Facile and rapid access to linear and truncated microcystin analogues for the implementation of immunoassays†

G. Clavé,[a,b] C. Ronco,[a,b] H. Boutal,[c] N. Kreich,[c] H. Volland,[c] X. Franck,[a] A. Romieu*[a,b] and P.-Y. Renard*[a,b,d]

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A series of simplified microcystin-LR analogues based on Adda [(2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldecadienoic acid] or its corresponding aldol precursor linked to a polypeptide moiety have been synthesised and assessed for their binding affinity by the monoclonal antibody mAb MC159, an anti-microcystin-LR mAb recently selected by us for the detection of microcystins through various immunoassay formats. Some modifications have been brought to the enantiospecific synthesis of *N*-Boc-Adda developed by Pearson *et al.* (*Org. Lett.*, 2000, **2**, 2901) which enabled us to access in an economical and time-saving manner a small library of MC-LR linear analogues. Among which Adda was chosen to synthesise, as an illustrative example, a fluorescent probe derived from this β-amino acid. This probe was subsequently solid-phase immobilised by means of oxime ligation in order to lead to biochips suitable for microcystin detection through the SPIT-FRI method.

Introduction

Microcystins (MCs) are well-known toxic cyclic heptapeptides produced by the cyanobacterial genera *Microcystis*, *Oscillatoria*, *Anabaena* and *Nostoc* found in fresh and brackish waters.[1] These cyanotoxins show potent hepatotoxicity and tumor-promoting activity through inhibition of protein phosphatases PP-1 and PP-2A. Thus, in countries where freshwater (*i.e.*, drinking and surface water) is prone to contamination by cyanobacterial blooms, MCs have been responsible for a variety of adverse effects on animal and human health.[2] The chemical structure of MCs comprises two variable L-amino acids X and Y, three D-amino acids (alanine, methylaspartic acid and glutamic acid) and two unusual amino acids, *N*-methyldehydroalanine (Mdha) and (2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldecadienoic acid (**Adda**) which play an important role in their biological activity.

MCs differ primarily in the two L-amino acids X and Y (Fig. 1), and over 80 variants have been reported to date. Among the variants fully identified, microcystin-LR (MC-LR, X = Leu, Y = Arg), also known as the "fast death factor", is among the most frequent and toxic microcystin congeners.[3] Thus, fast quantitative detection of this toxin is essential to ensure water quality, and therefore

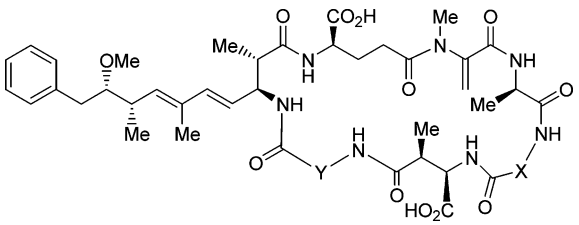

Fig. 1 General structure of microcystins (X and Y: variable L-amino acids).

human health. Currently, several detection methods based on analytical separation methods (*i.e.*, reversed-phase HPLC (RP-HPLC) coupled with mass spectrometry and/or UV detection),[4] bio-assays,[5] biochemical assays[6] and immunoassays (*i.e.*, direct competitive ELISA using monoclonal or polyclonal antibodies (mAbs or pAbs))[7] are in use. Recently, new technologies employing recombinant antibodies[8] and molecularly imprinted polymers[9] have been exploited to develop bioassays and biosensors for MCs. All these methods have their own advantages and drawbacks depending on their sensitivity, specificity, cross-reactivity (for other MCs in the context of immunoassays), cost and implementation procedure but most of them are slow, technically demanding and often require extensive sample processing prior to analysis.[10] To circumvent this latter deficiency, Kim *et al.* have developed an ultra-rapid one-step fluorescence immunochromatic assay which was used in portable biosensor systems suitable for checking the presence of MCs in drinking or surface water.[11a] We further developed such an immunochromatic assay yet further improved by the use of immunoliposomes. Interestingly, samples can be analysed for MCs at the test site in real-time within 15 min and in a sensitive and reproducible manner. The weakness of this method is that the antibody-immobilised and microcystin-immobilised systems exhibit poor stability over time due to the non-covalent immobilisation of these peptides and proteins

[a]*Equipe de Chimie Bio-Organique, COBRA-CNRS UMR 6014 & FR 3038, rue Lucien Tesnière, 76131 Mont-Saint-Aignan, France. E-mail: pierre-yves.renard@univ-rouen.fr, anthony.romieu@univ-rouen.fr; Fax: +33 2-35-52-29-71; Tel: +33 2-35-52-24-14 (or 24-15)*
[b]*Université de Rouen, Place Emile Blondel, 76821 Mont-Saint-Aignan, France*
[c]*iBiTecS, Laboratoire d'Etudes et de Recherches en Immuno-analyse, Commissariat à l'Energie Atomique, Gif-sur-Yvette, F-91191, France*
[d]*Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005, Paris, France*

