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Solid-Phase Synthesis of Chlorofusin Analogues

Esther C. Y. Woon,[†] Mariangela Arcieri, Andrew F. Wilderspin, John P. Malkinson, and Mark Searcey*,[†]

Department of Pharmaceutical and Biological Chemistry, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX

m.searcey@uea.ac.uk

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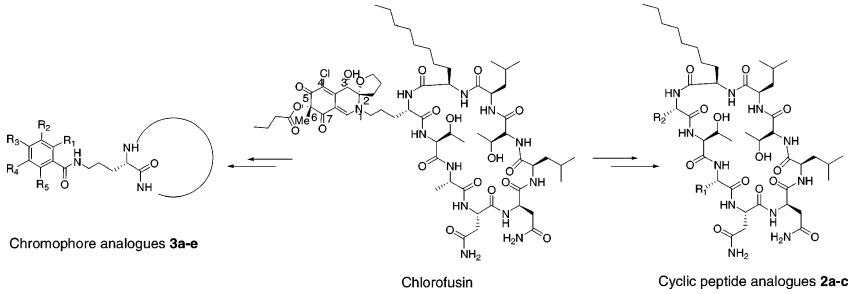

We report an efficient and versatile solid-phase synthesis through which two series of chlorofusin analogues, one bearing varying chromophores and the other with various amino acid substitutions in the cyclic peptide, were synthesized. These peptides were prepared using a strategy involving side-chain immobilization, on-resin cyclization, and postcyclization modification. The success of these syntheses demonstrates the broad utility of the method. Both series of analogues were evaluated for their inhibitory activity against the p53/MDM2 interaction but were shown to be inactive in the concentration range tested. This suggests that the full chromophore structure may be required for activity.

Introduction

The tumor-suppressor protein p53 plays a crucial role in the development of cancer,[1−3] with around 50% of all tumors containing a mutated, inactivated form.[4] However in about 7% of tumors where wild type p53 is retained, its function is compromised by an overexpression of MDM2 (its main negative regulator).[5] An inhibition of p53/MDM2 binding in these tumor cells would reactivate the p53 pathway, leading to cell-cycle arrest or apoptosis, hence providing a promising strategy for anticancer chemotherapy.[6−9] Research efforts in the quest for p53/MDM2 inhibitors initially focused on short peptides mimicking the primary structure of p53[10−12] in order to validate the target and identify key interactions between the two proteins. More recently, small molecules, such as derivatives of chalcones,[13] benzodiazepinediones,[14] and particularly *cis*-imidazolines[15] have moved toward demonstrating clinical utility as inhibitors of this key protein−protein interaction.

* Author to whom correspondence should be addressed. Tel +44-1603592026, fax +44-1603592003.

[†] Current address: School of Chemical Sciences & Pharmacy, University of East Anglia, Norwich NR4 7TJ.

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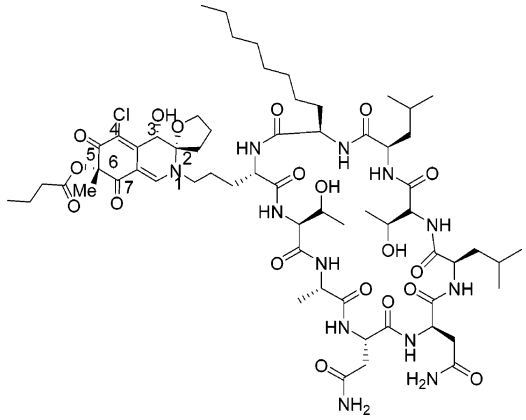


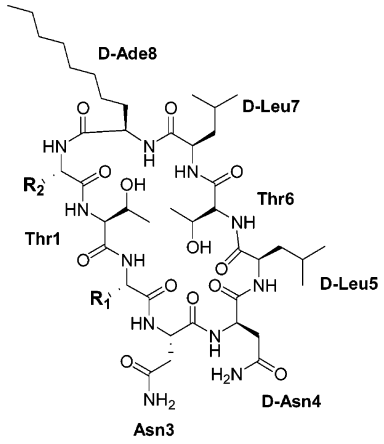
FIGURE 1. The structure and relative stereochemistry of chlorofusin.[16]

Chlorofusin is the first natural product to be identified as an inhibitor of the p53/MDM2 interaction.[16] It is a secondary metabolite isolated from the fermentation of *Microdochium caespitosum*, with an IC_{50} for the inhibition of MDM2 binding to p53 of 4.6 μM. To date, its molecular structure remains largely unexploited for drug discovery, and very little is known about its actual binding interaction with MDM2, other than the fact that it binds to the N-terminus.[17] In order to study the structure−activity relationship between chlorofusin and MDM2, there is a requirement for the development of versatile synthetic methods for the generation of a library of chlorofusin analogues. The synthesis of the chlorofusin peptide was disclosed simultaneously by two groups in 2003,[18,19] using both solution and solid-phase methods to verify the natural product structure. Herein, we describe the synthesis of a number of exemplar chlorofusin analogues containing either variations in the ornithine side-chain or "point mutations" in the peptide ring, using the side-chain immobilization methodology, and demonstrate the synthetic utility of this approach as a general route to chlorofusin analogues.

