

Enzyme-Labile Protecting Groups for the Synthesis of Natural Products: Solid-Phase Synthesis of Thiocoraline**

Judit Tulla-Puche,* Miriam Góngora-Benítez, Núria Bayó-Puxan, Andrés M. Francesch, Carmen Cuevas, and Fernando Albericio*

Nature creates extremely complex molecules which commonly present pharmaceutical properties of interest.^[1] A particularly intriguing class of these molecules are cyclic peptides isolated from marine species.^[2] The development of these peptides as drug candidates is hampered by the difficulty in isolating sufficient amounts of material from the natural sources. Despite the huge development of chemical methods during the past decade, the synthesis of this kind of compound remains a challenge, with numerous reaction and purification steps. In the case of peptides, many of these steps correspond to protection/deprotection steps, and limitations in a synthetic strategy often arise as a result of the incompatibility of protecting groups. Protecting groups should preferably be orthogonal,^[3] meaning that they can be removed in any order and in the presence of the other protecting groups. Such orthogonal removal is required in the case of cyclic peptides, where an intramolecular reaction takes place once the peptide chain has been constructed. It is best to make these kinds of molecules using solid-phase synthesis, although very often it is necessary to carry out a few solution reactions at the end of the synthesis. Enzyme-labile protecting groups add a new orthogonal level to the synthesis of complex molecules for which strategies based only on chemical methods have shown limitations. This is exemplified herein by the first solid-phase synthesis of the complex cyclothiopeptide thiocoraline^[4,5] (Figure 1). Isolated in

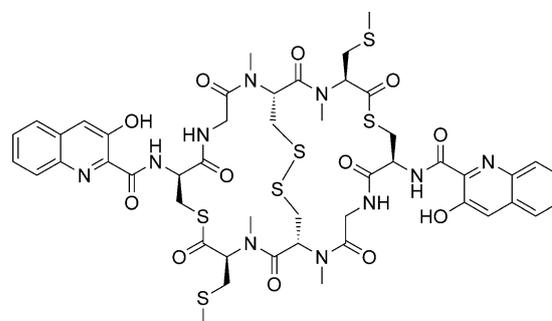


Figure 1. Chemical structure of thiocoraline.

1997 off the coast of Mozambique, this unique and potent antitumor agent has several features that make its structure extremely complex, namely a sequence rich in Cys, the presence of consecutive *N*-methylamino acids, and a bicyclic structure formed by a disulfide bridge flanked by two thioester moieties.

In addition to protection of the α -amino group, the presence of six Cys residues (four of them *N*-methylated and the remaining two in the *D* configuration, which mask a DNA bisintercalating chromophore) requires different protecting groups for the thiol groups and mild removal conditions for all of them. In this regard, the phenylacetamidomethyl^[6] (Phacm) group is suitable, because it can be cleaved by the enzyme penicillin G acylase (PGA). Therefore, an orchestrated scheme of protecting groups becomes the cornerstone for the synthesis of thiocoraline.^[7]

Although attempts were made to apply three distinct strategies developed by our group to other analogues of thiocoraline that do not have thioester moieties, our efforts were unsuccessful.^[8] The formation of these extremely delicate functional groups needs to be postponed until the final steps of the synthesis, and usually thioester formation is incompatible with the formation of the disulfide bridge. As a result, the *N*Me-Cys(Me) residue, which is the *C*-carboxyl component of the thioester, was chosen as a starting point for the synthesis. One drawback of this approach was the β -elimination side reaction that this residue undergoes when placed at the anchoring position and piperidine is used to remove the 9-fluorenylmethoxycarbonyl (Fmoc) group. This problem was overcome using the milder allyl-based chemistry during most of the elongation of the peptide chain. Synthesis of the allyloxycarbonyl (Alloc)-protected *N*-methylated Cys was accomplished starting from Boc-Cys(Me)-OH and Boc-Cys(trityl,Trt)-OH, by carrying out an *N*-methylation reaction with NaH and MeI, then introducing the Phacm moiety in the