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onto the nitrocellulose membrane used as the bioassay support. Recently, we have reported the Solid-Phase Immobilised Tripod for Fluorescent Renewable Immunoassay (SPIT-FRI), a new promising method for sensitive detection and quantification of various analytes that possesses the same advantages as described above for the immunochromatic assay.[12] One of the main advantages is that the SPIT-FRI assay has been conceived in order to be used in continuous flow, with a localised analysis point, and thus could easily be multiplexed. With that goal in mind to improve the stability of the immunosensor, we have developed a novel heterotrifunctional peptide-based cross-linking reagent aimed at the covalent immobilisation of the fluorescent "tripod" which supports an epitope similar or close to the target analyte and recognised by a specific antibody, on functionalised aldehyde surfaces *via* a highly chemoselective oxime ligation.[13] This method was validated using a clinically relevant neuropeptide, substance P, as a model of target analyte to be measured. At present, we want to apply SPIT-FRI to the most challenging and important targets such as MC-LR. Since implementation of such an immunoassay requires the production and selection of mAbs directed against this microcystin, we have recently started a research program devoted to the synthesis of linear and truncated MC-LR analogues that possess the unusual side-chain of **Adda**. Indeed, in the course of the evaluation of the SPIT-FRI immunoassay FRET-based system (Fig. 2), it is essential to have access to a small library of MC-LR structural analogues which are recognised by mAbs with a representative range of cross-reactivites (10–80%). Since the SPIT-FRI assay is based on the efficient immobilisation of the mAb on the solid surface through strong binding onto the toxin analogue covalently grafted on the solid-phase, this analogue should display a high affinity towards mAb. Yet, if the toxin is present in the medium, the fluorescent signal in uncovered by the competitive displacement of the mAb from the solid surface through stronger binding of the mAb onto the toxin itself, the differential binding of the mAb (cross-reactivity) between the toxin and its analogue is thus one of the key features of the SPIT-FRI system. In order to be able to evaluate the SPIT-FRI with a wide range of analogues, ideally, each compound of this small library must be synthesised in reasonable amounts (~10 mg), rapidly and inexpensively. However, the structural complexity of the **Adda** scaffold makes this objective not trivial despite the number of syntheses of this unusual β-amino acid already reported in the literature over the past decade.[14]

In this paper, we describe an efficient asymmetric synthesis of **Adda** by a stepwise linear approach allowing a rapid and straightforward addition of polypeptides on the **Adda** scaffold; some practical improvements have been brought to the 13-step synthesis reported by Pearson *et al.*[15] aimed at significantly reducing reagent costs and synthesis time. The corresponding *N*-Boc-protected derivative ***N*-Boc-Adda** and aldol precursor **10** were synthesised and used to rapidly prepare four additional linear and truncated MC-LR analogues through standard peptide-coupling reactions or *trans*-amidification reactions. In addition to these synthetic aspects, cross-reactivity assays towards mAb anti-MC MC159 binding affinity have enabled us to select one of these MC-LR analogues which was fluorescently labelled and subsequently immobilised on an aldehyde-functionalised glass surface. Indeed, such peptide microarrays are required for detecting MC-LR by SPIT-FRI.

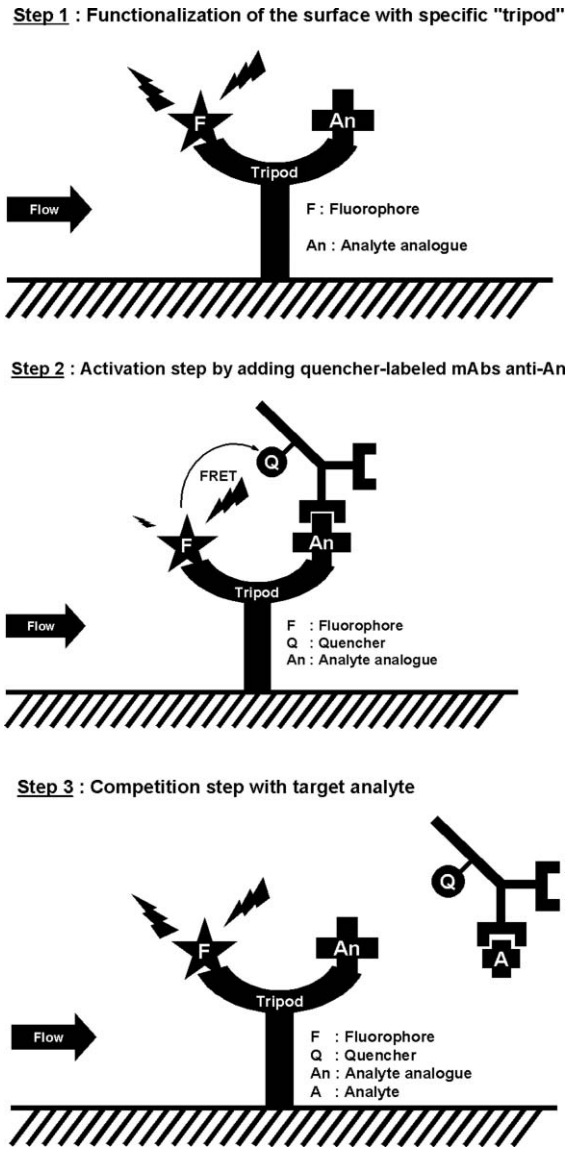


Fig. 2 Different steps of the SPIT-FRI procedure.