Analogue Design

Based on current spectroscopic data,[16] the chemical structure and relative stereochemistry of chlorofusin is believed to consist of two main components: a highly functionalized azaphilone chromophore and a cyclic peptide composed of nine amino acid residues **1**. The nitrogen atom bonded to the δ-carbon of the side-chain of Orn9 is directly incorporated into the bicyclic core of the chromophore, linking the two components together (Figure 1). The structure of the cyclic peptide was established by synthesis using both solid- and solution-phase methods and demonstrated *S* and *R* absolute stereochemistry for Asn3 and Asn4, respectively.[18,19] The solid-phase route involved side-chain immobilization of a protected aspartic acid *via* a Rink amide linker that would generate the β-carboxamide of Asn3 on release from the resin. This approach to the synthesis is amenable to the rapid parallel synthesis of analogues of the cyclic peptide containing single point mutations. Studies of peptides analogous to the N-terminal domain of the p53 protein established that p53 binds to MDM2 with an α-helical conformation, forming three critical contacts through the side-chains of Phe19, Trp23, and Leu26.[20] Recent studies of β-hairpin constructs demonstrated that cyclodecapeptides could be constructed based upon these observations with extremely high binding affinities for MDM2.[21] We suspected that Ala2, Orn9, and D-Ade8 of the chlorofusin peptide may interact in a similar manner with the p53 binding site on MDM2. In order to test the utility of the solid-phase synthesis, a small subset of compounds containing modifications at Orn9 and Ala2 (**2a**−**c**, Figure 2) were synthesized.

To explore the role of the chromophore in p53/MDM2 binding, analogues of the structure **3a**−**e** (Figure 3) were



1 (chlorofusin peptide)
: R_1 = Ala2 R_2 = Orn9
2a: R_1 = Phe2 R_2 = Orn9
2b: R_1 = Ala2 R_2 = Trp9
2c: R_1 = Phe2 R_2 = Trp9

FIGURE 2. The structures of proposed analogues **2a**−**c**.

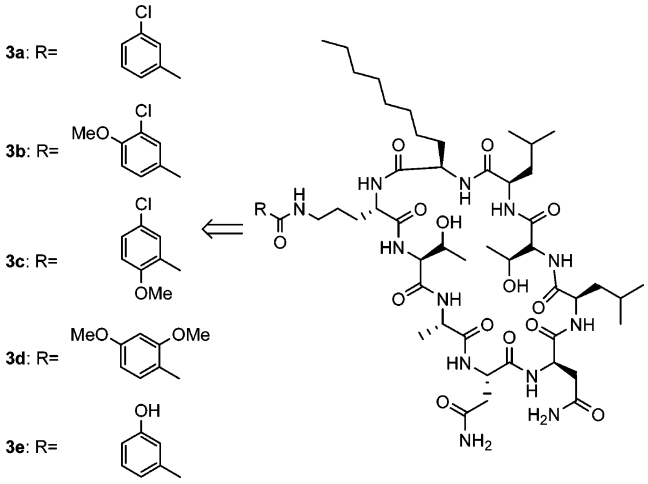


FIGURE 3. The structures of proposed analogues **3a**−**e**.