[*] Dr. J. Tulla-Puche, Dr. M. Góngora-Benítez, Dr. N. Bayó-Puxan, Prof. F. Albericio
Institute for Research in Biomedicine Barcelona, CIBER-BBN
Baldiri Reixac 10, 08028 Barcelona (Spain)
E-mail: judit.tulla@irbbarcelona.org
albericio@irbbarcelona.org

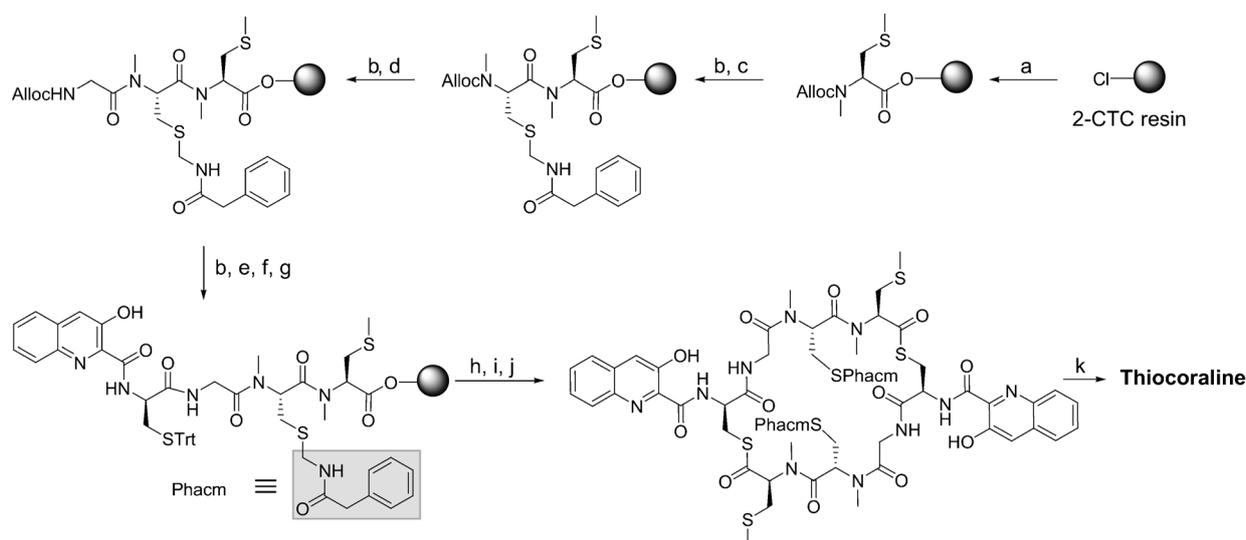
Prof. F. Albericio
Department of Organic Chemistry, University of Barcelona
Martí i Franquès 1, 08028 Barcelona (Spain)

Prof. F. Albericio
University of KwaZulu Natal
4001-Durban (South Africa)

Dr. A. M. Francesch, Dr. C. Cuevas
PharmaMar S. A.
Avda. de los Reyes 1, 28770 Colmenar Viejo, (Spain)

[**] This study was funded by the CICYT (CTQ2012-30930), the Generalitat de Catalunya (2009SGR1024), and the Institute for Research in Biomedicine Barcelona (IRB Barcelona). We thank Dr. M. Gairí and Dr. M. Royo from the Barcelona Science Park for NMR technical support and for helpful discussions, respectively, and Dr. T. Bruckdorfer (Iris Biotech) for encouraging us to use Phacm.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201301708>.



Scheme 1. Synthetic strategy for the synthesis of thiocoraline; a) Alloc-*N*-Me-Cys(Me)-OH, DIEA, CH₂Cl₂; MeOH; b) [Pd(PPh₃)₄], PhSiH₃, CH₂Cl₂; c) Alloc-*N*-Me-Cys(Phacm)-OH, HATU, HOAt, DIEA, DMF; d) Alloc-Gly-OH, HATU, HOAt, DIEA, DMF; e) Fmoc-*D*-Cys(Trt)-OH, DIPCDI, HOBT, DMF; f) piperidine-DMF (1:4); g) 3-hydroxyquinaldic acid, DIPCDI, HOBT, CH₂Cl₂; h) TFA/Me₂S/TIS/CH₂Cl₂ (2:0.5:0.5:97); i) TFA/TIS/CH₂Cl₂ (10:2.5:87.5); j) DIPCDI, HOAt, DMF/CH₂Cl₂; k) immobilized PGA enzyme, H₂O/DMSO (9:1), pH 6.7.

next step, and finally incorporating the Alloc protecting group.^[9] Following this strategy, Alloc-*N*-Me-Cys(Me)-OH and Alloc-*N*-Me-Cys(Phacm)-OH were obtained in good purities (> 95 %) and were used without further purification.

