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Oxathiocoraline: Lessons to be Learned from the Synthesis of Complex *N*-Methylated Depsipeptides

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Investigations into the synthesis of oxathiocoraline, a bicyclic depsipeptide with C_2 symmetry, revealed a number of unexpected side-reactions that could not be circumvented by classical or standard means. This cyclodepsipeptide has a large number of *N*-methyl amino acids coexisting with two ester bonds and also shows a branched structure; these features hinder its synthesis. In addition, complexity is further increased because of the presence of a large number of non-commercially available cysteines and heterocyclic moieties. Here we describe the general points that should be addressed when attempting the synthesis of a cyclodepsipeptide, such as strategies to prevent or minimize diketopiperazine formation, β-elimination, and oxidation byproducts. The optimum design includes a suitable solid support, the choice of the best starting point for the synthesis (first amino acid to anchor to the resin) and a set of compatible and/or protecting groups and coupling reagents.

Introduction

Cyclic depsipeptides of marine and terrestrial origin are frequently found to be novel potent compounds.[1–3] The development of the chemical synthesis of these compounds is required in order to verify their structures and to provide enough material for preclinical studies. Moreover, control of their syntheses allows researchers to examine the potential to produce new analogues with improved pharmaceutical and pharmacokinetic properties. Given the complex structures of this class of compounds, their synthesis implies the fine-tuning of standard peptide chemistry procedures. In this regard, the ester bonds present in depsipeptides present a double drawback: low reactivity of alcohols in the coupling reactions and fragile break points in the synthetic approach.

Oxathiocoraline[4] is a bicyclic depsipeptide with C_2 symmetry. It is made up of two antiparallel tetrapeptide chains linked by two ester bonds and an additional disulfide bridge in the central part of the molecule (Figure 1). From a synthetic point of view, the presence of a large number of *N*-methyl amino acids coexisting with two ester bonds is a bottleneck because of side-reactions associated with these two features.

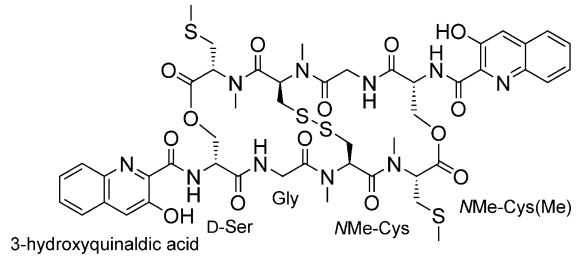

Figure 1. Structure and composition of oxathiocoraline.

In addition, the synthetic difficulty is further increased as a result of its branched structure and the presence of a large number of non-commercially available cysteines and heterocyclic moieties. Oxathiocoraline thus represents a challenge that requires the re-evaluation of standard peptide procedures. Recently, the first synthesis of oxathiocoraline has been achieved by an optimized solid-phase approach, through a key intermolecular disulfide bond formation on solid-support.[4] Together with its amide analogue,[5,6] oxathiocoraline was designed with the aim of producing a more resistant compound displaying antitumour activity comparable to that of the natural compound thiocoraline.[7,8] Here we discuss and analyse the procedures followed to achieve the synthesis of oxathiocoraline. Our strategy was based on the following steps: firstly, the design and evaluation of the possible synthetic approaches, taking into account the C_2 symmetry of the molecule and the distinct starting points, and secondly, an exhaustive explora-

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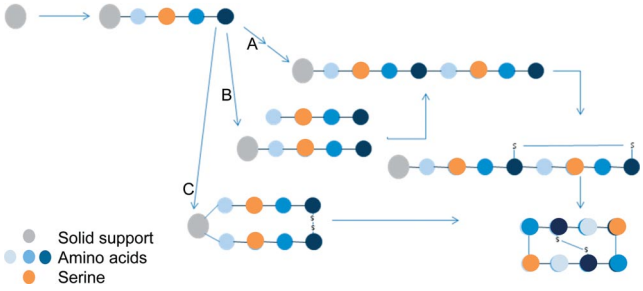
tion of the most feasible synthetic approaches, paying special attention to the most relevant side reactions: diketopiperazine (DKP) formation, ester stability and Cys-based side-reactions. Valuable information from the side-reactions encountered should be usable to establish a set of guidelines for subsequent research in cyclic depsipeptide chemistry.

Results and Discussion

1. Analysis of Possible Synthetic Approaches

1.1. Symmetry in the Synthetic Peptide Design

By consideration of the symmetry of oxathiocoraline, the number of synthetic steps was reduced, and the number of side reactions minimized. Oxathiocoraline should thus be better constructed by fragment coupling (Scheme 1, B) or by fragment dimerization (Scheme 1, C) than by a stepwise approach (Scheme 1, A). In the first two cases, only one ester bond within the peptide chain would need to be constructed, because the second one would be circumvented by fragment coupling. All the drawbacks associated with the presence of ester bonds would therefore be reduced.



Scheme 1. Possible strategies for the construction of a symmetrical peptide: A) stepwise approach, B) 4+4 fragment coupling, or C) 4+4 fragment dimerization.

1.2. Choice of the Amino Acid Attached to the Solid Support

Given the bicyclic structure of oxathiocoraline, the first amino acid will be involved in three crucial steps: in correct peptide chain elongation, in fragment coupling and in the final cyclization as the *C*-terminal amino acid. In a first analysis, none of the four amino acids that make up the oxathiocoraline can either be discounted or chosen as an obvious starting point, and an accurate evaluation of each is required. Each possibility must be analysed in terms of

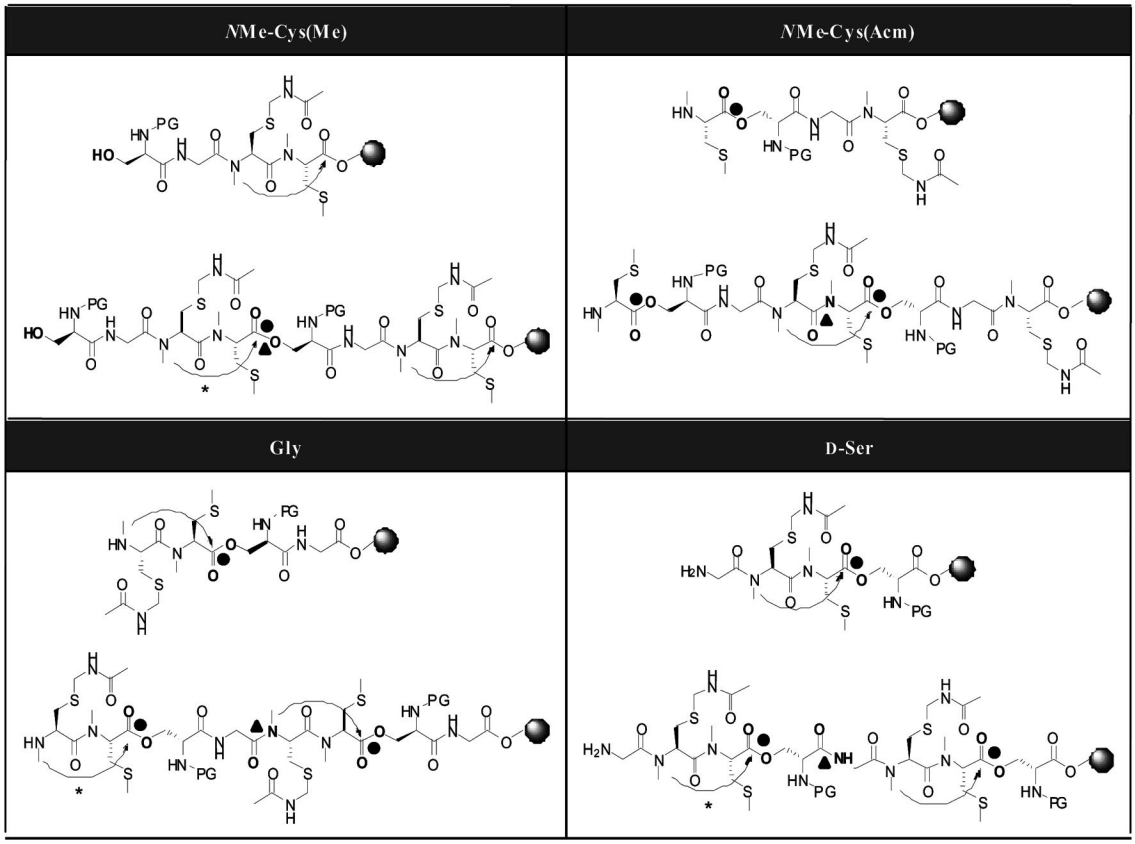


Figure 2. Graphic representation of potential side-reactions for each starting point in oxathiocoraline synthesis. ● indicates the ester bond; ▲ indicates the fragment coupling bond. * DKP formation will take place only when a total stepwise elongation is carried out; if a convergent approach is used this DKP formation does not occur.

the risk of DKP formation, racemization and – when Cys is the *C*-terminal amino acid – racemization and β-elimination, as well as difficulties both in fragment coupling and in cyclization. Figure 2 illustrates the four starting points and their associated risks.

NMe-Cys(Me)

Disadvantages: Cys as *C*-terminal amino acid is associated with racemization and β-elimination as side-reactions.[9–12] Moreover, two *N*Me-amino acids at the C terminus are highly prone to give DKP formation.[13] *Advantages*: Having D-Ser at the last position allows the introduction of the heterocycle after elongation of the tetrapeptidic chain. A further advantage is that the ester bond, which is highly labile, is constructed in the last step.

NMe-Cys(Acm)

Disadvantages: The same problems as those associated with the presence of a Cys at the C terminus. Moreover, a fragment coupling approach or a cyclization requires coupling between two *N*Me-amino acids. *Advantages*: There should be slightly less DKP formation because there are no consecutive *N*-methyl amino acids, although the Gly-*N*Me-AA sequence is also prone to give DKP.

Gly

Disadvantages: The main limitation of this approach is DKP formation, which can occur in the two key steps: 4+4 fragment coupling and cyclization. *Advantages*: No racemization will occur when loading the amino acid on the resin or in a 4+4 fragment coupling or cyclization. No DKP formation will occur at the resin level because the second residue (D-Ser) is elongated through its side chain.

D-Ser

Disadvantages: The ester bond is constructed at a very early stage in the sequence and is therefore more exposed to protecting group removal conditions that may break it. *Advantages*: The fragment coupling and the cyclization step should proceed smoothly because the two residues involved are the non-methylated amino acids (D-Ser and Gly). Furthermore, because the depsipeptide is elongated through the side chain, no DKP should be formed at the resin level.

Since none of the four possibilities is a priori exempt from possible side-reactions, the four starting points were explored in various synthetic stages: tetrapeptide synthesis, octapeptide elongation, cyclization and heterocycle moiety incorporation.