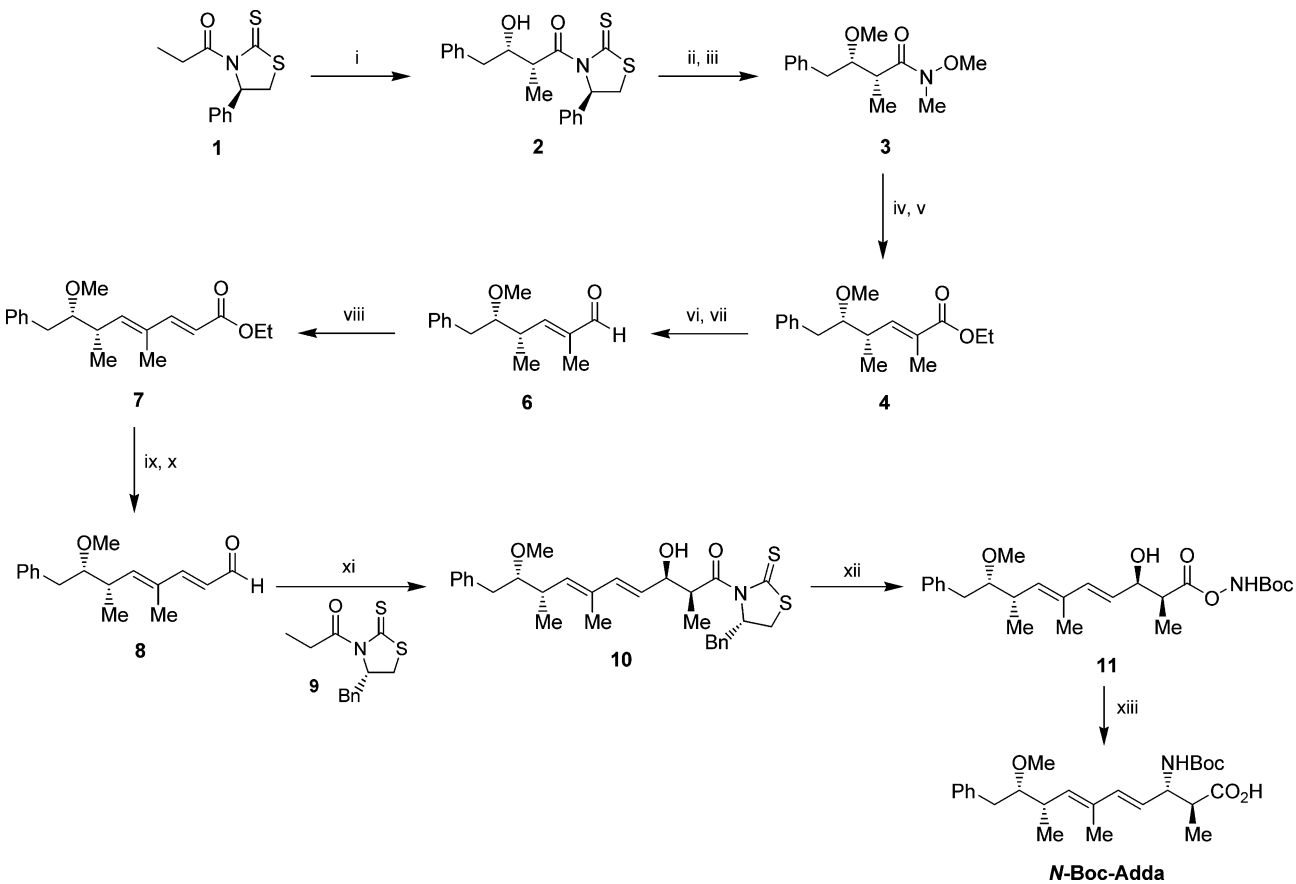
Results and discussion

Structural features of targeted MC-LR analogues

We recently described a procedure that allowed us to produce, isolate and characterise 52 different mAbs anti-MC.[16] The best results were obtained with mAb MC159 that recognises different variants of MCs indicating that the Adda unit plays an essential role in the recognition by this latter antibody. We decided to synthesise linear MC-LR analogues derived from the "north-western" part of this macrocycle which according to our preliminary screening results defines the minimum epitope required for antibody recognition. Thus, these Adda-containing peptides should be readily accessible through a convergent synthetic strategy based on solution-phase peptide couplings or related reactions and involving suitable protected derivatives of D-glutamic acid, *N*-methylalanine (*i.e.*, the saturated analogue of Mdha found in MCs) and **Adda**.

Synthesis of *N*-Boc-Adda

During the past 20 years, significant efforts have been devoted to the asymmetric synthesis of both **Adda** (as a single stereoisomer) and Adda conjugates to prepare MCs, MC analogues and Adda related specific protein phosphatase inhibitors. Since one of the current research interests of our group is the development of new synthetic methodologies involving stereoselective aldol reactions,[17] we decided to prepare ***N*-Boc-Adda** using the high-yield and convenient synthetic route developed by Pearson *et al.* The published protocols are efficient, yet, taking into account the experienced fragility of Adda derivatives, with both goals in mind to have milder derivatisation conditions, and cheaper access to significant quantities of ***N*-Boc-Adda** we proposed practical modifications. Those modifications are especially focused on the two Evans aldol reactions chosen to elaborate the functionalised chiral side-chain and to set the four stereocenters of this β-amino acid. Instead of using a chiral auxiliary belonging to the family of oxazolidinones, we preferred thiazolidinethione derivatives, which display a better leaving group ability which greatly facilitates their removal and/or nucleophilic displacement under mild conditions.[17,18] Consequently, direct access to original MC analogues by *trans*-amidification of fragile Adda aldol precursor **10** by free N-terminal peptides might be considered (*vide infra*). Such asymmetric aldol additions were achieved using chlorotitanium enolates of *N*-acyl thiazolidinethiones under the conditions reported by Crimmins and She (*i.e.*, combination of $TiCl_4$, DIEA and NMP),[19] and proceeded with high diastereoselectivity for the "Evans *syn*" product. Furthermore, titanium chloride is 60 times less expensive than dibutylboron triflate (Bu_2BOTf), the most popular reagent used to generate the boron enolates involved in stereoselective aldol condensations. Thus, such reactions could be carried out on a large scale (100 mmol) without causing prohibitive costs. *N*-Propionyl-thiazolidin-2-thiones **1** and **9** were prepared by cyclisation of the corresponding chiral β-aminoalcohols with carbon disulfide, following a literature procedure,[20] and subsequent *N*-acylation with propionyl chloride. ***N*-Boc-Adda** was synthesised through the 13-step synthetic procedure depicted in Scheme 1. (*R*)-*N*-Propionyl-4-phenylthiazolidine-2-thione **1** (prepared from D-phenylglycine) was allowed to react with phenylacetaldehyde in the presence of $TiCl_4$, DIEA and NMP, producing *syn* aldol **2** in quantitative yield with complete control of diastereoselectivity. Purification by simple liquid–liquid extractions provided this compound with satisfactory purity. Thereafter, thanks to the good leaving group ability of thiazolidinethione moiety, **2** was readily converted into the corresponding Weinreb amide under