The use of 2-chlorotrityl chloride (2-CTC) resin^[10] as solid support was compulsory, because the two first residues are *N*-Me-amino acids, which are extremely prone to producing diketopiperazine (DKP).^[11] Therefore, the synthesis began by anchoring Alloc-*N*-Me-Cys(Me)-OH onto the 2-CTC resin (Scheme 1).

Following removal of the Alloc group by treatment with [Pd(PPh₃)₄] and PhSiH₃, Alloc-*N*-Me-Cys(Phacm)-OH was coupled using 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU)/1-hydroxy-7-azabenzotriazole (HOAt)/diisopropylethylamine (DIEA) for one hour. The coupling was repeated to ensure complete reaction of the amino acid. Although 2-CTC had been used, removal of the protecting group was accompanied by DKP formation. We therefore optimized the method to minimize this side reaction, which consisted of performing the coupling under almost neutral conditions by adding fewer equivalents of base (4.9 equiv) with respect to the incoming amino acid and coupling reagents (5.0 equiv). At this point, the purity (89 %) and the absorbance spectra of the protected tripeptide showed no indication of DKP formation.^[12] To introduce the next residue (*D*-Cys), which has a 3-hydroxyquinaldic unit, either Boc or Fmoc could be used as the protecting group for the α -amino group of the Cys residue to later introduce the heterocycle. There is an advantage in the use of the Fmoc group in that the heterocycle can be introduced during the solid-phase synthesis, where coupling proceeds faster than in solution^[13] and gives better yields, even when using few equivalents of the precious chromophore. A Trt group is suitable to protect the thiol, because it can be removed under mild conditions and allows

subsequent formation of the thioester. Therefore, after removing the Alloc group of Gly, Fmoc-*D*-Cys(Trt)-OH was easily coupled using DIPCDI/HOBT in DMF for one hour. To minimize dehydration of the *N*-Me-Cys(Me) residue, the Fmoc group was removed with two treatments of piperidine/*N,N*-dimethylformamide (DMF) (1:4), each lasting two minutes. Coupling of 3-hydroxyquinaldic acid was achieved under mild conditions [*N,N'*-diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBT)] and few equivalents (1.1 equiv) to prevent overacylation at the free hydroxy group. After one hour, a ninhydrin test showed completion of the coupling. With the tetrapeptide of thiocoraline fully assembled, we detached it from the solid support. Owing to a large amount of oxidation on the Cys residues, especially *N*-Me-Cys(Me), alternative methods to the standard cleavage conditions for 2-CTC resin [trifluoroacetic acid (TFA)/CH₂Cl₂, 1:99], were examined. In this regard, cleavage of the tetrapeptide under mild reductive conditions [TFA/Me₂S/triisopropylsilane (TIS)/CH₂Cl₂, 2:0.5:0.5:97] afforded the protected tetrapeptide in 91 % purity as shown by analytical HPLC (see the Supporting Information).

Next, two possible methods were devised (Figure 2). The first consisted of forming an intermolecular disulfide dimer, which led to synthesis of the analogue oxathiocoraline.^[8b] However, although the formation of the dimer was complete, as indicated by HPLC and HPLC-MS, removal of the Trt groups to perform the double cyclization destabilized the dimer because of the presence of free thiol groups. This strategy was thus abandoned.