2. Difficulties and Successes in Peptide Elongation

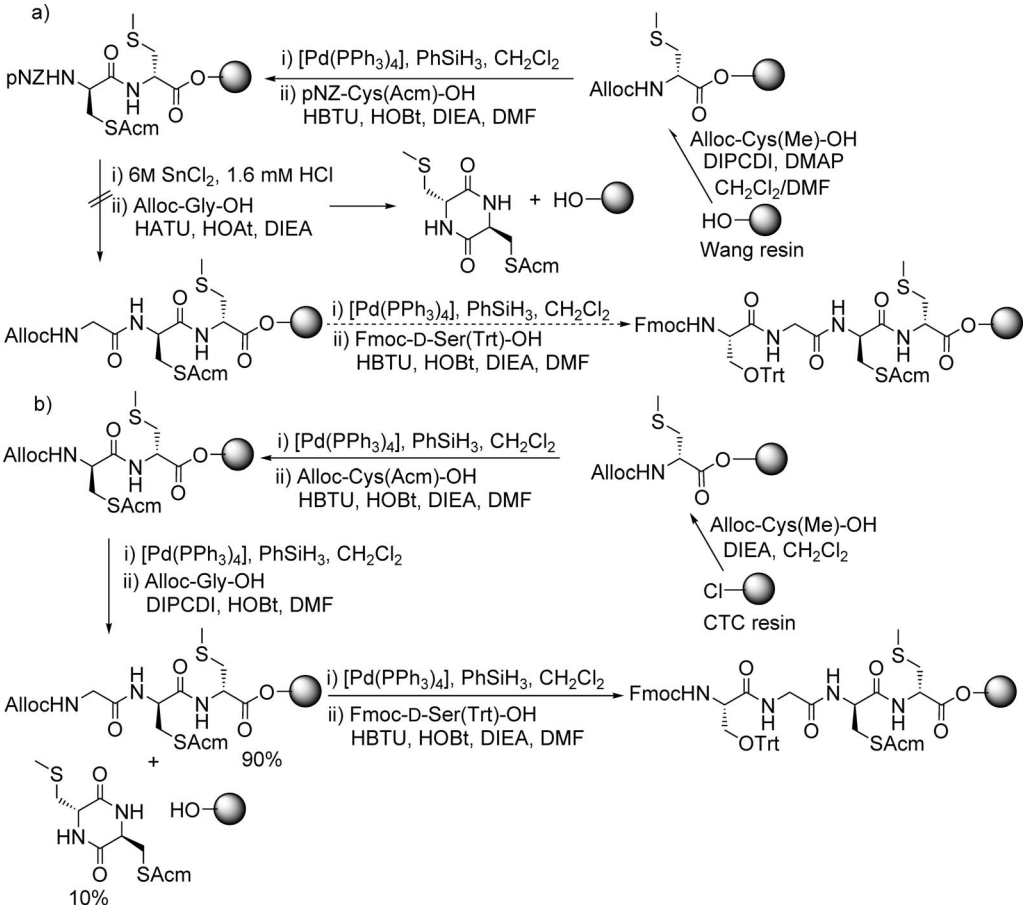
2.1. DKP Formation Throughout the Sequence

DKP formation is a side-reaction frequently encountered at the unprotected dipeptidyl-resin level. This secondary reaction is highly sequence-dependent, although some trends for its formation have been described.[13] Combinations of L- and D-amino acids, as well as the presence of one *N*-methylamino acid – or, worse still, two *N*-methylamino acids – favour DKP formation and must be treated carefully. A number of general precautions have been described, such as the use of highly steric hindered resins (e.g., CTC resin, which protects the carboxylic function[14–16]) or protecting groups that are removed under acidic or almost neutral conditions.[17–20] In depsipeptide syntheses, the presence of labile ester bonds in the peptide chain provides another potential point for DKP formation. This process is favoured by the flexibility of the growing chain together with the absence of protection from the solid-support steric hindrance. A general approach to achieving long peptides or complex sequences is the use of the fragment coupling approach. In this case, the active ester of the fragment to be coupled to the peptidyl resin is formed in situ in solution. However, this active fragment can also undergo DKP formation under specific conditions, as we encountered in this synthetic exploration. DKP formation can therefore occur at the resin level, at the intrachain level and at the interchain fragment activation level.

a) At the Resin Level

When Cys(Me) is the first amino acid [Figure 2, *N*Me-Cys(Me)], DKP formation can occur on deprotection of the dipeptide, because the sequence *N*Me-Cys(Acm)-*N*Me-Cys(Me) (two consecutive *N*Me-AAs) is highly prone to give DKPs. The formation of the tetrapeptides was thus first examined with use of non-methylated Cys, both on Wang resin and on CTC resin.[21] Two strategies were used (Scheme 2). For Wang resin, Alloc-Cys(Me)-OH (Scheme 2, a) was introduced as the first amino acid, and the pNZ group was used for protection of the amino group of the second residue, Cys(Acm), to keep the amino group protonated and therefore to minimize DKP formation.[20] Alloc-Gly-OH was introduced as a third amino acid, and finally Fmoc-D-Ser(Trt)-OH completed the tetrapeptide. In this approach, cleavage of the Fmoc group was used to quantify the resin loading. For CTC resin (Scheme 2, b), the tripeptide was elongated through Alloc chemistry, because pNZ acidic removal conditions result in partial detachment of the peptidic chain. As in the previous strategy, Fmoc-D-Ser(Trt)-OH was also used as fourth amino acid.

For the synthesis with Wang resin, Fmoc quantitation showed 100% DKP formation, thereby confirming that DKPs form very rapidly and that protonation of the amino group is not sufficient to prevent this process. A high degree of DKP formation was also observed for the synthesis with CTC resin, although it was minimized by an optimized *Neutral Removal Coupling Step*: the rapid removal of the Alloc group (2 × 10 min) followed by two consecutive fast couplings of Alloc-Gly-OH with HATU/HOAt with fewer equiv. of base. The most reactive aminium salt, HATU (5 equiv.), which is specially indicated for *N*Me-amino acid coupling, was used, although with a smaller amount of base (4.8 equiv.) than used in standard procedures (10–15 equiv.). In this case, we suspect that 4.8 equiv. of active ester was formed and that it reacted more rapidly with the

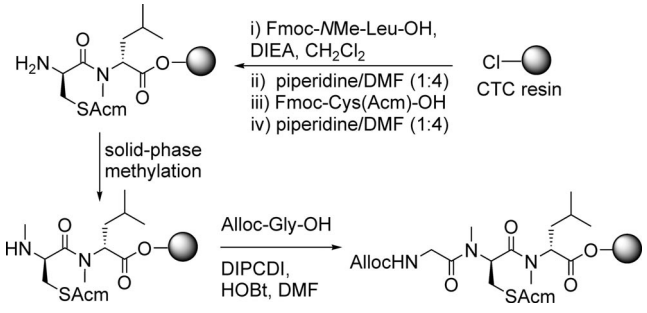


Scheme 2. Study of DKP formation on Wang and CTC resin.

*N*Me-amino acid. To ensure completion of the coupling, a re-coupling was performed under the same conditions. A negative De Clercq test[22] indicated total conversion.

When the synthesis was attempted with *N*Me-amino acids and Wang resin, a further limitation was encountered: the acidic treatment (50% TFA in CH_2Cl_2) applied to release the protected dipeptide from the resin resulted in loss of the *N*Me-amino acid at the *C*-terminal position. This side-reaction had been described in the case of Boc chemistry.[23,24] When CTC resin was used, the dipeptide Alloc-Gly-*N*Me-Cys(Acm)-OH was coupled to H-*N*Me-Cys(Me)-*O*-CTC. The coupling gave the desired tripeptide although a high degree of racemization at the *N*Me-Cys(Acm) site was observed; Cys at the C terminus is prone to give racemization with a further increase if the residue is *N*-acylated.

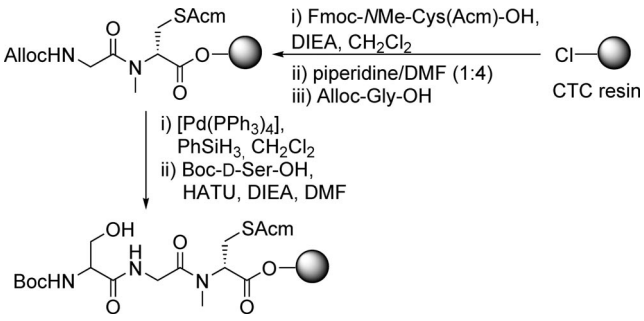
Finally, a stepwise approach was attempted by application of Alloc chemistry and by minimization of reaction time and the basicity. Shorter treatments for Alloc removal were therefore applied (2×10 min), and a deficit of DIEA was used (0.8 equiv.) for the coupling step. The tripeptide Alloc-Gly-*N*Me-Cys(Acm)-*N*Me-Cys(Me)-OH was obtained as a major compound in 70% yield. It is well known that DKP formation is highly sequence-dependent. Previous assembly of the tripeptides Alloc-Gly-*N*Me-Leu-*N*Me-Leu-OH by standard Fmoc chemistry and Alloc-Gly-*N*Me-Cys(Acm)-*N*Me-Leu-OH by solid-phase *N*-methylation[25] had provided the tripeptides in high yields. Solid-phase *N*-methylation, which requires a long period of time in basic media, was carried out on the dipeptidyl resin H-Cys(Acm)-*N*Me-Leu-*O*-CTC, and Alloc-Gly-OH was then introduced without any loss of yield (Scheme 3).



Scheme 3. Synthesis of the tripeptide Alloc-Gly-*N*Me-Cys(Acm)-*N*Me-Leu-*O*-CTC resin with use of methylation on solid phase at the second amino acid.

When *N*Me-Cys(Acm) was used as the first amino acid [Figure 2, *N*Me-Cys(Acm)], DKP formation occurred at the Gly deprotection step. In this case, the use of the Alloc

group to protect the second amino acid, combined with preactivation of the third amino acid (Boc-D-Ser-OH), ensured high yields of the desired tripeptide (Scheme 4).

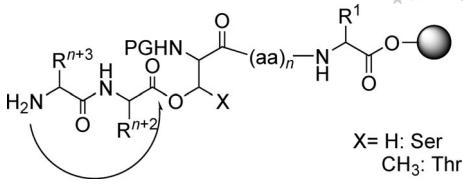


Scheme 4. Synthesis of tripeptide Boc-D-Ser-Gly-*N*Me-Cys(Acm)-*O*-CTC resin.

