all eight diastereomers were fully assigned using COSY, HMQC, HMBC and ROESY NMR data and *N,O*-ketal equilibration studies defined the related syn/anti pairs in each enantiomeric series. Notably, the equilibration studies requiring 5% TFA-HOAc (25 °C) revealed that the interconversions, like those in the **33** and **37** series, are slow even under these strongly acidic conditions and that the *N,O*-spiroketals are remarkably robust. Additionally, resubjecting **40B** or **40C** to the oxidative spirocyclization reaction conditions did not provide isomerized products suggesting that the syn products are both thermodynamically and kinetically favored and that the anti products generated are stable to the reaction conditions.

By using the diagnostic spectroscopic distinctions observed in the **40** and **42** series, having assigned the C4 absolute stereochemistry by CD (see Fig. 5),⁴⁰⁻⁴² and enlisting *N,O*-ketal equilibrations to define the syn/anti pairs in each enantiomeric series, the complete relative and absolute stereochemical assignments for all eight diastereomers were unambiguously established. Moreover, only diastereomer **40D** (4*R*,8*S*,9*R*) matched all the spectroscopic properties reported for the chlorofusin chromophore. In short, the two syn and two anti diastereomers in each enantiomeric series are readily distinguishable by the diagnostic ¹H NMR (C10-H, C11-H, C12-H, and C8-OH) and ¹³C NMR (C2 and C9-C12) chemical shifts and supported by kinetic and thermodynamic predominance of the syn isomers. The two anti diastereomers in each enantiomeric series are most readily distinguished by the diagnostic ¹H NMR (C8-H, C13-H, and C8-OH) and ¹³C NMR (C3, C7, C8, and C13) chemical shifts although there are more subtle spectroscopic distinctions as well. In both series, it was the anti diastereomers **40D** and **42D** (chromophore enantiomers) that most closely matched chlorofusin. Additionally, the (4*R*,8*S*,9*R*)-diastereomer **40D** provided a near perfect match with the spectroscopic properties reported for the chlorofusin chromophore, whereas the (4*S*,8*R*,9*S*)-diastereomer **42D** (chromophore enantiomer) proved readily distinguishable by both the ¹H NMR chemical shift and multiplicity of the ornithine CH₂^δ adjacent to the chromophore (δ 3.45, m, 2H for **40D** vs δ 3.41 and 3.52, two m, 1H each for **42D**; chlorofusin = δ 3.42, t, 2H). This final multiplicity distinction between **40D** and **42D** allowed the absolute stereochemical assignment for the chlorofusin chromophore. This assignment of the absolute configuration for chlorofusin is especially significant given that both C4 configurations are found in azaphilone natural products with roughly equivalent representations.

An important ramification of the conformational features of the syn and anti diastereomers where each possess an equatorial C8-H is the counterintuitive observation that all four diastereomers possess nearly equivalent C4-Me to C8-H distances (5.42-5.55 Å) and that it is actually closest (5.42 Å) for the anti isomer assigned to chlorofusin. Thus, regardless of the relative stereochemistry of the chromophore, a NOE is seen between C4-Me and C8-H making the reassignment of the chromophore relative stereochemistry consistent with Williams' spectroscopic characterization of the natural product. Importantly, the NOE data collected and reported by Williams is impeccable including that of the long range C4-Me/C8-H NOE, but this latter NOE did not prove diagnostic of a C4-Me/C8-H cis stereochemistry.

3.5 Total synthesis of chlorofusin and its 4*R*-Diastereomers

With the chromophore stereochemistry confidently assigned, the (4*R*,8*S*,9*R*)-diastereomer **40D** was incorporated into a total synthesis of chlorofusin. Peptide **44**, synthesized in a convergent sequence, was protected in a manner that avoids treatment of late stage

chromophore derived intermediates with strong acid that might promote C9 *N,O*-spiroketal isomerization. Fmoc deprotection of **40D** (piperidine) and coupling of the free amine **43** with heptapeptide **44** (EDCI, HOAt, 55%) provided **45** (Scheme 4). Concurrent benzyl ester deprotection and Cbz removal (H₂, Pd/C) followed by macrocyclization of **46** (EDCI, HOAt, 60%) afforded synthetic chlorofusin (**1**), which displayed spectroscopic properties indistinguishable from that reported for natural chlorofusin. Among the most notable of these spectroscopic correlations was the single signal for the ornithine CH₂^δ (δ 3.42, t, 2H for natural and synthetic chlorofusin) that readily distinguishes the natural diastereomer from the (4*S*, 8*R*, 9*S*)-diastereomer incorporating the chromophore enantiomer (δ 3.42 and 3.50, two m, 1H each) that was prepared and miscorrelated by Yao in related studies.⁴⁴