The second option, which a priori was more difficult to handle, involved removing the Trt groups and then performing the double thioesterification in a concentrated (18 mM) solution. To this end, the Trt groups were removed with a TFA/TIS/CH₂Cl₂ (10:2.5:87.5) solution at 4 °C for ten minutes, and then co-evaporated with TBME at low temper-

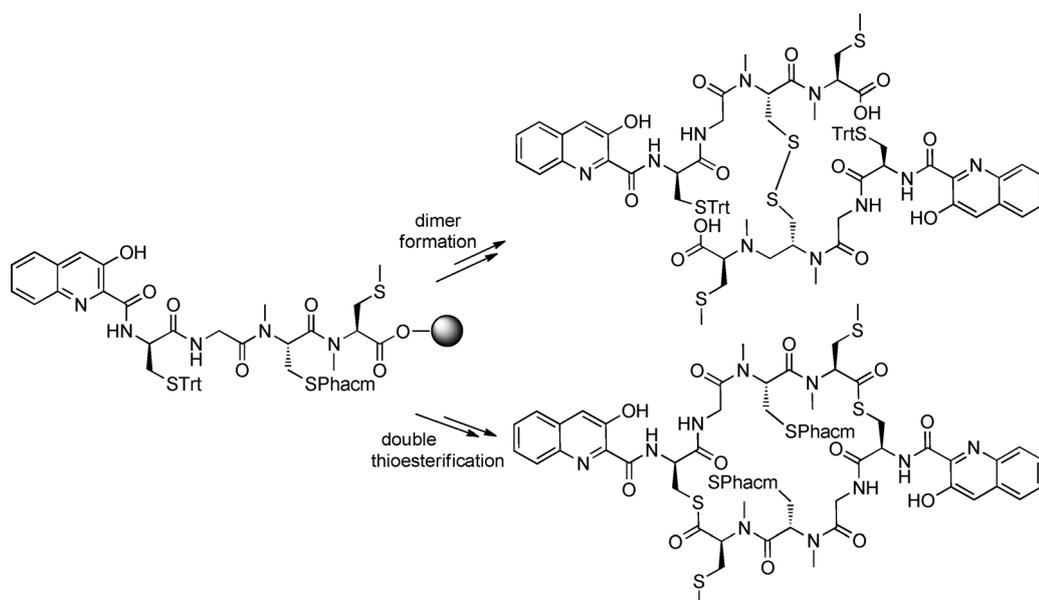


Figure 2. Possible routes from the tetrapeptide: dimer formation and double thioesterification.

ature to remove the remaining acid. The unprotected tetrapeptide was dissolved in CH_2Cl_2 and the solution was cooled to 4°C . DIEA was added until the pH was neutral. The addition of DIPCDCI and HOAt started the cyclization reaction,^[14] which was monitored by HPLC. After two hours, HPLC showed that the reaction had gone to completion, and no monomeric thioesterification was detected. The crude solution was then washed twice with H_2O and pre-purified with an ISCO instrument equipped with a reversed-phase C_{18} column to remove coupling reagents and other impurities. The cyclic thioester was obtained in 85% purity. To remove the Phacm protecting groups and form the disulfide bond, a portion of this crude product (5 mg) was dissolved in dimethylsulfoxide (DMSO; 1.5 mL), and H_2O was added to a final volume of 15 mL. The immobilized PGA enzyme was then added, resulting in a final pH of 6.7, and the suspension was incubated at 37°C and monitored by HPLC. After 56 hours, the immobilized enzyme was filtered and the reaction solution was lyophilized.^[15] Final purification of thiocoraline was performed using semipreparative HPLC. Co-elution with the natural product using a smooth analytical HPLC gradient (Figure 3), HR-ESMS, and NMR confirmed the identity of the synthetic thiocoraline (Supporting Infor-