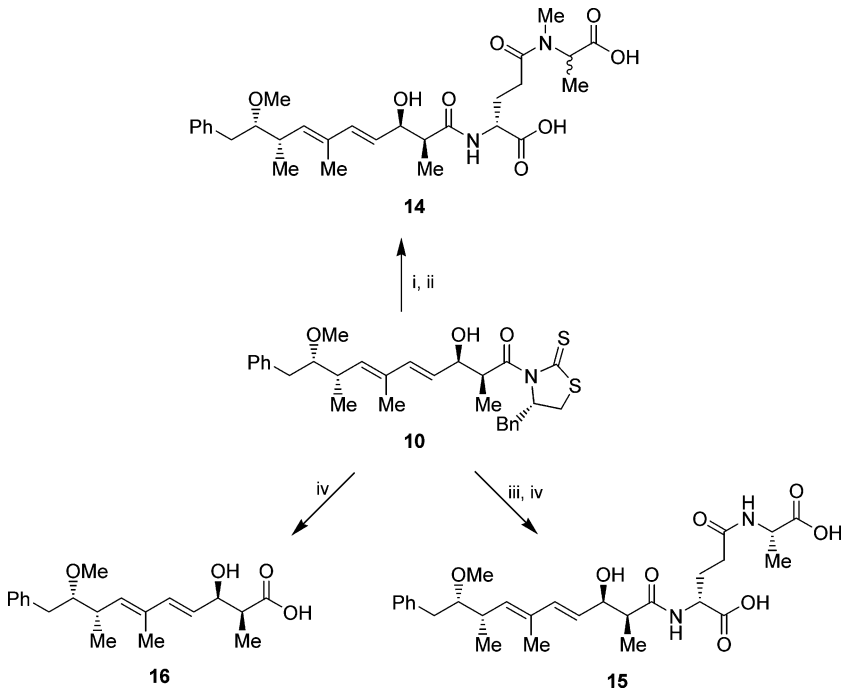


Scheme 1 Synthetic route to ***N*-Boc-Adda**. *Reagents and conditions*: i) $TiCl_4$, DIEA, NMP, phenylacetaldehyde, CH_2C_2, –78 °C; ii) *N*,*O*-dimethylhydroxylamine hydrochloride, imidazole, CH_2Cl_2; iii) NaH, MeI, THF, –78 °C; iv) DIBAL-H, THF, –78 °C; v) (carbethoxyethylidene)triphenylphosphorane, toluene, reflux; vi) DIBAL-H, THF, –78 °C; vii) MnO_2, toluene, 50–55 °C; viii) (carbethoxymethylidene)triphenylphosphorane, toluene, reflux; ix) DIBAL-H, THF, –78 °C; x) MnO_2, toluene, 50–55 °C; xi) $TiCl_4$, DIEA, NMP, CH_2Cl_2, –78 °C; xii) *N*-Boc hydroxylamine, imidazole, CH_2Cl_2, rt. xiii) Ph_3P, DIAD, THF, –78 °C then sodium naphthalide, THF, –78 °C.