In addition to the natural (4*R*, 8*S*, 9*R*)-diastereomer constituting chlorofusin, the (4*R*, 8*R*, 9*R*)-diastereomer **3** proposed by Williams as well as the (4*R*, 8*S*, 9*S*)- and (4*R*, 8*R*, 9*S*)-diastereomers of chlorofusin (**48** and **49**) were also prepared by this route from **40A-40C**. Notably, this entailed simply the three steps of Fmoc deprotection of the chromophore dipeptide precursor and EDCI-promoted coupling with the heptapeptide **44**, concurrent Cbz and benzyl ester deprotection, and a final macrolactamization to provide diastereomers **3**, **48** and **49** with purification of a single intermediate in addition to the final products (Scheme 5). This late stage divergence in a convergent total synthesis facilitated the assembly of the collective set of diastereomers and the anticipated non-correlation of their spectroscopic properties with that reported for the natural product provided further support for the new structural assignment.

Following the completion of this work and the chlorofusin structural reassignment, the comparison of a sample of authentic chlorofusin (ca. 1 mg, aged and of unknown quality) provided by Dr. Stephen Wrigley in 2003 exhibited a CD spectrum indistinguishable (sign and magnitude) from synthetic **1** confirming the absolute configuration assignment (Fig. 9B). Moreover, its CD spectrum was readily distinguishable from the two possible syn diastereomers (4*R*, 8*R*, 9*R* and 4*R*, 8*S*, 9*S*) and more subtly distinguishable from the alternative anti (4*R*, 8*R*, 9*S*)-diastereomer **49** (Figure 10A).

Just as significantly, the sample provided an ¹H NMR spectrum of a sufficient quality to confirm that it represented the authentic natural product. Illustrated in Fig. 10 is the spectral overlay of a diagnostic region of the ¹H NMR spectra of this sample of natural chlorofusin with the four 4*R* diastereomers illustrating the clear correlation with only the synthetic (4*R*, 8*S*, 9*R*)-diastereomer **1** and the clear distinctions with any of the alternative 4*R* diastereomers in this enantiomeric series, including the syn diastereomer **3** proposed by Williams. Additionally, the comparisons in this region of the ¹H NMR spectra confirmed the stereochemical purity and integrity of each isomer indicating that each, including the less stable anti diastereomers, is unaffected by the synthetic sequence required for their incorporation into the final products.

3.6 Synthesis of all four 4*S*-diastereomers of chlorofusin

With the disclosure by Yao of the synthesis and miscorrelation of the (4*S*, 8*R*, 9*S*)-diastereomer **52** with chlorofusin,⁴⁴ studies were extended to include the preparation and characterization of all four 4*S*-chromophore diastereomers of chlorofusin including **52**. The preparation of this enantiomeric chromophore series simply required the late stage individual incorporation of **42A-42D** into the full chlorofusin structure and again benefited from the convergent nature of the total synthesis providing diastereomers **2**, **50**, **51** and **52** in four steps of which **52** corresponds to the Yao structure (Scheme 6).

With **2**, **50**, **51** and **52** in hand, their spectroscopic properties proved readily distinguishable from the natural product. Each displayed a long wavelength negative Cotton effect opposite that observed with chlorofusin. Moreover, each 4*S*-diastereomer provided an essentially

identical, but of opposite sign, CD spectrum (250-500 nm) to the corresponding 4*R*-diastereomer indicating that it is the chromophore, and not the cyclic peptide, that establishes the sign and magnitude of the CD spectrum in this region.

Similarly, the ¹H NMR and ¹³C NMR spectroscopic properties of each diastereomer **2**, **50**, **51** and **52** were distinguishable from those of chlorofusin. This is most apparent in the region represented with the spectral overlay with the natural product in Fig. 11. The Yao diastereomer **52**, bearing the enantiomer of the chromophore found in natural chlorofusin, is most readily distinguishable by its ornithine CH₂^δ signal which appears as two multiplets of 1H each (δ 3.41 and 3.52), but also exhibits more subtle differences including C8-OH (δ 6.22 vs 6.26), Orn9 α-CH (δ 4.56 vs 4.59), Leu5 α-CH (δ 4.45 vs 4.48), Thr1 α-CH (δ 3.68 vs 3.66), Asn3 β-CH² (δ 2.57, dd vs 2.62, app t), C10-H (δ 2.40, m vs 2.38, br m) and C11-H (δ 2.04, m vs 2.0-2.2, br m). Similarly, the alternative syn diastereomer **2** proposed by Williams and in this enantiomeric series also exhibited spectroscopic properties readily distinguishable from the natural product.