Starting the synthesis at Gly (Figure 2, Gly) has the advantage that it prevents DKP formation at the resin level because the elongation of the peptide chain takes place through the side chain of D-Ser. The tetrapeptide [H-*N*Me-Cys(Acm)-*N*Me-Cys(Me)&][Boc-D-Ser(&)-Gly-*O*-CTC][26] was thus obtained in good yield and purity. However, the problem is that DKPs can form through the Ser ester bond, as further explained in the next section.

b) At the Ester Level in the Peptide Chain

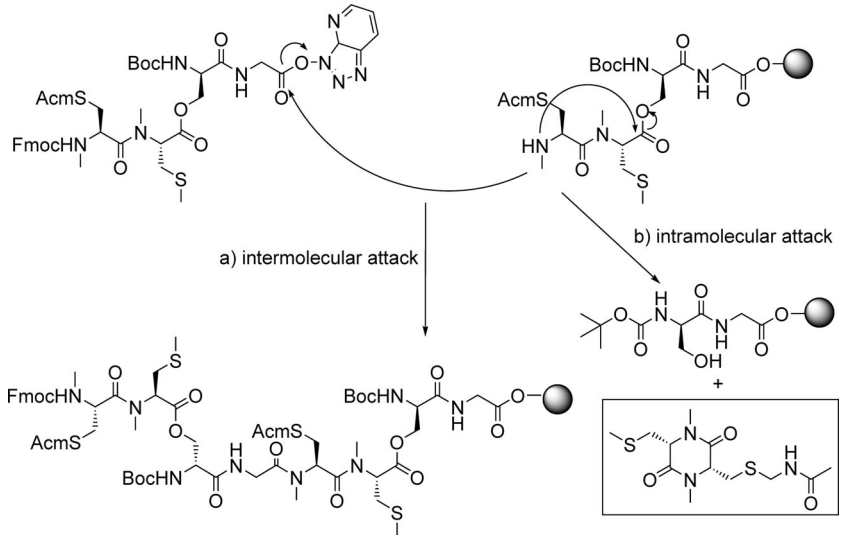
The synthesis of depsipeptides involves the presence of an ester bond in the sequence. In many synthetic approaches, this bond is located within the growing peptide chain and there is a risk of DKP formation after deprotection of the second amino acid after the ester bond (Scheme 5). The growing peptide chain is now more flexible and the effect of hindrance protection from the solid support is no longer noticeable.



Scheme 5. DKP formation by attack on the ester bond within the peptide chain during depsipeptide synthesis.

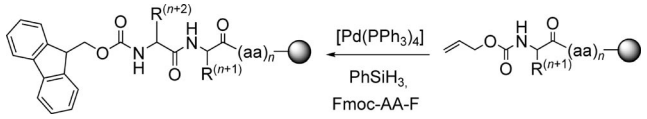
Starting the tetrapeptide at Gly (Figure 2, Gly) prevents DKP formation at the resin level. However, DKP formation at the D-Ser ester bond can occur during fragment coupling. We tested two sets of coupling conditions: DIPCDI/HOAt and PyOAP/DIEA. The neutral conditions were used to minimize DKP formation and to preserve ester integrity through the use of non-basic media, whereas the harsher conditions were assayed to favour a rapid coupling reaction.[27] In all cases, coupling with DIPCDI/HOAt resulted in the recovery of the unreacted tetrapeptide. For coupling with PyOAP/DIEA, low levels of conversion were achieved because of the basic conditions. Treatment with the protected Fmoc fragment resulted in a preferential intramolecular attack (DKP formation), rather than the desired intermolecular coupling (Scheme 6). Analysis of the peptidyl resin after a 24 h coupling period thus showed the presence of the octapeptide but also the appearance of the dipeptide Boc-D-Ser-Gly-OH. Analysis of the coupling solution showed the presence of the DKP [&*N*Me-Cys(Acm)-*N*Me-Cys(Me)&].

When the synthesis was started at D-Ser (Figure 2, D-Ser) and Alloc chemistry was used, only a 60% yield was obtained for the tetrapeptide {[Alloc-Gly-*N*Me-Cys(Acm)-*N*Me-Cys(Me)&][Boc-D-Ser(&)-*O*-CTC]}. The byproducts indicated DKP formation through the D-Ser ester bond. To suppress DKP formation, we used the tandem deprotection/coupling approach described by Thieriet et al.[19] This ap-



Scheme 6. Fragment coupling products starting at Gly.

proach uses an Alloc removal cocktail in which the incoming Fmoc-protected amino acid is included in fluoride-activated form (Scheme 7).



Scheme 7. Deprotection/coupling tandem reaction described by Thieriet et al. (Ref. 19).

However, the use of this approach was incompatible with CTC resin. The peptidyl resin bond was unstable to the HF generated from the tandem reaction and the incoming activated Fmoc-Gly-F ended up attached to the resin, so only Fmoc-Gly-OH was recovered at the cleavage step. A second approach was assayed by use of the optimized *Neutral Removal Coupling step* described earlier (Figure 3).

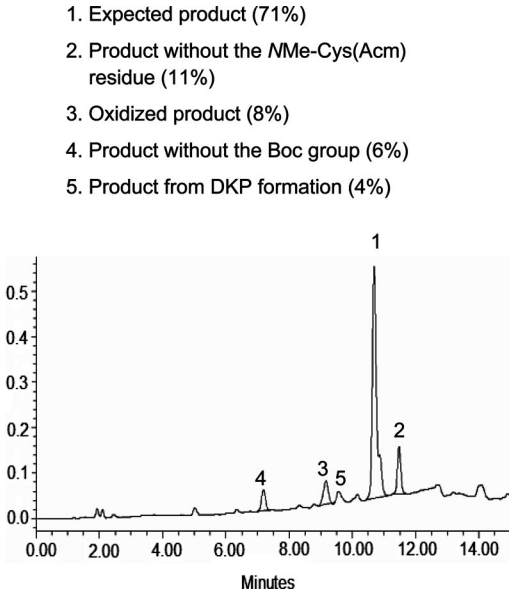


Figure 3. HPLC analysis from Fmoc-tetrapeptidyl resin starting at D-Ser. HPLC conditions: gradient from 8:2 to 0:10 in 15 min (A: H_2O with 0.045% TFA and B: ACN with 0.036% TFA).

We performed another synthesis using Alloc-*N*Me-Leu-OH instead of both Alloc-*N*Me-Cys(Me)-OH and Alloc-*N*Me-Cys(Acm)-OH. This approach revealed the effect of chain flexibility. The first tetrapeptide {[Alloc-Gly-*N*Me-Leu-*N*Me-Leu&][Boc-D-Ser(&)-*O*-CTC]} was obtained as a pure compound, thereby indicating the absence of DKP formation. After elongation of the peptide chain by a stepwise approach, it was found that the second ester was more labile, and the final octadepsipeptide was achieved in 50% yield, with the other 50% corresponding to the hexapeptide arising from the loss of the DKP (Figure 4). In this case the second ester bond showed more flexibility and so more DKP was formed.

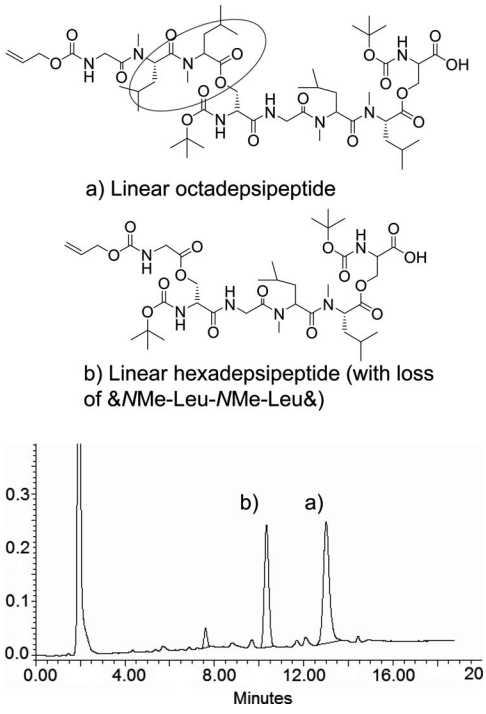


Figure 4. HPLC analysis and structures of linear octadepsipeptide and the byproduct resulting from loss of DKP [&*N*Me-Leu-*N*Me-Leu&]. HPLC conditions: gradient from 7:3 to 0:10 in 15 min (A: H_2O with 0.045% TFA and B: ACN with 0.036% TFA).

c) At the Activated C-Terminal End

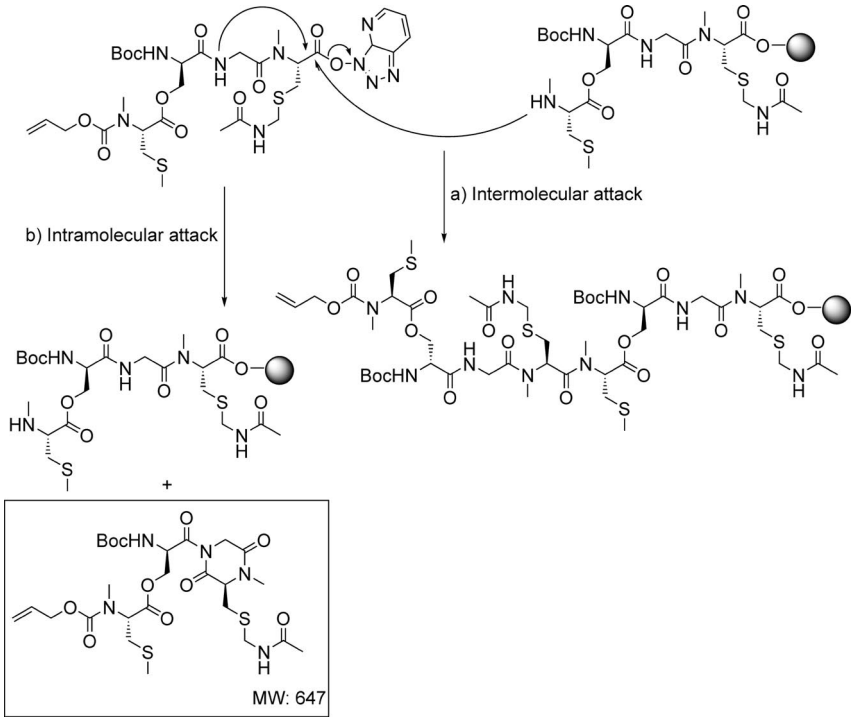
Although the evaluation of *N*Me-Cys(Acm) as a starting point concluded that this approach is not associated with DKP formation either at the resin or at the peptide chain level, it was surprising to detect intramolecular DKP formation at the amide bond level during fragment coupling. The protected tetrapeptide underwent an intramolecular reaction between the Gly amide bond and the activated carboxylic function at the C terminus. There was competition between intermolecular (fragment coupling) and intramolecular (DKP formation) attack, which resulted in a moderate coupling reaction yield of 75% (Scheme 8).

2.2. Protection Scheme and Side-Reactions Associated with Ester Stability

Depsipeptide synthesis is associated with two main problems: reduced reactivity of the hydroxy group in the formation of the ester bond and at the same time high lability of this newly formed bond. In the process of developing the synthesis of oxathiocoraline, we explored the dependence of these two key points on sequence and on protecting group chemistry. Several side-reactions were identified.