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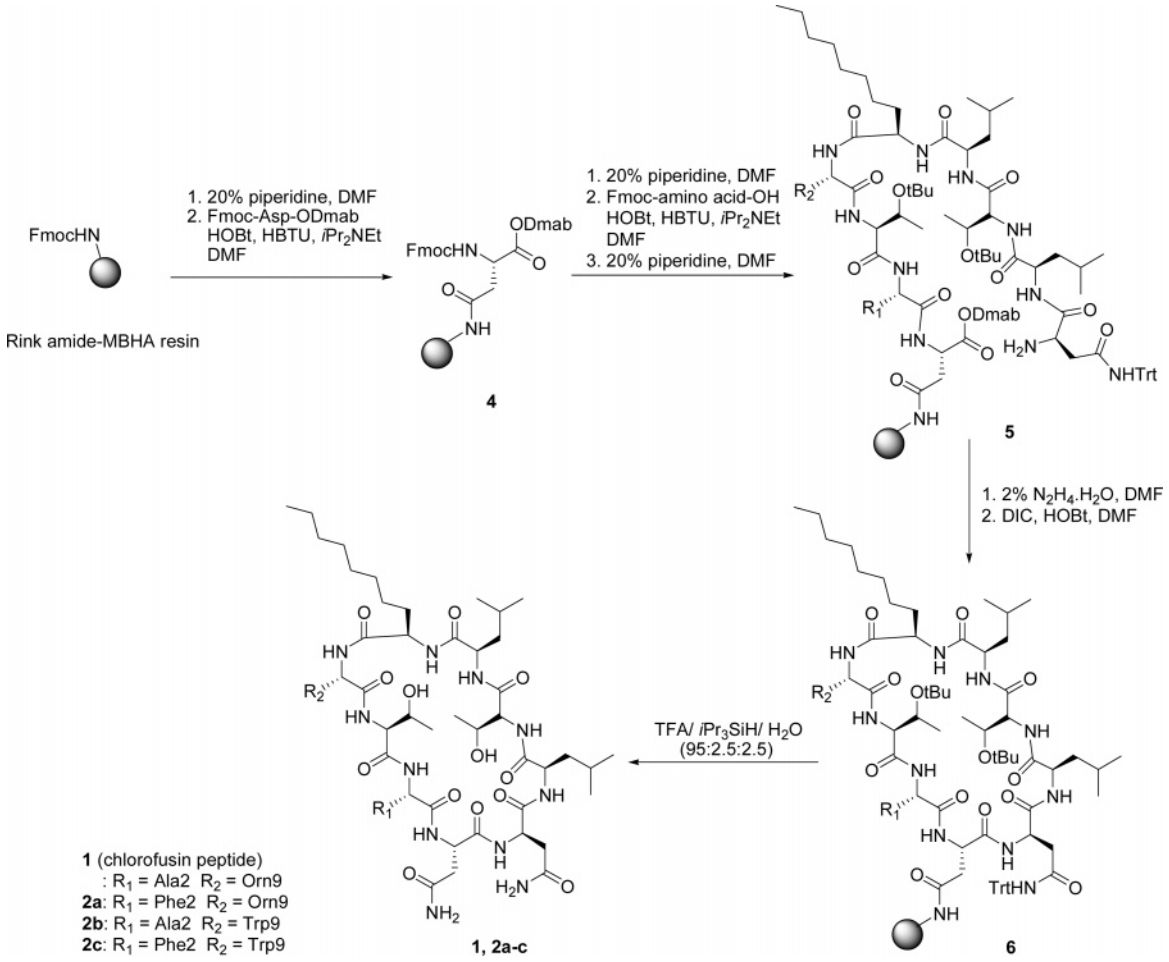
SCHEME 1. Synthesis of 1 and Analogues 2a−c



proposed. In this design, the chromophore is replaced with a simplified substituted benzamide ring, which serves to mimic any possible interactions involving the 4-, 5-, 6-, and 7-positions of the azaphilone ring with MDM2, while retaining the planar conformation of the cyclohexane-1,3-dione ring. With an orthogonal Mtt protecting group on the ornithine side-chain, it is possible to carry out the synthesis entirely on solid phase and deprotect and react selectively at this position prior to cleavage from the resin. This route also has potential in parallel synthesis.

Results and Discussion

The synthesis of cyclic peptides posed a synthetic challenge as a much-strained conformation is imposed on a linear peptide through cyclization. Our lab has recently developed an innovative side-chain immobilization solid-phase strategy for the synthesis of the chlorofusin peptide **1** (Scheme 1, Route A).[19] Essentially, the synthesis involved three key steps: (1) attachment to the solid support *via* the side-chain β-carboxyl of Fmoc-Asp-ODmab, (2) linear chain formation, and (3) on-resin head-to-tail cyclization through amide bond formation between the α-carboxyl group of Asn3 and the α-amino group of D-Asn4.