4 Conclusions

The first total synthesis of chlorofusin was achieved resulting in the reassignment of its chromophore relative stereochemistry and the establishment of its chromophore absolute stereochemistry. Careful spectroscopic characterization of each of the four chlorofusin model chromophore diastereomers in two distinct series suggested the initial Williams assignment might not prove accurate and revealed that the key spectroscopic feature leading to the original assignment does not distinguish the four possible diastereomers. Subsequent preparation and characterization of all four diastereomers of a dipeptide conjugate with both enantiomers of the chlorofusin chromophore confirmed the required relative stereochemistry reassignment and permitted an assignment of its absolute configuration. This was unambiguously established with the total synthesis of not only chlorofusin, which displayed spectroscopic properties indistinguishable from the natural product, but also all seven alternative chlorofusin chromophore diastereomers, each of which displayed spectroscopic properties readily distinguishable from the natural product. This included the diastereomer advanced by Yao⁴⁴ as being identical with spectroscopic properties reported for natural chlorofusin as well as both enantiomeric chromophores of the original Williams assignment.²³ This study with the comprehensive characterization of all possible isomers was accomplished enlisting a convergent synthetic strategy with a key late stage divergent introduction of the eight chromophore-dipeptide conjugates requiring only four steps for completion of the synthesis and facilitating the total synthesis of the full series of eight chromophore diastereomers. One of the most interesting aspects of the contrathermodynamic *N,O*-ketal stereochemistry found in the chlorofusin chromophore is the prospect now that that other chlorofusin chromophore diastereomers, e.g. its more stable syn isomer **48**, may likely be identified as natural products. The results of such studies as well as efforts to probe the mechanism by which chlorofusin disrupts the interaction of p53 and MDM2 will be facilitated by the work summarized herein.

5 Acknowledgments

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Abbreviations

ADA, 2-aminodecanoic acid
[α]²³_D, specific rotation at 23 °C and wavelength of sodium D line
CD, circular dichroism

COSY, Correlation Spectroscopy (usually ^1H , ^1H)
 CSA, camphorsulfonic acid
 DMAP, *N,N*-dimethylaminopyridine
 DMF, *N,N*-dimethylformamide
 DMSO, dimethylsulfoxide
 EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
 ee, enantiomeric excess
 ELISA, Enzyme Linked Immunosorbent Assay
 Fmoc, 9-fluorenylmethoxycarbonyl
 fod, 6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3, 5-octanedionate
 Hep G2, human hepatocellular carcinoma cell line
 HBTU, *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
 HDM2, human double minute 2 protein
 HMBC, heteronuclear multiple bond correlation (usually ^{13}C , ^1H)
 HMQC, heteronuclear multiple quantum correlation (usually ^{13}C , ^1H)
 HOAt, 1-hydroxy-7-azabenzotriazole
 IBX, *o*-iodoxybenzoic acid
 MAPH, methylaluminium bis(2,6-diphenylphenoxide)
 MDM2, murine double minute 2 protein
 Mtt, 4-methyltrityl
 NBS, *N*-bromosuccinimide
 NCS, *N*-chlorosuccinimide
 NOE, nuclear Overhauser effect
 NOESY, nuclear Overhauser effect spectroscopy
 NMO, *N*-methylmorpholine oxide
 PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
 ROESY, rotating Overhauser effect spectroscopy
 SES, 1-[(trimethylsilyl)ethyl]sulfonyl
 TBDPS, *t*-butyldiphenylsilyl
 Tf, trifluoromethanesulfonyl
 TFA, trifluoroacetic acid
 TIPS, triisopropylsilyl
 TMS, trimethylsilyl
 TMSE, trimethylsilylethyl
 Tol, *p*-tolyl
 Trt, trityl
 TsOH, *p*-toluenesulfonic acid

6 References

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Fig. 1.
Chlorofusin.

2, (4*S*,8*S*,9*S*)-chlorofusin **3, (4*R*,8*R*,9*R*)-chlorofusin**

Fig. 2.
Original chromophore relative stereochemical assignment. No assignment of absolute stereochemistry was made.

Fig. 3.
Chlorofusin carbons with enhanced ^{13}C NMR signals derived from ^{13}C labelled acetate.

Fig. 4.
Searcey chlorofusin cyclic peptide analogues.

Fig. 5. CD spectra (0.2 mM in MeOH) and α_D^{23} for azaphilones **26** and **29** (R = butyrate).