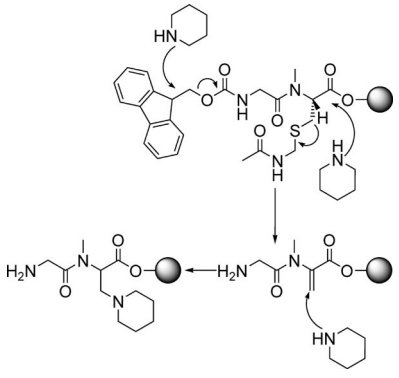
a) β-Elimination at the C Terminus

Acidity of the α-proton of an amino acid residue in combination with the presence of a leaving group at the β-position favours a β-elimination reaction. Nature has translated this into the formation of didehydroalanine from Ser and Cys residues, of the didehydro-α-aminobutyric acid moiety



Scheme 8. Fragment coupling products starting at *N*Me-Cys(Acm).

from Thr, and of didehydrophenylalanine from β-hydroxyphenylalanine.[28–31] During the synthetic process, this β-elimination can be enhanced by the presence of withdrawing groups at the *N*-function (protecting groups).[11,32] β-Elimination byproducts were detected when synthetic approaches began with either D-Ser or Cys residues. When a Fmoc-based strategy was followed, further incorporation of piperidine through a Michael addition to give a 3-(piperidin-1-yl)alanine residue was observed (Scheme 9).[11] The extent of this side-reaction was greater with further Fmoc removal steps. When the peptide chain began with Fmoc-Cys(Acm)-OH, analysis of the corresponding dipeptide H-Gly-Cys-(Acm)-OH and tetrapeptide [Boc-D-Ser(&)-Gly-Cys(Acm)-OH][H-Cys(Me)-&] showed the presence of the *N*Me-3-(piperidin-1-yl)alanine residue instead of *N*Me-Cys(Acm), increasing after each Fmoc treatment.



Scheme 9. Mechanism of *N*Me-3-(piperidin-1-yl)alanine formation.

When the synthesis was repeated with use of a Fmoc protecting group only for the first amino acid, with Alloc groups for Gly and *N*Me-Cys(Me), the tetrapeptide {[Boc-D-Ser(&)-Gly-*N*Me-Cys(Acm)-OH][Alloc-*N*Me-Cys(Me)&]} was obtained free of *N*Me-3-(piperidin-1-yl)alanine. This result indicated that this side-product was formed exclusively when the amino function was *N*-acylated and not during the removal of the Fmoc of the *C*-terminal Cys. The Fmoc-protected group can thus be used for the first amino acid, which is also useful for determination of the initial functionalization, but Alloc chemistry should be used for the following steps.

When the peptide chain began at D-Ser, β-elimination plus piperidine addition was also observed (Figure 5). Analysis of protected and free dipeptide revealed quantitative conversion into the compound QNA-3-(piperidin-1-yl)-alanine. In this case, the combination of the electron-withdrawing *N*-acyl form with the ester bond substantially increased the acidity of the α-proton.

To prevent this side-reaction, a second attempt using a carbamate-protecting group, such as the Boc group, was made (Scheme 10).

Analysis of an aliquot of the final tetrapeptide revealed the presence of the desired compound and the absence of the 3-(piperidin-1-yl)alanine residue. However, another by-product, resulting from the loss of the *N*Me-Leu residue, was detected. This side-reaction was associated with the instability of the ester bond inside the peptide chain, as described in next section. Therefore, D-Ser and Cys residues at the C terminus are subject to two reactions: i) β-elimination with subsequent piperidine incorporation, and

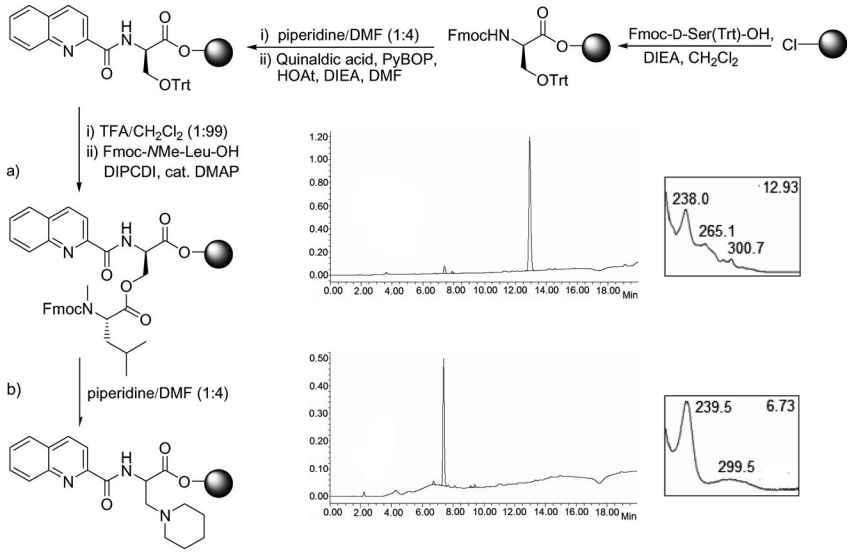
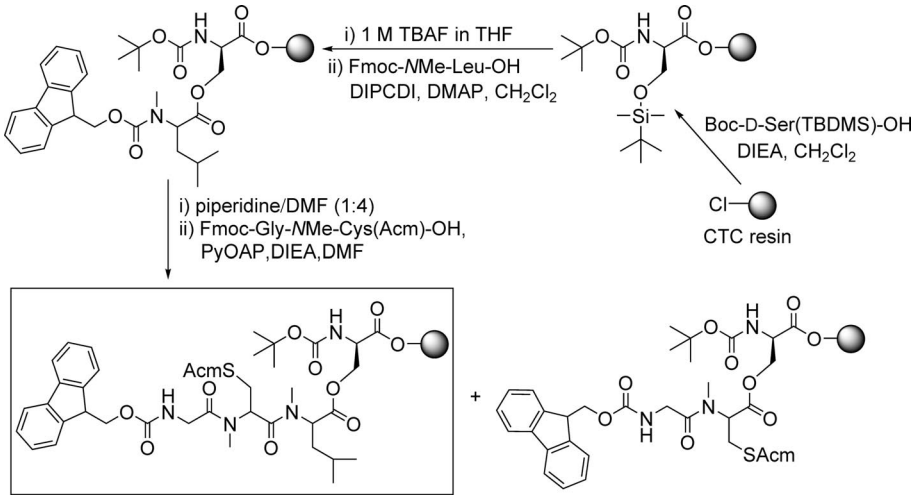


Figure 5. Synthesis of tetrapeptide starting at D-Ser with coupling of the heterocycle on solid support. a) dipeptide {[QNA-D-Ser(&)-*O*-CTC][Fmoc-*N*Me-Leu&]}, and b) compound obtained after piperidine/DMF (1:4) treatment. HPLC conditions: gradient from 7:3 to 0:10 in 15 min (A: H_2O with 0.045% TFA and B: ACN with 0.036% TFA).



Scheme 10. Synthesis of tetrapeptide starting at D-Ser.

ii) loss of the adjacent unprotected amino acid on piperidine treatment once the ester bond is formed.

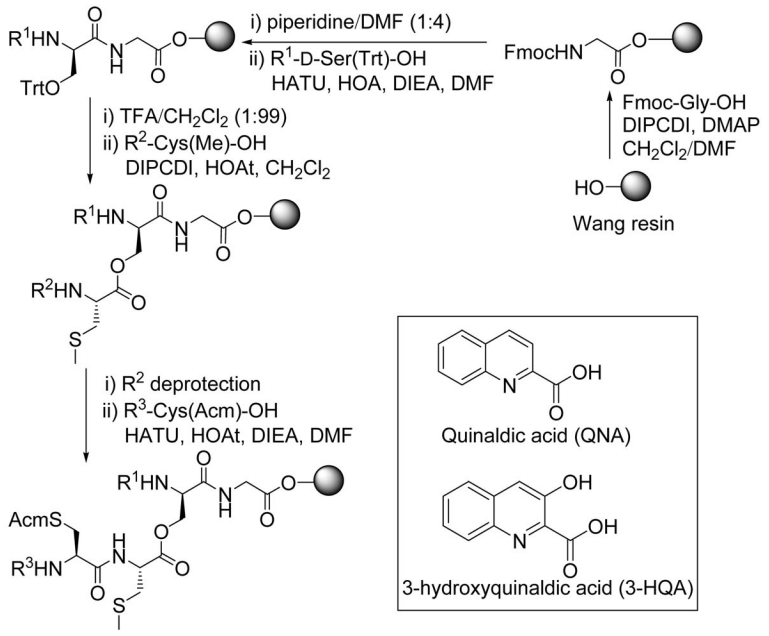
By using Alloc chemistry instead of Fmoc chemistry, we obtained the tetradepsipeptides {[Alloc-Gly-*N*Me-Leu-*N*Me-Leu&][Boc-D-Ser(&)-OH]} and {[Alloc-Gly-*N*Me-Cys(Acm)-*N*Me-Cys(Me)&][Boc-D-Ser(&)-OH]} in high yields and purities.

b) Ester Stability within the Peptide Chain

Natural cyclic depsipeptides generally incorporate a branching point at a Ser/Thr residue, through the associated hydroxy group. The stability and reactivity of the hydroxy group can be affected by protecting group chemistry, coupling conditions and the step at which the branching point is created. When synthesis was started at Gly on Wang resin, several protection strategies (Table 1) were examined. The main limitation was found to reside in ester lability in response to the protecting group removal conditions. In these first attempts, either 3-hydroxyquinaldic acid (3-HQA) or commercial quinaldic acid (QNA) were used (Scheme 11).

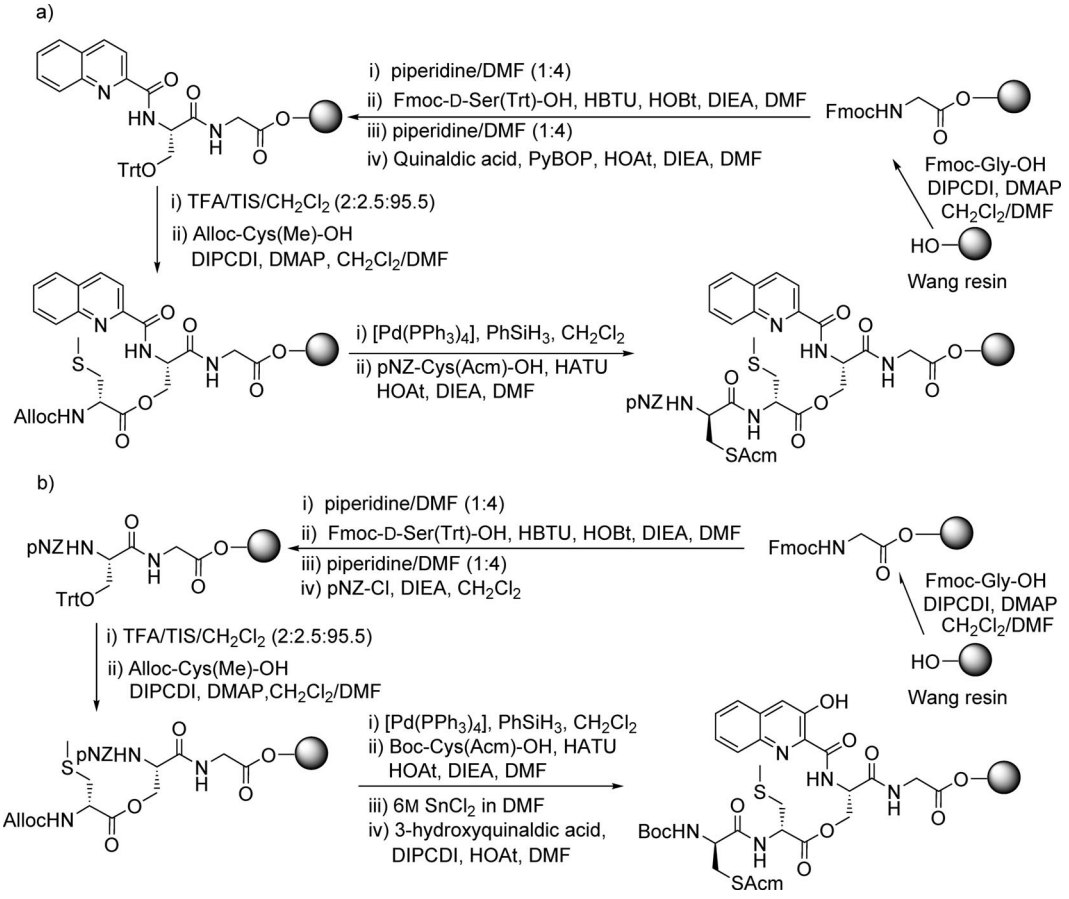
Table 1. Exploring diverse chemistries based on initiation at Gly to afford the tetradepsipeptide.