To initiate the synthesis, N^{α}-Fmoc-Asp-ODmab was immobilized onto a Rink amide MBHA resin *via* its carboxylic acid side-chain using HBTU/HOBt activation in the presence of DIPEA in DMF. Selective N^{α}-Fmoc deprotection of the resin-bound aspartic acid residue **4** was followed by standard N^{α}-Fmoc-based chain extension (i.e., incorporating Fmoc-Ala-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Orn(Boc)-OH, Fmoc-D-Ade-OH, Fmoc-D-Leu-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-D-Leu-OH and then Fmoc-D-Asn(Trt)-OH employing cycles of Fmoc deprotection with 20% v/v piperidine in DMF and HBTU/HOBt/DIPEA-mediated coupling). The completion of each coupling cycle was monitored after 30 min of agitation by means of the Kaiser test.[22] The linear peptide chain **5** thus assembled was then subjected to intramolecular head-to-tail cyclization on-resin to furnish the macrocycle **6**. This was achieved by first deprotecting the N^{α}-Fmoc protecting group of Asn4 and the α-carboxyl Dmab protecting group of Asn3 using 20% piperidine in DMF and 2% hydrazine monohydrate in DMF, respectively, followed by coupling with a 5-fold excess of DIC and HOBt in DMF. Subsequent treatment of the resin with 95% TFA led to global deprotection and cleavage of the peptide from the solid support. The crude product was then dissolved in 50% acetic acid and lyophilized before purification by semipreparative reverse-phase HPLC using a gradient of acetonitrile in water to furnish cyclic peptide **1** in 27% yield. The above solid-phase strategy was then applied to the synthesis of analogues **2a−c** (Scheme 1). These targets have specific point mutations applied

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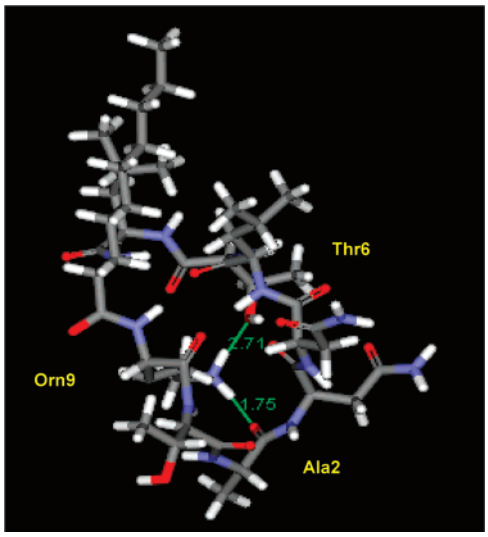


FIGURE 4. The solution structure of chlorofusin peptide **1** determined by NMR.[19] This view shows the folding of Orn side-chain into the hydrophilic core, forming two intramolecular hydrogen bonds: one with the hydroxyl group of Thr6 and the other with the carbonyl oxygen of Ala2.

to the cyclic peptide **1** to mimic the binding interactions between p53 and MDM2. Their preparations were very similar to that of **1**, and they took advantage of the on-resin cyclization technique which provided a pseudo-dilution effect that helped to minimize (cyclo)oligomerization. It also allowed the use of excess reagents which is important in driving the lactam ring formation toward completion, improving both the rate of reaction and the yield. The appropriate sequence modifications were made during the assembly of the linear peptides, replacing either Ala2 with Phe2 (**2a**) or Orn9 with Trp9 (**2b**), or making both substitutions simultaneously (**2c**). All three syntheses proceeded smoothly to give the desired compounds **2a**−**c** in good yield (13−24%), with >95% purity.