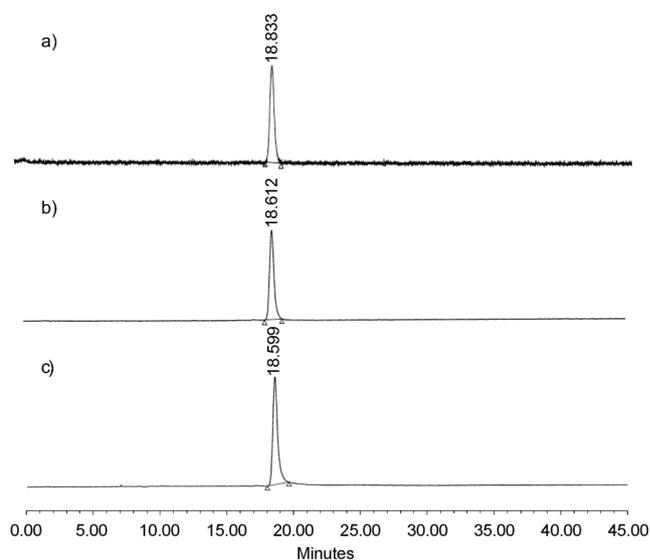


Figure 3. Analytical HPLC of a) natural thiocoraline; b) synthetic thiocoraline; and c) co-elution of natural and synthetic thiocoraline. HPLC conditions: linear gradient of ACN (0.036% TFA)/ H_2O (0.045% TFA) 62:38 to 65:35 over 45 min at a flow rate of 1 mL min^{-1} .

Table 1: In vitro results for natural and synthetic thiocoraline.

Cell line	Breast MDA-MB-231	NSCLC ^[a] A549	Pancreas PSN1	Colon HT-29	
Natural thiocoraline	GI_{50} ^[b]	2.07E-9	6.22E-9	1.47E-9	4.15E-8
	TGI_{100} ^[c]	9.50E-9	2.42E-8	4.23E-9	2.68E-8
	LC_{50} ^[d]	3.46E-8	9.50E-8	1.30E-8	1.38E-7
Synthetic thiocoraline	GI_{50}	1.04E-9	4.29E-9	1.56E-9	1.90E-9
	TGI	4.75E-9	1.90E-8	3.63E-9	1.73E-8
	LC_{50}	1.73E-8	9.50E-8	9.50E-9	4.06E-7

[a] NSCLC = non-small-cell lung cancer. [b] GI_{50} = growth inhibition 50%. [c] TGI = total growth inhibition. [d] LC_{50} = lethal concentration 50%.

of the monomer was done on a solid-phase support using 2-CTC resin and Alloc as the protecting group for the α -amino group of the first three residues, which prevented two side-reactions: β -elimination to form a dehydroAla residue and DKP formation. Macrocyclization was carried out successfully through the thioester without racemization, taking advantage of the high reactivity of the thiols, in a double reaction. This unprecedented reaction could be favored by the presence of the two *N*-methyl residues, which tend to favor the *cis*-conformation of the peptide bond. Finally, the Phacm groups were removed with concomitant formation of

mation). Finally, the *in vitro* activity of the synthetic compound in four human tumor cell lines showed very similar activity to thiocoraline isolated from its natural source (Table 1).

In summary, the enzyme-labile Phacm protecting group has proven to be key in synthesizing the complex antitumor agent, thiocoraline. Additionally, all synthetic steps were carried out using mild conditions to remove the protecting groups. The elongation

the disulfide bridge to give the synthetically difficult thiocoraline in just a few steps and with only two purification steps.

Received: February 27, 2013

Published online: ■ ■ ■ ■ ■, ■ ■ ■ ■ ■

Keywords: antitumor agents · cysteine · peptides · protecting groups · thiocoraline