mild conditions by treatment with *N*,*O*-dimethylhydroxylamine (HCl salt) in the presence of imidazole and DMAP. Therefore, the enantiopure thiazolidine-2-thione moiety, contrary to the corresponding oxazolidinone derivative used by Pearson *et al.*, can be considered both as a chiral auxiliary and as an activated amide (also named "activated ester" in the terminology of peptide synthesis), preventing both the use of drastic conditions for its removal and the subsequent activation of the resulting carboxylic acid. Subsequent methylation of the free secondary alcohol performed with methyl iodide and NaH, and DIBAL-H reduction of Weinreb amide moiety led to the corresponding aldehyde which was engaged in a first Wittig reaction with (carbethoxyethylidene)triphenylphosphorane to provide olefin **4**. This latter α,β-unsaturated ester was then subjected to a two-step reduction–oxidation sequence to give the aldehyde **6**. The reduction step with DIBAL-H was achieved under the same conditions as used for the Weinreb amide. MnO_2-mediated oxidation was optimised because in our hands, the reaction conditions reported by Pearson *et al.* (*i.e.*, 0 °C in CH_2Cl_2) failed to yield **6**. Due to the lack of information about the source of MnO_2, we screened several batches from different providers to find the most active reagent. In our hands, the best results were obtained using MnO_2 from Alfa Aesar (activated, tech., Mn 58% min) in suspension in dry toluene at 50–55 °C for 2 h, to obtain the targeted aldehyde **6** in a quantitative yield. These conditions prevented its subsequent partial polymerisation observed with other sources of activated MnO_2, or at higher temperatures or with longer reaction times when less activated MnO_2 sources were used. A second Wittig reaction between **6** and (carbethoxymethylidene)triphenylphosphorane provided ester **7**, completing the diene system of **Adda**. A second reduction–oxidation sequence performed under the same conditions as stated above, gave aldehyde **8**. Aldol reaction between **8** and (*S*)-*N*-propionyl-4-benzyl-thiazolidine-2-thione **9** was again achieved according to the Crimmins protocol to provide *syn* aldol **10**. For this second asymmetric aldol reaction, the chiral auxiliary derived from L-phenylalanine was preferred because this latter amino acid was two times less expensive than L-phenylglycine. Thereafter, the thiazolidinethione moiety of **10** was displaced by *N*-Boc hydroxylamine to give *N*-protected hydroxamic acid **11** in 70% overall yield (for the last four steps). This latter compound underwent an intramolecular Mitsunobu reaction which resulted in the formation of an isoxazolidin-5-one intermediate whose N–O bond was reductively cleaved with sodium naphthalide to give ***N*-Boc-Adda**. Purification of this *N*-protected amino acid was achieved by silica gel column chromatography. All spectroscopic data, especially NMR and mass spectrometry were in agreement with the structure assigned. It is important to note that the 5-step synthetic sequence used for the conversion of ester **7** to ***N*-Boc-Adda** must be performed within a short period of time because we observed significant degradation of the intermediate compounds **8**, **10** and **11**, even after few months of storage at −20 °C when kept under an argon atmosphere. Surprisingly, the observed poor stability of these latter intermediates was not reported by Pearson *et al.* To sum up, the present synthetic approach has enabled us to prepare ***N*-Boc-Adda** on a 100 mg scale with a reasonable 5% overall yield; practical modifications brought to the Pearson's synthesis allowed this total synthesis to be achieved within two weeks (if chiral auxiliaries are already available), in a perfectly reproducible manner whatever the experimental skill of the chemist (from undergraduate student to post-doctoral fellow). Yields given were obtained on a significant larger scale than the reactions conditions described by Pearson *et al.*, and have been counterchecked by two independent chemists (*i.e.*, GC and CR). Moreover, we faced unreported stability/purification issues, which can also explain the significantly lower global yield.

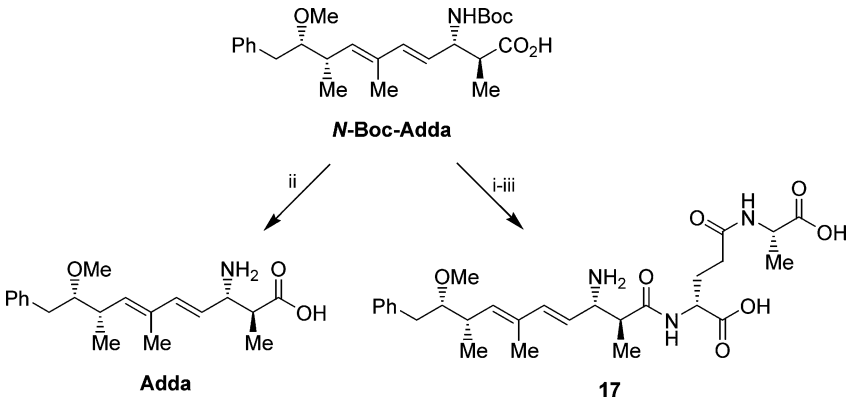
Synthesis and antibody cross-reactivity of MC-LR analogues

As previously mentioned, we have recently produced and characterised monoclonal antibodies directed against MC-LR. Among the 52 mAb obtained, mAb MC159 was selected due to its broad specificity to develop a sensitive competitive enzyme immunoassay (EIA) aimed at quantifying MCs and nodularins in water samples and an innovative immunoliposome-based immunochromatographic assay.[11b,16] To simplify the implementation of SPIT-FRI, which requires the production step of "tripod" in substantial amount, structurally simpler analogues of the whole targeted toxins are desirable. Moreover, since one of the questions remaining to be addressed in the SPIT-FRI assay is the required cross-reactivity for binding between the analogue grafted on the tripod and the toxin, in order to have an efficient specific displacement of the mAbs by the toxin itself, a small library of easily accessible analogues of the toxins was required. Thus, we first planned the synthesis of a tripeptide derived from **Adda**, D-glutamic acid and *N*-methylalanine. In order to widen the size of the library, without introducing too many structural differences in the global structures, before starting the total synthesis of this Adda conjugate according to standard peptide synthesis protocols, we explored an original coupling strategy based on the aminolysis of aldol **10** (Scheme 2). Indeed, this intermediate in the synthesis of **Adda** possesses almost all structural attributes of the unusual side-chain of this β-amino acid (except for the stereochemistry of C-3 which is inverted and for the nature of substituent at C-3: the amino group is replaced by a hydroxyl one). This intermediate can also be used as an activated ester as we previously described, thanks to the good leaving group ability of the thiazolidine-2-thione moiety. This heterocyclic system has been successfully used in solution-phase peptide synthesis in the early 80's,[21] but since, few applications of this peptide coupling additive have been reported.[22] To demonstrate its usefulness and to obtain rapidly two simplified MC-LR analogues **14** and **15**, we have investigated the *trans*-amidification of aldol **10** by dipeptides H-D-Glu(MeAla-OMe)-OMe **12** and H-D-Glu(Ala-OMe)-OMe **13**. These latter dipeptide methyl diesters were readily synthesised by coupling between the commercial amino acids Boc-D-Glu-OMe and H-MeAla-OMe (or H-Ala-OMe) and subsequent Boc removal by acidolysis with TFA. To overcome the coupling difficulties generally encountered with *N*-methylated amino acids, PyBrOP/DIEA activation was used for the preparation of **14**.[23] The more conventional peptide coupling reagent BOP was effective for synthesising **15**.[24] By analogy with the synthesis of intermediates **3** and **11** involved in the preparation of ***N*-Boc-Adda**, *trans*-amidification of aldol **10** with the TFA salt of primary amine **14** (or **15**) was carried out in THF at room temperature, in the presence of imidazole (2 equiv.) and a catalytic amount of DMAP. Contrary to oxazolidinone derivatives, the reaction does not require the use of a Lewis acid such as $AlMe_3$ which may have deleterious effects on the structure of peptide partner used. Purification by silica gel column chromatography