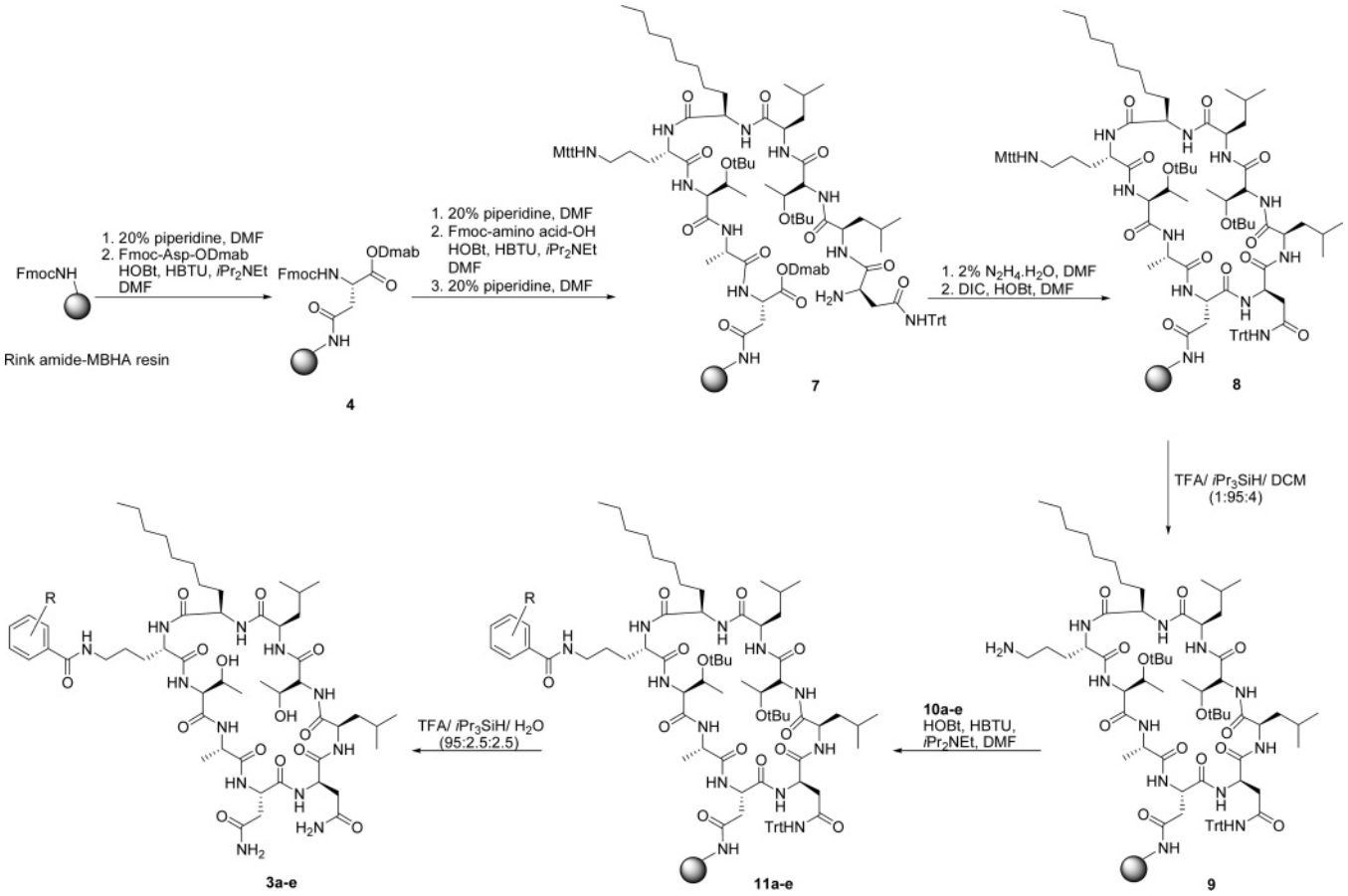
In adapting the same tactic to the synthesis of analogues **3a**−**e**, two approaches were considered. First we examined the possibility of coupling **1** directly with the chromophore using solution synthesis. The advantage of this method is that it involves using relatively mild reaction conditions compared to those employed in Fmoc-solid-phase chemistry, in particular the basic and strongly acidic conditions required for Fmoc-deprotection and peptide release respectively. The latter would not be suitable for chromophores with acid- or base-sensitive functional groups. To investigate this method, **1** was treated with 3-chlorobenzoic acid using various coupling conditions, such as stirring in acetonitrile at room-temperature both in the presence and absence of CSA, microwave irradiation at various temperatures in DMF, or employing a mixture of solvents. However, all attempts were unsuccessful (data not shown). Apparently, factors such as the presence of unprotected side chains could have resulted in the failure of the reaction. Careful examination of the solution structure of the chlorofusin peptide **1**,[19] previously determined using NMR spectroscopy, also suggests the possibility of the Orn9 side-chain folding into the hydrophilic core, forming two putative intramolecular hydrogen-bonds with the hydroxyl group of Thr6 (2.77 Å) and the carbonyl of Ala2 (1.75 Å) (Figure 4). This could potentially reduce the availability of its amino group for reaction, thus further hindering the reaction.