Chemical Shift Comparison with Williams' Values²³

Isomer	Total ^a Avg ¹ H Δδ	Diagnostic Avg ¹ H Δδ	Total ^a Avg ¹³ C Δδ	Diagnostic Avg ¹³ C Δδ
33A	0.16	0.26 ^b	1.0	1.8 ^b
33B	0.19	0.31 ^b	0.9	1.9 ^b
33C	0.11	0.01 ^c	0.5	0.8 ^c
33D	0.09	0.03 ^c	0.3	0.2 ^c

^aC1–C13, C8–OH ^bC2, C9–C12 ^cC3, C7, C13 Chemical Shift Comparison with Williams' Values²³

Isomer	Total ^a Avg ¹ H Δδ	Diagnostic Avg ¹ H Δδ	Total ^a Avg ¹³ C Δδ	Diagnostic Avg ¹³ C Δδ
37A	0.14	0.22 ^b	0.9	2.0 ^b
37B	0.14	0.23 ^b	0.9	2.1 ^b
37C	0.05	0.08 ^c	0.3	0.7 ^c
37D	0.03	0.00 ^c	0.2	0.2 ^c

^aC1–C18, C8–OH, Ornδ ^bC2, C8–OH, C9–C12 ^cC3, C7, C8, C8–OH, C13**Fig. 6.**

Model chromophore diastereomers **33A–33D** and **37A–37D**. Tables show the average variation in total and diagnostic chemical shifts in proton and carbon spectra when compared with Williams' original data.²³

Fig. 7.
X-Ray structures of **37A** and **37D**.

Fig. 8.

Synthesis of all eight L-Orn⁹-L-Thr¹ conjugated chromophore diastereomers. Tables show the average variation in total and diagnostic chemical shifts in proton and carbon spectra when compared with Williams' original data.²³

Fig. 9. (A) CD spectra (0.2 mM in MeOH) of **3**, **48**, **49** and **1**, and (B) CD spectra of synthetic versus natural chlorofusin.

Fig. 10.
 ^1H NMR overlay of natural chlorofusin with all four C4-R chlorofusin chromophore diastereomers.

Fig. 11.
 ^1H NMR overlay of natural chlorofusin with all four C4-S chlorofusin chromophore diastereomers.

Scheme 1. Boger solution phase synthesis of the chlorofusin cyclic peptide.^{29a}

Scheme 2.

Searcey solid phase synthesis of the chlorofusin cyclic peptide.

Scheme 3.
Boger second generation azaphilone synthesis.

Scheme 4.
Completion of the total synthesis of chlorofusin.

40A–40C

1. piperidine
2. EDCI–HOAt, **44**
3. H₂, Pd/C
4. EDCI–HOAt

3 (4*R*,8*R*,9*R*)
(syn isomer proposed by Williams)

48 (4*R*,8*S*,9*S*)

49 (4*R*,8*R*,9*S*)

Chemical Shift Comparison with Williams Values²³

Isomer	Total ^a Avg ¹ H Δδ	Diagnostic Avg ¹ H Δδ	Total ^a Avg ¹³ C Δδ	Diagnostic Avg ¹³ C Δδ
3 , Williams	0.15	0.26 ^b	0.9	1.8 ^b
48	0.13	0.24 ^b	0.9	1.7 ^b
49	0.04	0.08 ^c	0.3	0.5 ^c
1 , synthetic	0.00	0.00 ^c	–	–

^aC1–C18, C8–OH, Ornδ ^bC2, C8–OH, C9–C12, Ornδ ^cC3, C7, C8, C8–OH, C13, Ornδ

Scheme 5.

Synthesis of the three remaining 4*R*-diastereomers of chlorofusin. The table shows the average variation in total and diagnostic chemical shifts in proton and carbon spectra when compared with Williams' original data.²³

Chemical Shift Comparison with Williams Values²³

Isomer	Total ^a Avg ¹ H Δδ	Diagnostic Avg ¹ H Δδ	Total ^a Avg ¹³ C Δδ	Diagnostic Avg ¹³ C Δδ
2 , Williams	0.14	0.24 ^b	0.9	1.7 ^b
50	0.13	0.24 ^b	0.9	1.9 ^b
51	0.03	0.05 ^c	0.3	0.6 ^c
52 , Yao	0.02	0.04 ^c	0.1	0.2 ^c

^aC1–C18, C8–OH, Omδ ^bC2, C8–OH, C9–C12, Omδ ^cC3, C7, C8, C8–OH, C13, Omδ

Scheme 6.

Synthesis of the four 4*S*-diastereomers of chlorofusin.