	R^1	R^2	R^3	Peptide purity	Observations
1	Fmoc	Alloc	pNZ	good	Fmoc cannot be changed at a later stage because of piperidine cleavage
2	QNA	pNZ	pNZ	poor	cleavage of the ester with removal of pNZ at position 2
3	QNA	Alloc	pNZ	good	upon deprotection of the tetrapeptide, partial cleavage of ester occurs
4	QNA	Alloc	Boc	good	the unprotected tetradepsipeptide is obtained
5	3-HQA	Alloc	pNZ	poor	elongation through the OH
6	pNZ	Alloc	Boc	good	



Scheme 11. Synthesis of tetrapeptide starting at Gly.

Table 1 examines the purities of protected tetradepsipeptides obtained when starting at Gly. With use of a combination of Fmoc for D-Ser, Alloc for Cys(Me) and pNZ for Cys(Acm) (Entry 1), a tetradepsipeptide of good purity was obtained. pNZ was introduced at this position in order to minimize DKP formation. However, this strategy has the



Scheme 12. Final strategies for tetradepsipeptides containing a) quinaldic acid, and b) 3-hydroxyquinaldic acid.

drawback that Fmoc can not be removed after the ester has been formed because of cleavage of the ester bond upon treatment with piperidine. Switching to quinaldic acid after the introduction of Fmoc-D-Ser(Trt)-OH solves this problem. Elongation of the peptide by use of pNZ chemistry (Entry 2) gave a protected tetradepsipeptide of low purity because of cleavage of the ester when $SnCl_2$ was used to remove the pNZ group from Cys(Me). When the Alloc group was used instead of pNZ at this position, removal of the Alloc group with $[Pd(PPh_3)_4]$ was clean, without cleavage of the ester functionality. pNZ-Cys(Acm)-OH was coupled as a fourth amino acid, providing the tetradepsipeptide in good purity (Entry 3). Although this strategy can be continued, partial cleavage of the ester bond occurred with the $SnCl_2$ treatment, probably because of DKP formation. This strategy is therefore not appropriate when using *N*Me-AAs, because DKP formation is more favoured. A fourth strategy using Boc-Cys(Acm)-OH was also examined. Cleavage of the peptide from the resin simultaneously removed the Boc group. In this case, the unprotected tetradepsipeptide was obtained in good purity.

To obtain the natural target 3-hydroxyquinaldic-protected tetradepsipeptide, the heterocycle was introduced at the dipeptide level, as in the previous strategy, because this quinolinic OH was found to be unreactive in solution reactions under mild coupling conditions (EDC·HCl/HOSu). The introduction of the third and fourth amino acids, however, resulted in additional elongation through the hydroxy function (Entry 5), and so it was necessary to change the protection scheme in order to couple the heterocycle at a later stage. With this in mind, the dipeptide was treated with piperidine to remove the Fmoc group, and the amino function was reprotected with pNZ-Cl. Ester formation was accomplished as before, by the introduction of Alloc-Cys(Me)-OH, but Boc was used as a protecting group for Cys(Acm). At this stage, the pNZ group was removed, and coupling with 3-hydroxyquinaldic acid was accomplished with DIPCDI/HOAt in DMF. The tetradepsipeptide was obtained in good purities (Entry 6). The final strategies used to obtain the two tetradepsipeptides are thus summarized in Scheme 12. HPLC traces are shown in Figure 6.

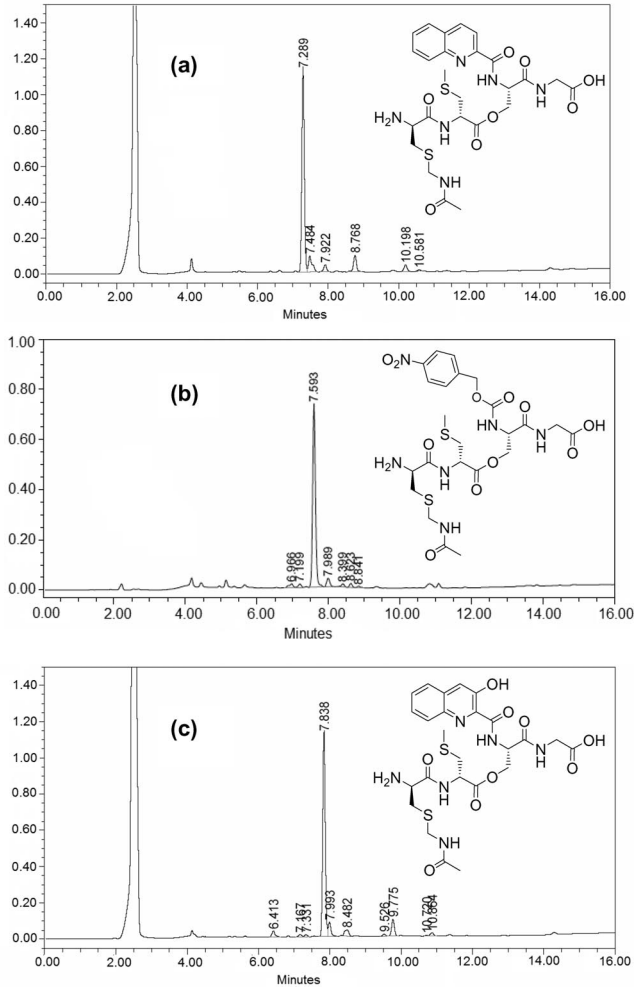


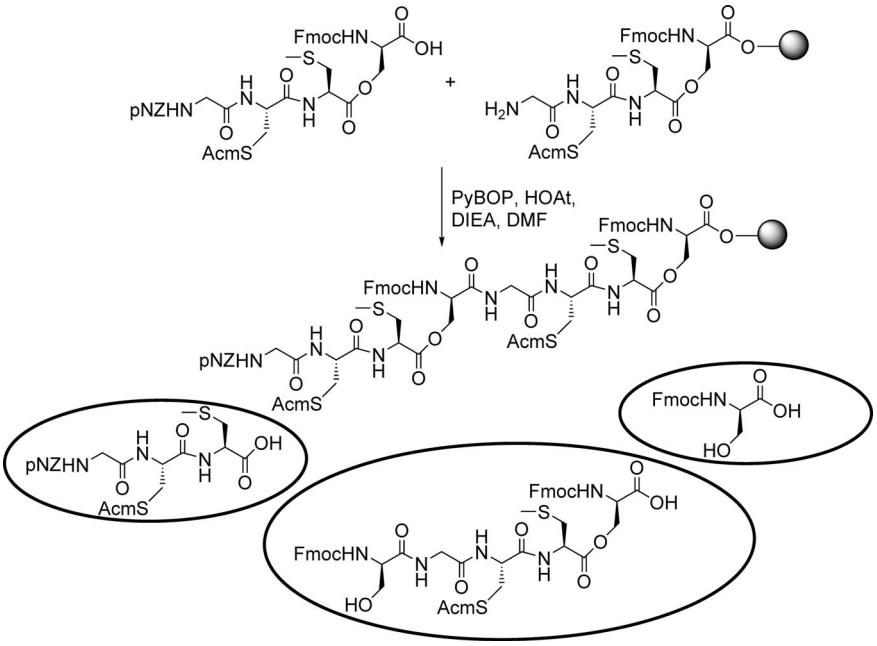
Figure 6. HPLC profiles of a) quinaldic-, b) pNZ-, and c) 3-hydroxyquinaldic-protected tetradepsipeptides.

c) TBDMS as a Convenient Protecting Group for Ser

In the synthesis of cyclic depsipeptides, Ser is commonly used as branching point in the middle of the peptide chain and rarely at the C or N terminus. Usually, Ser is introduced with its free hydroxy group, with careful selection of mild coupling reagents such as DIPCDI/HOBt to prevent acylation of the hydroxy functionality. However, it is sometimes convenient to have a protected Ser: when it is used as a first amino acid, for instance, or if is coupled to a *N*Me-amino acid. These couplings require stronger conditions, and use of the Trt group has been described in these cases. We also explored the use of the *tert*-butyldimethylsilyl (TBDMS) group as a convenient hydroxy moiety protecting group compatible with CTC resin. Although TBDMS is widely used in organic and sugar chemistry,[33] its use in Ser or Thr protection has not been addressed.[34] TBDMS can be incorporated easily and can be removed on solid phase by treatment with commercial TBAF solution. These conditions appear to be compatible both with the peptidyl resin bond and with the other protecting groups, such as Fmoc, Boc, Alloc and pNZ. Boc-D-Ser(TBDMS)-OH was prepared in solution from commercial Boc-D-Ser-OH and TBDMS-Cl in the presence of a base (imidazole or DMAP, 64% yield). The amino acid Boc-D-Ser(TBDMS)-OH was used in the tetradepsipeptide synthesis {[Alloc-Gly-*N*Me-Cys(Acm)-*N*Me-Cys(Me)&][Boc-D-Ser(&)-*O*-CTC]} and {[Alloc-Gly-*N*Me-Leu-*N*Me-Leu&][Boc-D-Ser(&)-*O*-CTC]}, thereby showing the convenience of the TBDMS hydroxy protecting group in SPPS.

d) D-Ser at the C Terminus in Fragment Coupling

When fragment coupling was performed with D-Ser at the C terminus (Scheme 13), three degradation products were recovered instead of the octapeptide, thereby showing the instability of the linear peptide chain. The byproducts obtained – Fmoc-D-Ser-OH, the pentapeptide {[Fmoc-D-Ser(OH)-Gly-Cys(Acm)-Cys(Me)-&][Fmoc-D-Ser(&)]} and the tripeptide pNZ-Gly-Cys(Acm)-Cys(Me)-OH – were a consequence of the breakage of the ester bonds. The second



Scheme 13. Byproducts obtained in the fragment coupling starting at D-Ser.