Accordingly, incorporating the chromophore on-resin should circumvent the problem of side −chain interactions as they all remain protected. In addition, the peptide, being still attached to the hydrophobic solid support, will presumably be prevented from assuming the conformation required for hydrogen-bond formation with Orn9. Hence, the original strategy used for the synthesis of **1** was modified to incorporate an on-resin, postcyclization coupling step. The principal advantage of this approach is that the entire synthesis is completed on-resin, without the need for further reaction in solution and necessitating only a single purification by preparative HPLC. A potential drawback, however, is that any resin-bound reactive or nucleophilic byproduct remaining after cyclization may also be acylated, resulting in a more complex mixture of similar (cyclic) peptides that may be more difficult to remove during purification. The attachment of the chromophore was intentionally carried out after cyclization (as opposed to prior to), as we desired to use intermediate **9** as a template through which a library of chlorofusin analogues could be rapidly assembled. Hence analogues **3a**−**e** were prepared by first immobilizing Fmoc-Asp-ODmab on Rink amide resin (Scheme 2). The linear peptide was then assembled as previously, incorporating Fmoc-Orn(Mtt)-OH instead of Fmoc-Orn(Boc)-OH. The Mtt group (4-methyltrityl) was chosen as it can be removed selectively and rapidly by very mild acidolysis using 1% TFA in DCM, leaving the peptide-resin linkage and the rest of the protecting groups, namely O*t*Bu and CONHTrt, unaffected. After on-resin cyclization, orthogonal deprotection of Mtt and selective coupling of the resulting free amino group with benzoic acids **10a**−**e**, the peptides were released from the solid support with simultaneous side-chain deprotection. Purification of the crude products with semipreparative reverse-phase HPLC gave the corresponding target compounds **3a**−**e** in 14−27% overall yield. In all cases, the desired compounds were the major products, hence demonstrating the high efficiency and utility of the synthesis.

Complete ^{1}H NMR resonance assignments for all peptides were determined *via* standard homonuclear and heteronuclear two-dimensional NMR techniques[23] (Tables 1−3, Supporting Information). Their chemical shifts compare well with that of chlorofusin and previously reported spectra of **1**. Their mass spectral analyses and HPLC retention times are also reported.

Biological Results. Preliminary biological evaluation of these analogues was conducted through an ELISA competition assay adapted from the method described by Kahne et al.[11] It involved a measurement of the ability of various analogues to disrupt the interaction between HDM2 (17−125) and biotinylated SGSG-p53 peptide (17−27), which was immobilized on a streptavidin-coated 96-well plate. The experimental procedure for the assay is given in Supporting Information. The control p53 peptide (SQETFSDLWKLLPEN) produces an IC_{50} value of 4.16 μM in this assay, comparing well with literature values. All the compounds tested had little or no p53/MDM2 inhibitory activity, with IC_{50} values outside the solubility range in this assay. Chlorofusin has been reported to have an IC_{50} of 4.6 μM in a similar assay. The overall reduction in potency compared to the parent compound (chlorofusin) suggests that interactions involving regions other than those replicated by the benzamide core, in particular, the cyclic ketal and the 3-hydroxyl group of the azaphilone are likely to be important for potency.

SCHEME 2. Synthesis of Analogues 3a−e



Conclusions

We describe, herein, an efficient and versatile solid-phase strategy through which a series of chlorofusin analogues, bearing various cyclic peptides **2** and varying chromophores **3**, were generated. These syntheses demonstrate the broad utility of the side-chain immobilization strategy previously devised for the formation of the chlorofusin peptide. We have also reported on the *in vitro* activities of these analogues on p53/MDM2 interaction. It appears that both the cyclic peptide and the full chromophore structure are essential for p53/MDM2 inhibitory activity.

Experimental Section

Chlorofusin Peptide 1 and Analogues 2a−c. Rink amide MBHA resin (700 mg, 0.46 mmol, substitution = 0.66 mmol/g) was suspended in DMF (15 mL) in a peptide synthesis vessel. The vessel was gently agitated to remove air bubbles, and the resin was allowed to swell for 30 min. Vacuum was then applied to remove DMF, and the N^{α}-Fmoc group was removed by treatment with 20% v/v piperidine in DMF (2 × 10 min). The resin was then thoroughly washed with DMF (4 × 20 mL) and treated with a DMF solution of Fmoc-Asp-ODmab (1.54 g, 2.31 mmol, 5 equiv), the latter being activated just before addition with HBTU (859 mg, 2.26 mmol, 4.9 equiv), HOBt (353 mg, 2.31 mmol, 5 equiv), and DIPEA (596 mg, 4.62 mmol, 10 equiv). After 30 min of agitation at room temperature, a small sample of resin was analyzed for free amines using the Kaiser test. Upon completion of the coupling reaction, as indicated by a negative color test, the resin was thoroughly washed with DMF, DCM, and then MeOH/DCM (1:1), and dried *in vacuo* over KOH to a constant weight (860 mg, 0.46 mmol). The loaded resin was then split into four equal portions (200 mg, 0.11 mmol) and transferred into separate vessels A, B, C, and D for the assembly of **1**, **2a**, **2b**, and **2c**, respectively.