Scheme 2 Synthetic reactions used for the preparation of acyclic microcystin analogues **14–16** from aldol **10**. *Reagents and conditions*: i) H-D-Glu(MeAla-OMe)-OMe **12**, imidazole, DMAP, THF, rt; ii) aq. LiOH, CH_3OH, rt; iii) H-D-Glu(Ala-OMe)-OMe **13**, imidazole, DMAP, THF, rt; iv) aq. LiOH, CH_3OH, rt.

has enabled us to isolate the resulting fully-protected tripeptides in moderate yields and proved smooth enough so that we were also able to recover the released chiral auxiliary. As the final step, the methyl esters were removed by short treatment with LiOH in H_2O–CH_3OH to give the target microcystin analogues **14** and **15** which were isolated in their pure form by semi-preparative RP-HPLC. Their structures were confirmed by detailed measurements, including ESI mass spectrometry and NMR analyses. However, if **15** proved to be present as a single diastereomer, this was not the case with its *N*-methylated analogue **14**. NMR analyses revealed that complete epimerisation of *N*-methylalanine residue had taken place giving rise to a 1 : 1 mixture of diastereomers for analogue **14**. In our case, the signal splitting observed in proton NMR spectrum of **14** cannot be assigned to a mixture of two amide rotamers because such spectral behaviour did not disappear on warming and was not observed with the parent methyl diester derived from the same peptide scaffold. Furthermore, the racemisation of *N*-substituted-*N*-methylamino acid methyl esters during saponification with LiOH has been already reported in the literature.[25] All our attempts to separate each one of the diastereomers by semi-preparative RP-HPLC failed, and using the mixture in a competitive EIA experiment, a significant cross-reactivity of around 25% (IC_{50} 219 pM) for binding to mAb MC159 as compared with MC-LR was obtained. This result clearly shows that the Adda side-chain plays a pivotal role in the recognition of MCs by mAb MC159. This structure/cross-reactivity relationship study was extended to microcystin analogue **15** and β-hydroxy acid **16**, this latter compound being obtained by direct saponification of aldol **10**. Monoclonal antibody MC159 recognises the greatly simplified MC analogues **15** and **16** with ~10% of cross-reactivity for binding affinity as compared to MC-LR (12% for **15** and 13% for **16**, IC_{50} 614 pM and 409 pM respectively). These results demonstrate that the methyl residue on the amino group of the alanine residue is important, but not pivotal, for the recognition of our analogues by the selected mAb. Conversely, the presence of the D-glutamic acid residue in the structure has no influence on the binding affinities of the selected analogues for the mAb MC159. These three acyclic analogues of MCs, which could be readily obtained from the ***N*-Boc-Adda** synthesis intermediate **10** in the yield range 30–70% (for one or two steps), offer a first small library of MC analogues. They will be further used to evaluate the SPIT-FRI scope and limitations, and could offer an economical alternative to Adda-based analogues of MCs. Next, we turned our attention to the synthesis and cross-reactivities studies of Adda peptidyl conjugates (Scheme 3) in order to widen the scope of cross-reactivities, but also since **14** and **15** do not perfectly fulfil all requirements of an useful epitope for the SPIT-FRI technology. Indeed, the presence of two carboxylic acid functions within their structure and availability of **14** only as a mixture of diastereomers makes more tricky the chemoselective conjugations to a fluorescent label and functionalised solid-phases in an efficient manner (*i.e.*, good yield and high degree of purity for the resulting tri-component conjugate). To avoid epimerisation of *N*-methylalanine residue occurring during the final ester deprotection step, tripeptide **17** derived from **Adda**, D-glutamic acid and alanine was preferred to Adda-containing tripeptide H-Adda-*iso*-D-Glu(MeAla)-OH initially considered. Furthermore, we thought that the negative effect of the lack of this methyl group on the recognition by mAb MC159 could be counterbalanced by the positive effect expected with the presence of **Adda** instead of its aldol precursor. As previously described, the conjugation of ***N*-Boc-Adda** to amino acids or