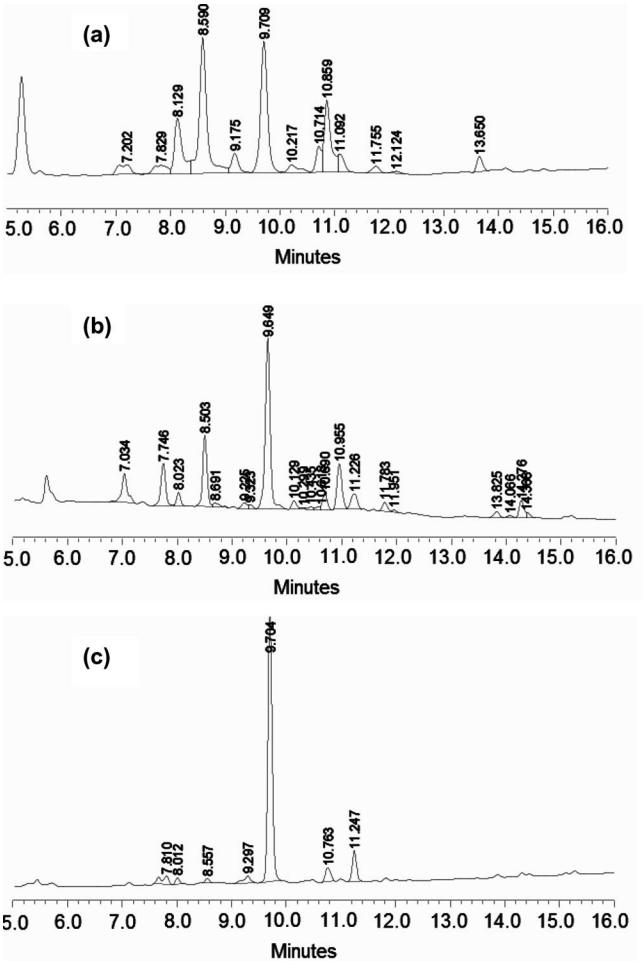


Figure 7. HPLC profiles for cleavage with a) TFA/CH_2Cl_2 (1:4), b) TFA/H_2O/CH_2Cl_2 (20:5:75), and c) TFA/H_2O/CH_2Cl_2 (2:1:7).

ester in particular was very labile, because no product containing this bond was recovered. Because the first ester bond is constructed at a very early stage, D-Ser is not a convenient option for the C terminus in cyclic depsipeptide synthesis because the basic media and long reaction times used in fragment couplings are incompatible with the stability of the linear chain.

2.3. Partial Oxidation of Cys(Me) – Cleavage Optimization

Partial oxidation of Cys(Me) was observed during the cleavage from the resin. Several cleavage cocktails were assayed with the goal of minimizing the amounts both of oxidized product and also of the Wang-linker derived product (Figure 7). Addition of H_2O to the cleavage cocktail improved the purity significantly. Cleavage of the tetradepsipeptide with TFA/CH_2Cl_2 (1:4) thus resulted in a purity of 24%, increasing to 37% by cleavage with TFA/H_2O/CH_2Cl_2 (20:5:75), and to 77% with TFA/H_2O/CH_2Cl_2 (2:1:7). The last cleavage protocol was therefore applied in subsequent syntheses on Wang resin.

3. Intermolecular Dimerization as a Peptide Chain Elongation Step

So far, although we have identified several features to improve the synthetic process, there is still no final strategy that allows the preparation of oxathiocoraline. On the basis of the purities of the obtained tetradepsipeptides, the use of the strategy starting at Gly is the most promising. In this approach, however, as well as in the rest of the strategies, the main problem that remains to be solved is DKP formation when the peptide chain is elongated. As mentioned ear-

lier, stepwise elongation entails a greater risk in terms of DKP formation because the second ester is positioned further away from the solid support and so is more flexible. In the case of 4+4 fragment coupling strategies, approaches that prevent ester bond formation during fragment coupling either have associated DKP formation because of the preference for an intramolecular reaction (i.e., when starting at Gly) or result in an unstable linear peptide (i.e., when starting at D-Ser). Since the use of distinct removal reagents for protecting groups in stepwise or 4+4 fragment coupling failed to prevent DKP formation, the only solution was to restrict the mobility of the peptide chain by making the *N*Me of Cys(Acm) unable to reach the carboxyl of *N*Me-Cys(Me). This was achieved by the formation of the interchain disulfide bridge. This dimerization through disulfide bond formation provided more rigidity and therefore conferred more stability on the peptide chain, thereby preventing DKP formation (Figure 8). Several optimizations were performed to explore the potential of this approach and to achieve high purities and yields in the dimer formation as the step prior to the final double cyclization.

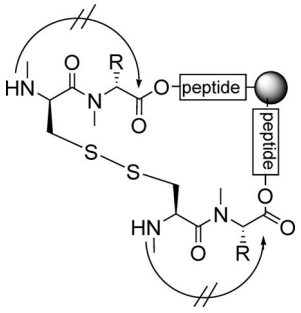


Figure 8. The intermolecular disulfide bond suppresses DKP formation by restricting the mobility of the peptide chain.

3.1. Protected versus Unprotected Tetrapeptide

When the tetradepsipeptide chain was started at Cys(Me), several options for the coupling of the last amino acid were tested, such as the introduction of unprotected Boc-D-Ser-OH and also of Fmoc-D-Ser(Trt)-OH, followed by the introduction of the quinaldic unit. We then examined the effect of the protecting group in the dimerization step. In all cases, the formation of the dimer was achieved through two treatments with I_2 (5 equiv., 10 min) in DMF, as shown in Scheme 14 and Figure 9.

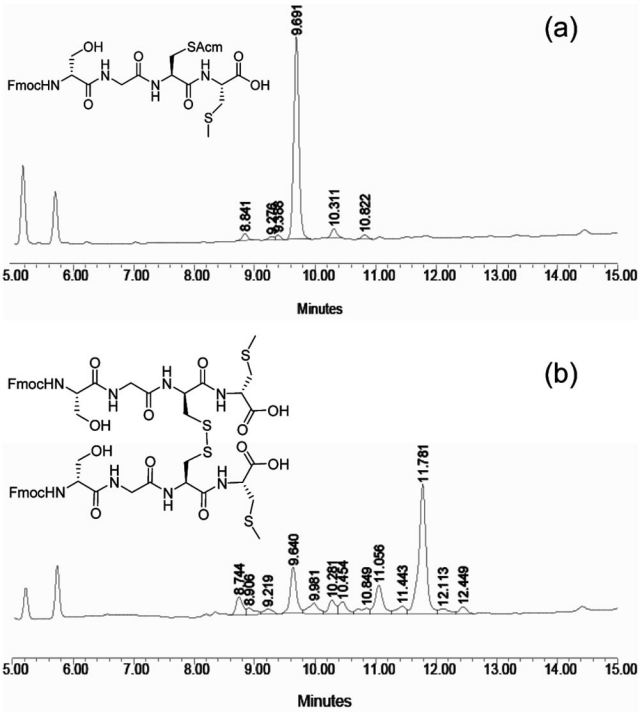
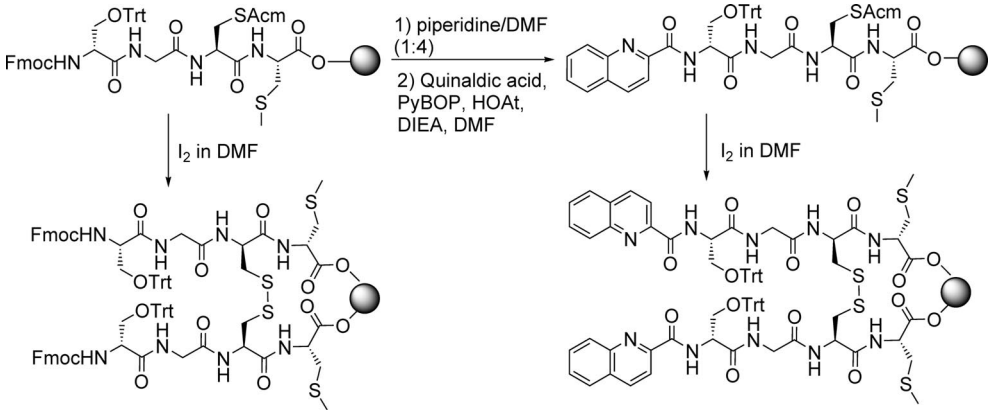
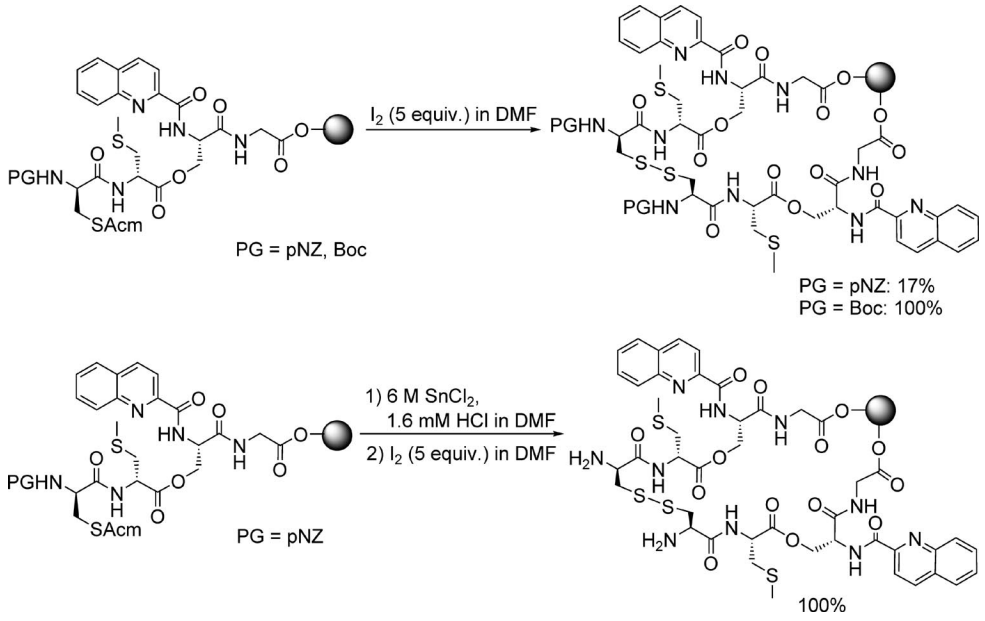


Figure 9. HPLC profiles of a) Fmoc-protected tetradepsipeptide, and b) tetradepsipeptide dimer.

We also evaluated dimer formation by the protected and unprotected tetrapeptide when the peptide chain was started with Gly. Scheme 15 shows that the first attempts were carried out with pNZ protection, Boc protection or use of *N*-unprotected Cys(Acm). For these purposes, one portion of the pNZ-protected resin-bound tetrapeptide was treated with $SnCl_2$ to cleave the pNZ group, and the resins were treated with I_2 (5 equiv.) in order to form the disulfide bridge. After two 10 min treatments, the degree of conversion to the protected dimer was only 17%, whereas full conversion to the unprotected dimer had been achieved. In



Scheme 14. Dimerization by disulfide bridge formation.