-
- [1] B. Mishra, V. K. Tiwari, *Eur. J. Med. Chem.* **2011**, *46*, 4769–4807.
- [2] G.-M. Suarez-Jimenez, A. Burgos-Hernandez, J.-M. Ezquerra-Brauer, *Mar. Drugs* **2012**, *10*, 963–986.
- [3] a) G. Barany, R. B. Merrifield, *J. Am. Chem. Soc.* **1977**, *99*, 7363–7365; b) G. Barany, F. Albericio, *J. Am. Chem. Soc.* **1985**, *107*, 4936–4942.
- [4] a) F. Romero, F. Espliego, J. Pérez Baz, T. García de Quesada, D. Gravalos, F. De La Calle, J. L. Fernández-Puentes, *J. Antibiot.* **1997**, *50*, 734–737; b) J. Pérez Baz, L. M. Canedo, J. L. Fernández Puentes, M. V. Silva Elípe, *J. Antibiot.* **1997**, *50*, 738–741.
- [5] A. Negri, E. Marco, V. García-Hernández, A. Domingo, A. L. Llamas-Saiz, S. Porto-Sandá, R. Riguera, W. Laine, M.-H. David-Cordonnier, C. Bailly, L. F. García-Fernández, J. J. Vaquero, F. Gago, *J. Med. Chem.* **2007**, *50*, 3322–3333.
- [6] a) M. Royo, J. Alsina, E. Giral, U. Slomczynska, F. Albericio, *J. Chem. Soc. Perkin Trans. 1* **1995**, 1095–1102; b) M. Góngora-Benítez, A. Basso, T. Bruckdorfer, M. Royo, J. Tulla-Puche, F. Albericio, *Chem. Eur. J.* **2012**, *18*, 16166–16176.
- [7] An elegant synthesis in solution of thiocoraline with 18 linear steps and 12 purifications was developed: D. L. Boger, S. Ichikawa, W. C. Tse, M. P. Hedrick, Q. Jin, *J. Am. Chem. Soc.* **2001**, *123*, 561–568.
- [8] Strategies developed for azathiocoraline, oxathiocoraline, or NMe-azathiocoraline could not be applied to the synthesis of thiocoraline: a) N. Bayó-Puxan, A. Fernández, J. Tulla-Puche, E. Riego, C. Cuevas, M. Álvarez, F. Albericio, *Chem. Eur. J.* **2006**, *12*, 9001–9009; b) J. Tulla-Puche, N. Bayó-Puxan, J. A. Moreno, A. M. Francesch, C. Cuevas, M. Álvarez, F. Albericio, *J. Am. Chem. Soc.* **2007**, *129*, 5322–5323; c) J. Tulla-Puche, E. Marcucci, E. Prats-Alfonso, N. Bayó-Puxan, F. Albericio, *J. Med. Chem.* **2009**, *52*, 834–839.
- [9] E. Marcucci, N. Bayó-Puxan, J. Tulla-Puche, J. Spengler, F. Albericio, *J. Comb. Chem.* **2008**, *10*, 69–78.
- [10] K. Barlos, D. Gatos, J. Kallitsis, G. Papaphotiu, P. Sotiriou, W. Yao, W. Schaefer, *Tetrahedron Lett.* **1989**, *30*, 3943–3946.
- [11] M. C. Khosla, R. R. Smeby, F. M. Bumpus, *J. Am. Chem. Soc.* **1972**, *94*, 4721–4724.
- [12] The same strategy carried out with the less hindered acetamidomethyl (Acm) group showed some diketopiperazine formation.
- [13] Coupling of the heterocycle in solution may take up to four days.
- [14] The low temperature and DIPCDI in the presence of HOAt in a rather apolar solvent such as CH₂Cl₂ accompanied by high efficiency of the double macrothiolactamization assured the absence of racemization.
- [15] The use of the Acm instead of Phacm in the diBoc-cyclic analogue was tested, but the I₂ treatment led to total destruction of the compound. Interestingly, similar results were found by Boger et al.^[7] in their synthesis trials.
-

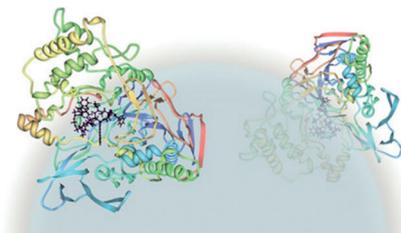
Communications



Natural Products

J. Tulla-Puche,* M. Góngora-Benítez,
N. Bayó-Puxan, A. M. Francesch,
C. Cuevas, F. Albericio* — ■■■■—■■■■

Enzyme-Labile Protecting Groups for the
Synthesis of Natural Products: Solid-
Phase Synthesis of Thiocoraline



Another (orthogonal) dimension: The solid-phase synthesis of thiocoraline was accomplished for the first time by a combined approach involving chemical and enzymatic methods. One-pot cleavage of the phenylacetamidomethyl protecting group using immobilized penicillin G acylase enzyme (see picture) and disulfide formation are the key steps of the synthetic strategy.