Each portion was allowed to swell in DMF for 30 min and was then thoroughly washed with DMF (4 × 20 mL). Following N^{α}-Fmoc deprotection, the resin portions in vessels B and D were coupled with Fmoc-Phe-OH (213 mg, 0.55 mmol, 5 equiv), while those in vessels A and C were coupled with Fmoc-Ala-OH (171 mg, 0.55 mmol, 5 equiv). All four were then deprotected and coupled with Fmoc-Thr(*t*Bu)-OH (219 mg, 0.55 mmol, 5 equiv), after which chain extension was continued for A and B using Fmoc-Orn(Boc)-OH (250 mg, 0.55 mmol, 5 equiv), while C and D using Fmoc-Trp(Boc)-OH (290 mg, 0.55 mmol, 5 equiv). Subsequent couplings were the same for all four and were performed in this order: Fmoc-D-Ade-OH (225 mg, 1.65 mmol, 5 equiv), Fmoc-D-Leu-OH (194 mg, 0.55 mmol, 5 equiv), Fmoc-Thr(*t*Bu)-OH (219 mg, 0.55 mmol, 5 equiv), Fmoc-D-Leu-OH (194 mg, 0.55 mmol, 5 equiv), and then Fmoc-D-Asn(Trt)-OH (328 mg, 0.55 mmol, 5 equiv). All couplings were carried out in the presence of HBTU (204 mg, 0.54 mmol, 4.9 equiv), HOBt (84 mg, 0.55 mmol, 5 equiv), and DIPEA (142 mg, 1.10 mmol, 10 equiv). Prior to cyclization, the N^{α}-Fmoc group of the terminal amino acid (Asn4) was removed with 20% v/v piperidine in DMF (2 × 10 min). This was followed by the removal of the α-carboxyl Dmab protection group of Asn3, using 2% hydrazine monohydrate in DMF (3 × 5 min). The resin was then washed with 5% DIPEA in DMF (4 × 20 mL) and treated with DIC (69 mg, 0.55 mmol, 5 equiv) and HOBt (84 mg, 0.55 mmol, 5 equiv) in a minimal volume of DMF (2 × 24 h). The resin was then washed thoroughly with DMF, DCM, and then MeOH/DCM (1:1) and dried *in vacuo* over KOH to constant weight.

Simultaneous side-chain deprotection and release of the peptides from the solid support was achieved by stirring with a cleavage mixture containing TFA/H_2O/TIPS (95:2.5:2.5), 10−25 mL/g resin at room temperature for 3 h, after which the TFA was removed under reduced pressure. The crude peptides were precipitated and washed with cold anhydrous Et_2O, extracted into 50% acetic acid, and lyophilized. Purification of the crude peptides **3a**−**e** was carried out using semipreparative reverse-phase HPLC performed on a Waters Prep LC C18 column (100 mm × 25 mm). Separation was achieved using a linear gradient of solvent A (water + 0.02% TFA) and solvent B (90% MeCN + 9.98% water + 0.02% TFA), eluting at a flow rate of 5 mL/min and monitoring at 214 nm: 0% B to 40% B over 60 min and then 40% B to 60% B over 60 min. In all cases, a purity of >95% was obtained as determined by analytical reverse-phase HPLC, which was performed on a Vydac C4 Protein column (250 mm × 4.6 mm), eluting at a flow rate of 1.2 mL/min using a linear gradient of 0% B to 60% B over 120 min.

1 (15 mg, 27% yield): MALDI-TOF MS *m/z*: 1011.4 $[M + H]^+$ (calcd for $C_{46}H_{82}N_{12}O_{13}$, 1010.6124); HPLC retention time = 18.583 min.

2a (15 mg, 13% yield): MALDI-TOF MS *m/z*: 1087.9 $[M + H]^+$ (calcd for $C_{52}H_{86}N_{12}O_{13}$, 1086.6437); HPLC retention time = 19.400 min.

2b (19 mg, 16% yield): ES+ MS *m/z*: 1105.7 $[M + Na]^+$ (calcd for $C_{52}H_{82}N_{12}O_{13}$, 1082.6124); HPLC retention time = 22.183 min.

2c (31 mg, 24% yield): ES+ MS *m/z*: 1181.7 $[M + Na]^+$ (calcd for $C_{58}H_{86}N_{12}O_{13}$, 1158.6437); HPLC retention time = 23.733 min.

For ^{1}H NMR data, see Table 1 and 3, Supporting Information.