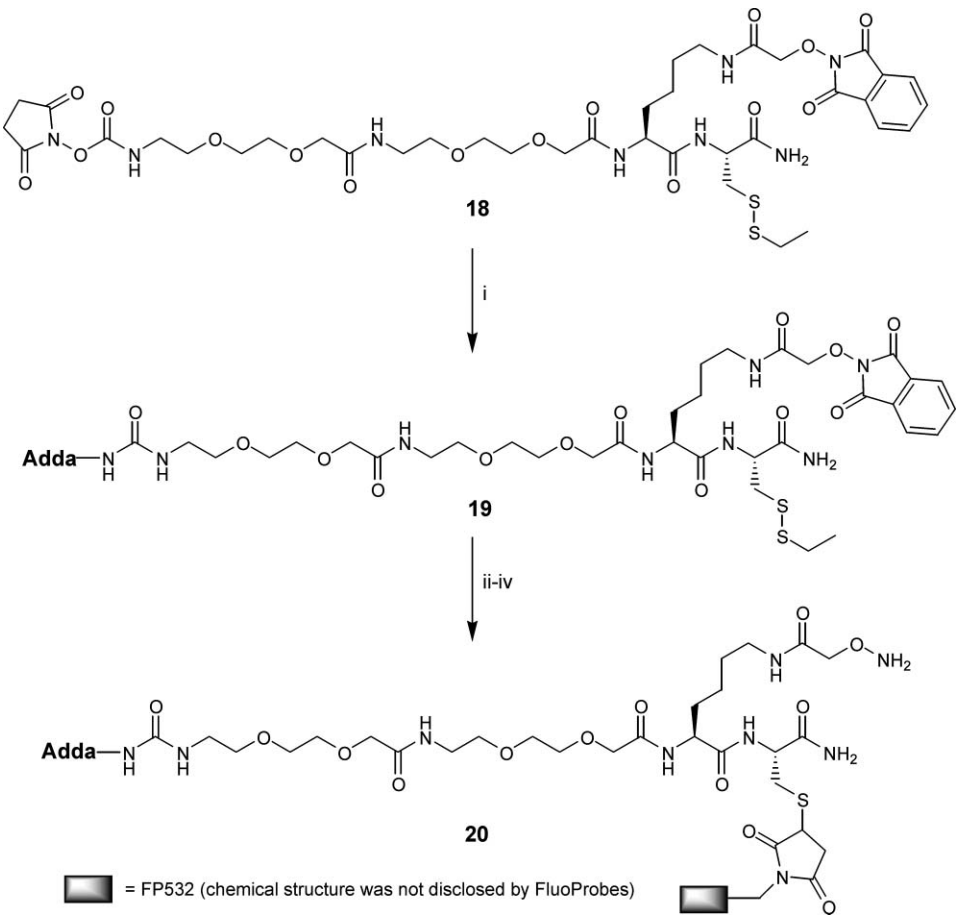


Scheme 3 Synthetic reactions used for the preparation **Adda** and tripeptide **17** from ***N*-Boc-Adda**. *Reagents and conditions*: i) HATU, 2,4,6-collidine, H-D-Glu(Ala-OMe)-OMe, DMF, rt; ii) TFA, CH_2Cl_2, reflux; iii) aq. LiOH, CH_3OH, rt.

related peptides through amidifcation reaction is not a trivial synthetic task and often requires non standard peptide coupling conditions. Representatively, satisfactory results to get the fully-protected tripeptide were only obtained through the use of the optimised protocol described by Gulledge *et al.*:[26] pre-activation of carboxylic acid of ***N*-Boc-Adda** with uronium salt HATU in the presence of 2,4,6-collidine followed by aminolysis of the formed OAt esters by H-D-Glu(Ala-OMe)-OMe **13**. Finally, the Boc and methyl ester groups were sequentially removed by treatment with TFA and LiOH respectively. Purification was achieved by semi-preparative RP-HPLC to give the novel acyclic MC analogue **17** in 24% overall yield for the three steps. Monoclonal antibodyMC159 binds to this analogue with 45% cross-reactivity as compared to MC-LR (IC_{50} 122 pM). This good result clearly shows that the stereostructural features of C-2 and C-3 positions of **Adda** amino acid are essential for designing simplified acyclic MC analogues well-recognised by mAb MC159. In addition to this result, we have also determined the cross-reactivity of mAb MC159 for binding MC-LR toward the simplest synthetic analogue of MCs, **Adda** itself. TFA-mediated deprotection of ***N*-Boc-Adda** followed by semi-preparative RP-HPLC purification provided this β-amino acid in a pure form. Cross-reactivity for binding mAb MC159 of this analogue relative to MC-LR is 58% (IC_{50} 94 pM). Thus, with these two additional Adda-based derivatives, we have a complete panel of MC-LR analogues with cross-reactivity for MC159 binding varying from 12% to 58% required for the implementation of SPIT-FRI. Since **Adda** is synthetically accessible more rapidly, it was preferred for the preparation of a first "tripod", the key reagent in this original competitive immunoassay, with the first goals in mind: (1) to evaluate the whole set of chemical steps required for the tripod synthesis using a fragile toxin analogue, (2) to have an "universal" tripod which could be applied for the detection of any Adda-based toxin (if the appropriate mAb is produced), and (3) evaluate the solid-phase attachment efficacy.

Preparation of the first "microcystin tripod" through the sequential derivatisation of a heterotrifunctional cross-linking reagent