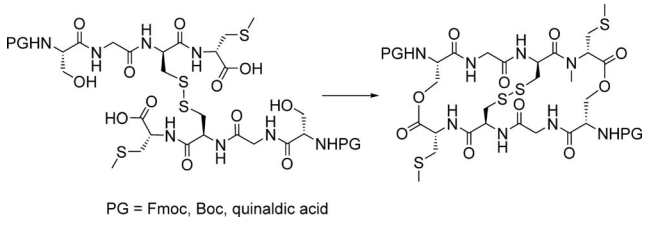


Scheme 15. Dimerization conversions with protected and unprotected tetrapeptides.

contrast, when the Boc-protected tetradepsipeptide was used, conversion was complete, thereby suggesting that the electronic behaviour of the pNZ group impedes the progress of the reaction. In view of the results, the best and safest option is to use the Boc-protected tetradepsipeptide. This approach presents three advantages: Boc-D-Ser-OH is commercially available, the dimer is quantitatively formed, and the cleavage from the resin simultaneously removes the Boc groups, thereby providing the unprotected dimer of tetrapeptides ready for the final cyclization step.

3.2. Double Lactonization – Reactivity in Ester Formation as a Function of the Amino Protecting Group of D-Ser

As described above, when the tetradepsipeptide was started at Cys(Me), the D-Ser residue was introduced as a fourth amino acid in several protected forms, and dimerization was carried out. The next step consisted of the double lactonization of the dimer to afford the bicyclic skeleton (Scheme 16). The three protected dimers (Fmoc, Boc and quinaldic acid) were tested to examine the influences of: a) carbamate versus amide functionality, and b) distinct protecting groups.



Scheme 16. Bis-lactonization from the dimer precursor.

Lactonization was tested with two coupling systems commonly used for ester formation: DIPCDI/DMAP and MSNT/DIEA. For the Fmoc- and Boc-protected dimers, the formation of bicyclic depsipeptide was observed with the DIPCDI/DMAP system, although the rate of cyclization was slow and complete cyclization was not achieved. Although MSNT/DIEA[35] is a more potent coupling system for esterification than DIPCDI/DMAP, cyclization was not detected. One possible explanation for this observation is that the excess of base used in this coupling breaks the ester functionality. This notion is supported by the observation that attempts to remove the Fmoc group from the pro-

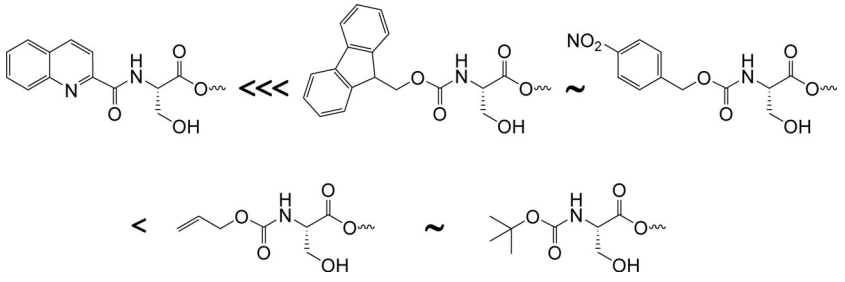


Figure 10. Reactivity in the formation of the ester bond depending on the group present at the amino terminus of D-Ser.

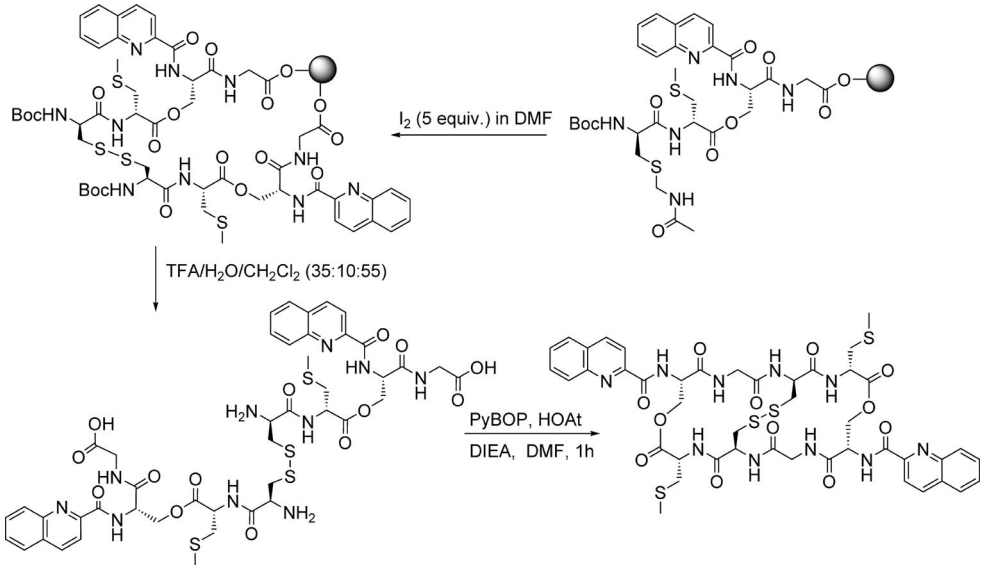
tected bicyclic depsipeptide with piperidine or diethylamine resulted in loss of the compound as a result of degradation. When quinaldic acid protection was present, no cyclization to form the bicyclic depsipeptide was observed in any coupling system.

Thus, from these results and from the behaviour observed during the elongation of the tetrapeptidyl chain with use of the different protection schemes, we conclude that the reactivity of the ester bond is greatly influenced by the nature of the group (amide or carbamate) present at the amino terminus of the Ser residue. With regard to the carbamate-type protecting groups, the electron-withdrawing capacity of the group determines the reactivity in the ester formation and also the difficulty in removing the protecting group at the D-Ser side-chain (Figure 10).

Given the problems of low reactivity encountered in the double lactonization, our efforts focused on strategies with lactamization as the final step.

3.3. Lactamization – Obtaining Demethylated Oxathiocoraline Analogues

When the peptide chain was started at Gly on Wang resin, Boc-Cys(Acm)-OH was introduced as the fourth amino acid. As described above, the two tetradepsipeptides (one bearing the quinaldic acid and the other the natural 3-hydroxyquinaldic acid) were obtained in good purities (Fig-



Scheme 17. Final steps in the synthesis of demethylated analogues of oxathiocoraline.

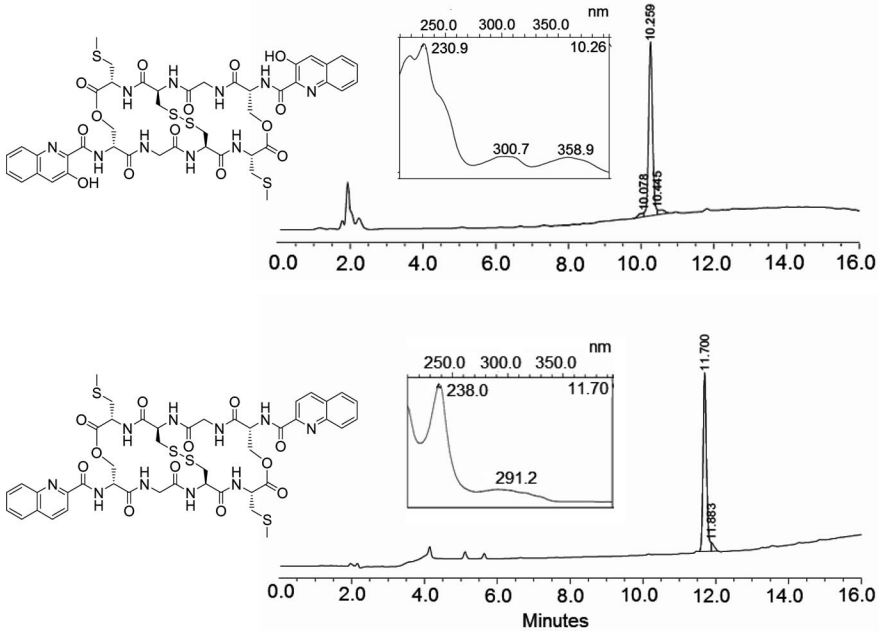
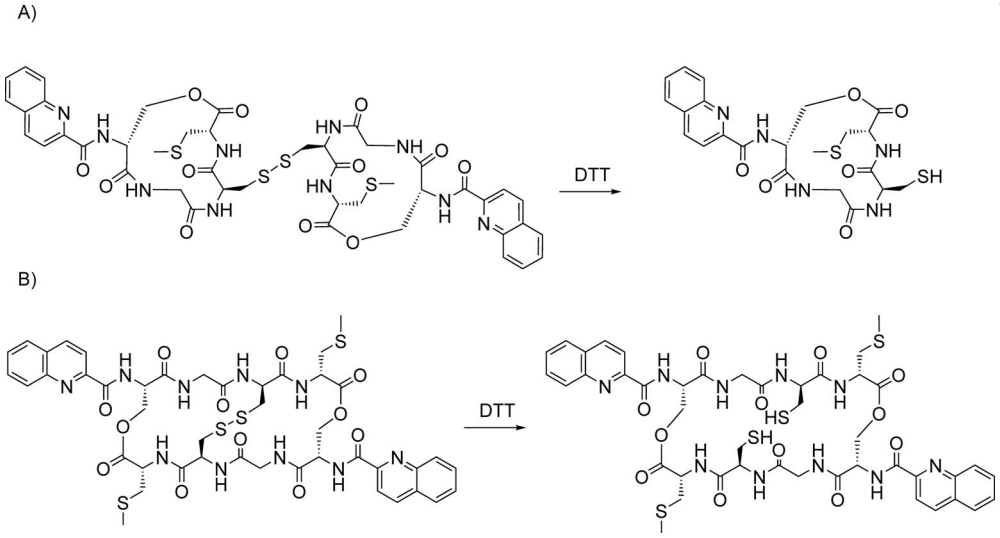


Figure 11. Structures, UV spectra and HPLC analysis of the final demethylated analogues of oxathiocoraline.



Scheme 18. Disulfide reduction by addition of DTT.

ure 6). The two tetradepsipeptides were subsequently dimerized, cleaved and subjected to lactamization by treatment with PyBOP and HOAt in DMF at pH 8 (DIEA) (Scheme 17). The lactamization proceeded smoothly and was complete in 1 h. After workup and purification by semipreparative HPLC, two analogues of oxathiocoraline, {[3-HQA-D-Ser(&[1])-Gly-Cys(&[2])-Cys(Me)&[3]] [3-HQA-D-Ser(&[3])-Gly-Cys(&[2])-Cys(Me)&[1]]} and {[QNA-D-Ser(&[1])-Gly-Cys(&[2])-Cys(Me)&[3]] [QNA-D-Ser(&[3])-Gly-Cys(&[2])-Cys(Me)&[1]]}, were obtained (Figure 11).

3.4. Reduction of the Disulfide Bridge with DTT

To rule out the possibility of two cyclotetradepsipeptides linked by a disulfide bridge (Scheme 18, A), the quinaldic acid cyclodepsipeptide was subjected to disulfide reduction by exposure to DTT in a denaturating buffer (6 M Gdn·HCl, 1 mM EDTA, 0.1 M Tris·HCl at pH 8.7) under N_2 (Scheme 18). Under these conditions, only a compound with two units of mass more than the bicyclic depsipeptide was observed, which corresponded to the reduced octacyclodepsipeptide (Scheme 18, B).