Chlorofusin Analogues 3a−e. Fmoc-Asp-ODmab (1.10 g, 1.65mmol, 5 equiv) was immobilized onto a Rink amide MBHA resin (500 mg, 0.33 mmol, substitution = 0.66 mmol/g) as described above. Upon completion of the coupling reaction, as indicated by a negative color test, the resin was thoroughly washed with DMF (4 × 20 mL) and the above coupling procedure was repeated with the following amino acids, in this order: Fmoc-Ala-OH (514 mg, 1.65 mmol, 5 equiv), Fmoc-Thr(*t*Bu)-OH (656 mg, 1.65 mmol, 5 equiv), Fmoc-Orn(Mtt)-OH (1.01 g, 1.65 mmol, 5 equiv), Fmoc-D-Ade-OH (675 mg, 1.65 mmol, 5 equiv), Fmoc-D-Leu-OH (583 mg, 1.65 mmol, 5 equiv), Fmoc-Thr(*t*Bu)-OH (656 mg, 1.65 mmol, 5 equiv), Fmoc-D-Leu-OH (583 mg, 1.65 mmol, 5 equiv), and then Fmoc-D-Asn(Trt)-OH (985 mg, 1.65 mmol, 5 equiv). Prior to cyclization, the N^{α}-Fmoc group of the terminal amino acid (Asn4) was removed with 20% v/v piperidine in DMF (2 × 10 min). This was followed by the removal of the α-carboxyl Dmab protecting group of Asn3 using 2% v/v hydrazine monohydrate in DMF (3 × 5 min). The resin was then washed with 5% DIPEA in DMF (4 × 20 mL) and treated with DIC (208 mg, 1.65 mmol, 5 equiv) and HOBt (252 mg, 1.65 mmol, 5 equiv) in a minimal volume of DMF (2 × 24 h). After cyclization, the resin was washed thoroughly with DMF, DCM, and then MeOH/DCM (1:1) and dried *in vacuo* over KOH to constant weight.

The dried resin was then split into five equal portions and transferred into separate peptide synthesis vessels. Each portion was allowed to swell in anhydrous DCM for 30 min and thoroughly washed with anhydrous DCM (5 × 20 mL). A mixture of TFA/DCM/TIPS (1:94:5) was then added to selectively remove the Mtt protecting group from Orn9. After standing for 2 min, the mixture was removed *via* vacuum and this step was repeated a few times until a colorless solution was obtained, indicating the absence of remaining Mtt protecting groups (a pronounced yellow color on addition of the 1% TFA mixture indicates the liberation of trityl cations). The resin portions were then separately coupled with 5 equiv of one of the following benzoic acids: 3-chlorobenzoic acid, 3-chloro-4-methoxybenzoic acid, 5-chloro-2-methoxybenzoic acid, 2,4-dimethoxybenzoic acid, and 3-hydroxybenzoic acid to give resin-bound peptides **11a**, **11b**, **11c**, **11d**, and **11e**, respectively. The benzoic acids were activated with HBTU (4.9 equiv), HOBt (5 equiv), and DIPEA (10 equiv) just before addition. The resin-bound peptides **11** were then washed, dried, removed from the solid support (with simultaneous side-chain deprotection), and purified as described by HPLC (0% B to 100% B over 20 min, and then 100% B for 5 min, and then 100% B to 0% B over 7 min).

3a (14 mg, 22% yield): MALDI-TOF MS *m/z*: 1171.8 $[M + Na]^+$ (calcd for $C_{53}H_{85}ClN_{12}O_{14}$, 1148.5997); HPLC retention time = 30.150 min.

3b (10 mg, 15% yield): MALDI-TOF MS *m/z*: 1202.0 $[M + Na]^+$ (calcd for $C_{54}H_{87}ClN_{12}O_{15}$, 1178.6102); HPLC retention time = 30.067 min.

3c (11 mg, 17% yield); MALDI-TOF MS *m/z*: 1201.6 $[M + Na]^+$ (calcd for $C_{54}H_{87}ClN_{12}O_{15}$, 1178.6102); HPLC retention time = 30.500 min.

3d (9 mg, 14% yield): MALDI-TOF MS *m/z*: 1175.7 $[M + H]^+$, 1197.7 $[M + Na]^+$ (calcd for $C_{55}H_{90}N_{12}O_{16}$, 1174.6598): HPLC retention time = 29.450 min.

3e (11 mg, 18% yield): MALDI-TOF MS *m/z*: 1154.3 $[M + Na]^+$ (calcd for $C_{53}H_{86}N_{12}O_{15}$, 1130.6336); HPLC retention time = 27.750 min.

For ^{1}H NMR data, see Tables 1 and 2, Supporting Information.

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Supporting Information Available: The NMR data and experimental procedure for the biological assay. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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