Conclusion and Final Strategy

A number of factors must be considered when attempting the synthesis of a sophisticated depsipeptide. The main limitations arise from the ester functionality, which, in combination with the presence of consecutive *N*Me-amino acids, numerous Cys residues and a disulfide bridge, requires careful examination and optimization of each step. DKP formation and approaches to overcome this process were explored in several situations. The main trends in the instability and reactivity of the ester bonds were studied, as well as the side-reactions associated with Cys. The new concept of restricting mobility to protect the ester bond against DKP formation was used first to obtain demethylated analogues of oxathiocoraline, and later for the synthesis of oxathiocoraline (Scheme 19).

In the course of the studies directed towards the synthesis of oxathiocoraline, we propose these guidelines for the assembly of other cyclodepsipeptides:

a) If possible, to simplify the synthetic approach by taking the symmetry of the molecule into account.

b) To minimize the risk of DKP formation by: i) avoiding Cys(Me) as the C terminus because it is very prone to give DKPs as a result of the acidity of its β-proton, ii) using Alloc and pNZ groups instead of the Fmoc group,[19,20] iii) using CTC resin, which because of its steric hindrance gives better results than Wang resin,[15,16] and iv) restricting the flexibility of the peptide chain by, for example, using the dimerization approach.[4]

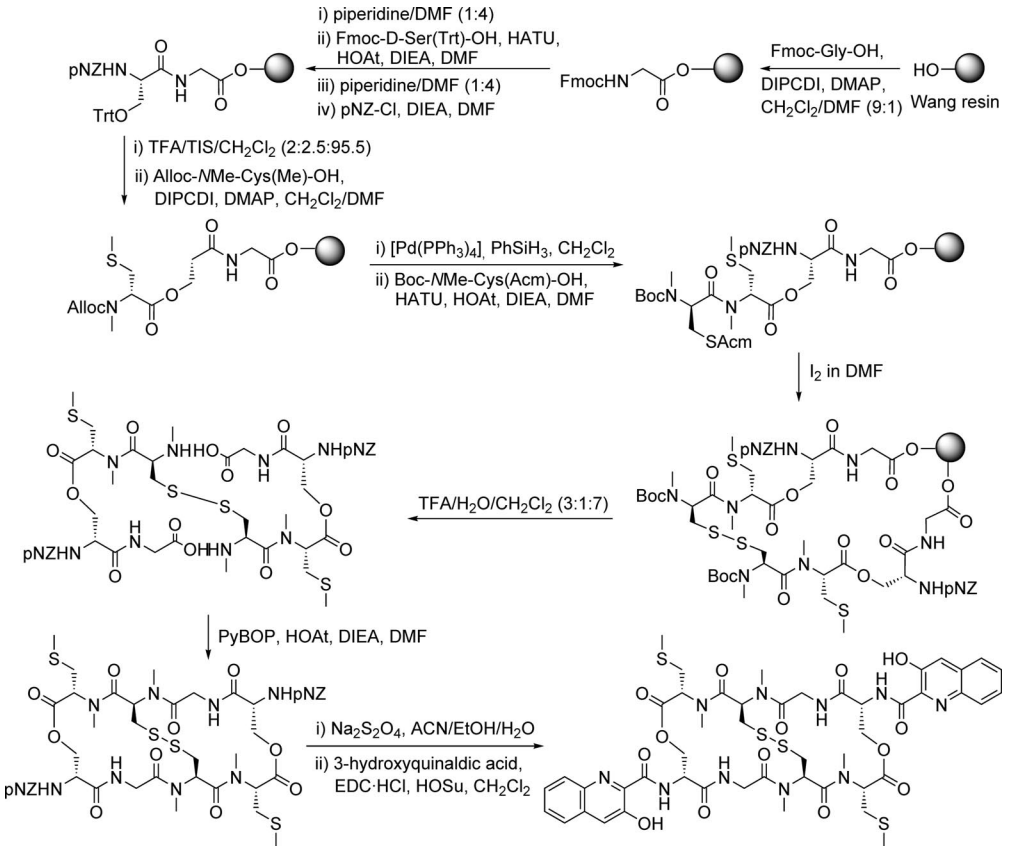
c) To choose the chemistry carefully after the ester bond has been formed. The Fmoc group should be avoided and the pNZ group can be used only at certain positions; it is not compatible, for instance, as a protecting group for the amino acid to be coupled as an ester because its removal results in hydrolysis of the ester bond. Neither is pNZ compatible as a protecting group for the first amino acid anchored to CTC resin.

d) Tandem deprotection/coupling with Alloc- and Fmoc-AA-Fs are not compatible with CTC resins because partial detachment of the peptide chain occurs.

e) *N*Me-Cys(Me) is not a good choice as first amino acid when using a Wang resin, which requires TFA (50%) for final cleavage, because these conditions favour de-*N*-methylation.

f) The protecting group for the α-amino functionality is relevant to the reactivity of the β-position because it may substantially enhance the acidity of the α-proton. The rate of esterification/lactonization of the Ser hydroxy group, the yields of the β-elimination reactions of both Cys and even Ser, and the oxidation of the thiol group are all dependent on the α-amino-protecting group. Acyl is the poorest choice.

g) Ser is not a suitable choice as C terminus because it makes the β-ester highly labile. This negative effect is fur-



Scheme 19. Synthesis of oxathiocoraline by a fragment dimerization approach.

ther increased when the protecting group of the *N*-terminal Ser is an acyl moiety.

h) The TBDMS group is an alternative to the usual Trt or *t*Bu groups that can be used in conjunction with CTC resin and Fmoc, pNZ and Boc protecting groups.[34]

i) Gly is not a suitable choice as a residue next to the C terminus in a peptide fragment to be coupled because its poor hindrance favours the formation of intramolecular DKPs. This side reaction will compete with the condensation, thereby resulting in a low reaction yield.

j) To couple a *N*Me-AA but at the same to minimize possible side-reactions, a reactive but neutral coupling (HATU with a deficit of base) gives the best yields.

k) Optimization of the cleavage cocktail should be carried out to reduce oxidation byproducts.

The points outlined above have been observed in other depsipeptides and form the basis of the design of synthetic approaches in our laboratory.

Experimental Section

Loading of the First Amino Acid onto CTC Resin: The resin was first washed with DMF (5 × 1 min) and CH_2Cl_2 (3 × 1 min) and a solution of Fmoc-AA-OH (1 equiv.) and DIEA (6.7 equiv.) in CH_2Cl_2 was added. After 10 min, more DIEA (3.3 equiv.) was added and the mixture was stirred for 50 min at room temperature. The reaction was quenched by the addition of MeOH (0.4 mL per g resin) and the mixture was stirred for a further 10 min.

Loading of the First Amino Acid onto Wang Resin: The resin was first washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Next, the Fmoc-AA-OH (8 equiv.) was dissolved in CH_2Cl_2/DMF (9:1) and DIPCDI (4 equiv.) was added. The mixture was then added to the resin, DMAP (1 equiv.) in DMF (0.2 mL) was finally added, and the mixture was allowed to react for 14 h at 25 °C.

Peptide Elongation: Protected amino acids (5 equiv.) were coupled either with HATU/HOAt or HBTU/HOBt (5 equiv. each) and DIEA (10 equiv.) in DMF for 1 h. In cases in which a neutral coupling was necessary, the coupling system DIPCDI/HOAt (5 equiv. each) in DMF was used.

Fmoc Group Removal: The peptide-resin was treated with piperidine/DMF (1:4, 2 × 1 min, 2 × 5 min) and washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min).

Alloc Group Removal: The peptide-resin was treated under Ar with $PhSiH_3$ (10 equiv.) and $[Pd(PPh_3)_4]$ (0.1 equiv.) dissolved in CH_2Cl_2 for 15 min, and was then washed with CH_2Cl_2 (3 × 1 min). The process was repeated three times.

pNZ Group Removal: The peptide resin was treated with $SnCl_2$ (6 M) and HCl (1.6 mM) in DMF for 30 min. The resin was then washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min), and the process was repeated once.

Trt Group Removal: The peptide resin was treated with TFA/TIS/CH_2Cl_2 (2:2.5:95.5, 5 × 1 min). The resin was then washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min).

TBDMS Group Removal: The peptide was treated with a solution of TBAF in THF (1 M, 3 × 10 min). The resin was then washed with THF (3 × 1 min), DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min).

Solid-Phase *N*-Methylation: After removal of the corresponding amino-protecting group, a solution of *o*-NBS-Cl (4 equiv.) and DIEA (10 equiv.) in CH_2Cl_2 was added to the resin and the mixture was stirred for 90 min. After filtration and washings with CH_2Cl_2 (3 × 1 min), DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and THF (3 × 1 min), a solution of PPh_3 (5 equiv.) and MeOH (10 equiv.) in THF and a solution of DIAD (5 equiv.) in THF were mixed and added to the peptide resin. After the resin had been stirred for 1 h, it was washed with THF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). After treatments (2 × 15 min) with DBU (5 equiv.) and 2-mercaptoethanol (10 equiv.) in DMF, the resin was washed with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min).

Tandem Deprotection/Coupling Reaction: The peptide-resin was treated with $PhSiH_3$ (12 equiv.), $[Pd(PPh_3)_4]$ (0.2 equiv.) and previously prepared Fmoc-AA-F (7 equiv.) in CH_2Cl_2 for 15 min, and more Fmoc-AA-F (7 equiv.) was then added. The resin was finally washed with CH_2Cl_2 (3 × 1 min).

Fast *Neutral Removal/Coupling* Step: To remove the Alloc group rapidly, the peptide-resin was treated under Ar for 10 min with $PhSiH_3$ (10 equiv.) and $[Pd(PPh_3)_4]$ (0.1 equiv.) dissolved in CH_2Cl_2, and was then washed with CH_2Cl_2 (3 × 1 min). The process was repeated once, and two consecutive fast couplings of the Alloc-AA-OH (5 equiv.) with HATU/HOAt (5 equiv. each) and DIEA (4.8 equiv.) in DMF (35 min) were then carried out.

Solid-Phase Dimerization: Formation of the intermolecular disulfide bridge was achieved by treatment of the resin-bound tetrapeptide with I_2 (5 equiv., 0.06 M) in DMF (2 × 10 min). The resin was then repeatedly washed with CH_2Cl_2 (10 × 1 min), DMF (10 × 1 min) and CH_2Cl_2 (10 × 1 min).

Reduction of the Disulfide Bridge: The peptide (1.9 μmol) was dissolved under N_2 in a denaturating buffer (6 M Gdn·HCl, 1 mM EDTA, 0.1 M Tris·HCl at pH 8.7, 1 mL). A dithiothreitol (DTT) solution (0.38 M, 0.5 mL) was then added, and the reaction mixture was stirred for 45 min at 25 °C.

Cleavage from CTC Resin: The peptide was cleaved from the resin by treatment with a TFA/CH_2Cl_2 solution (2:98, 5 × 1 min) and the filtrates were collected in the presence of H_2O (60 mL per g of resin), dried and lyophilized.

Cleavage from Wang Resin: The peptide was cleaved from the resin by treatment with TFA/H_2O/CH_2Cl_2 (3:1:6, 5 mL, 2 × 30 min), the solvents were removed from the mixture (N_2), and the product was lyophilized.

Supporting Information (see also the footnote on the first page of this article): Detailed experimental procedures and characterization material for Boc-D-Ser(TBDMS)-OH and demethylated analogues of oxathiocoraline.

Acknowledgments

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