Once an MC-LR analogue has been selected (*i.e.*, **Adda**), the next step for the implementation of the SPIT-FRI detection method is the design and synthesis of an Adda-derived fluorescent probe which will be able to react readily with an aldehyde-functionalised solid-phase (Scheme 4). Thus, it is essential to introduce two orthogonal reactive groups (for chemoselective fluorescent labelling and subsequent solid-phase immobilisation) within the Adda structure without altering its recognition by mAb MC159. To our knowledge, there are no reports in the literature for fluorescent labelling of this unusual β-amino acid. Several fluorescent probes of MC-LR have been prepared but the labelling strategies are based on the chemoselective derivatisation of the side-chain of an amino acid other than **Adda**: (1) conjugation under basic conditions of a tetramethylrhodamine 1,3-diketone derivative to the arginine side-chain[27] or (2) a two-step process involving a Michael type addition of an aminothiol (*i.e.*, cysteamine or cysteine) to the α,β-unsaturated carbonyl of the Mdha residue followed by the reaction of its amino group with *N*-hydroxysuccinimidyl (NHS) of a terbium cryptate[28] or isothiocyanate derivative of fluorescein (FITC).[29] Consequently, we have explored the synthesis of an aldehyde-reactive Adda-based fluorescent "tripod" through the four-step sequential derivatisation of an in-house-developed heterotrifunctional cross-linking reagent (Scheme 4).[13] Indeed, this generic bio-labelling reagent contains three orthogonal reactive groups: an amine-reactive *N*-hydroxysuccinimidyl carbamate moiety, an aldehyde/ketone-reactive aminooxy group (protected as phthalimide) and a thiol group (protected as disulfide) with a propensity to form urea, oxime and thioether linkages under mild conditions respectively. First, **Adda** readily reacted through its free amine, with a slight excess of reagent **18** in NMP in the presence of DIEA to obtain Adda-peptidyl conjugate **19**. As previously observed with other amine-containing compounds, this acylation reaction was found to be fast and efficient. Purification was achieved by RP-HPLC to give **19** in 48% yield. Such a reaction could be favorably performed in alkaline aq. buffers (*i.e.*, $NaHCO_3$ or borate buffer, pH 8–9) but the high degree of hydrophobicity of the Adda side-chain prevents its solubilisation in such a reaction medium. Thereafter, the phthaloyl and *S*Et protecting groups were cleanly removed by using the two-step sequential synthetic procedure previously described by us.[13] Finally, the chemoselective fluorescent labelling of intermediate free-sulfhydryl peptide was achieved by Michael addition of the newly formed thiol group to the maleimide moiety of a water-soluble analogue of rhodamine 6G (FluoProbe® 532, FP532). Noteworthily, as we previously described, no reaction of the aminooxy group was observed in these conditions, even with an excess of maleimide fluorescent



Scheme 4 Synthetic reactions used for the preparation of fluorescent "microcystin-tripod" **20**. *Reagents and conditions*: i) **Adda**, DIEA, NMP, rt; ii) hydrazine monohydrate, CH_3OH, rt; iii) DTT, 0.1 M borate buffer (pH 8.2), rt; iv) FluoProbes® 532A maleimide, 0.1 M $NaHCO_3$ buffer (pH 8.5), CH_3CN, rt.

thiol reagent. The resulting aminooxy-containing Adda-derived fluorescent probe **20** was isolated in a pure form by RP-HPLC. The modest 5% overall yield (for the three steps) is explained both by the non-quantitative yield of the aminooxy deprotection step (~50%) and by significant losses of intermediate conjugates during the chromatographic purification and lyophilisation steps. Indeed, this is the main unavoidable drawback of efficient bioconjugation reactions performed at the micromole scale. The structure of **20** was confirmed by ESI mass spectrometry (see ESI†). Further evidence in favor of the integrity of "microcystin tripod" **20**, especially its fluorescent label and the aminooxy functionality, was provided by immobilisation experiments. Thus, this probe was grafted on an aldehyde-functionalised glass surface using the same protocol as previously described for "substance P-tripod".[13] After washings, an intense fluorescence signal was observed and proved the efficacy of the oxime ligation to covalently graft Adda related "tripods" to a solid surface (Fig. 3a). A spotting experiment with the non-reactive oxime derivative (obtained by mixing **20** with acetone) gave no significant signal (Fig. 3b). Furthermore, the concentration of the aminooxy probe was varied (1–170 μM) and comparison of the intensities of fluorescence confirmed that the amount of anchored "tripod" increases with increasing tripod concentration until saturation of surface reactive sites is reached around 80 μM (Fig. 3c). This result proved the efficient and

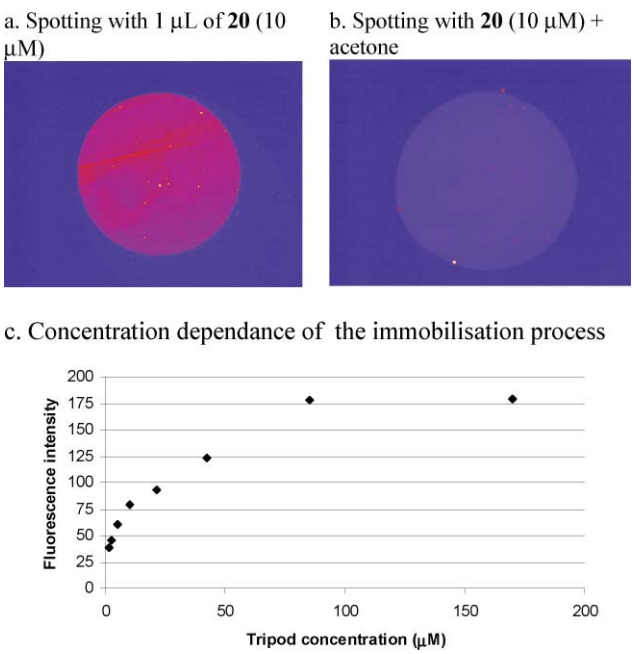


Fig. 3 Immobilisation of fluorescent "microcystin-tripod" **20** on aldehyde-functionalised silica surface by oxime ligation.

concentration-dependant immobilisation process of **20** involving its aminooxy group. We thus proved the efficacy of the solid-phase immobilisation of such an Adda-based fluorecent probe. Since the fluorescent Adda derivative **20** is significantly recognised by mAb MC159 (IC_{50} 211 pm, molar cross-reactivity compared to MC-LR 26%), the present microarrays composed of smaller Adda conjugates should be suitable to investigate activation and competition steps of SPIT-FRI, for instance, aimed at detection and quantifying MC-LR in continuous flow. The corresponding results will be reported in due course.

Conclusions

In this paper, we have described the synthesis of four novel acyclic analogues of microcystin-LR bearing Adda amino acid (or its aldol precursor) as the key component. Some practical changes have been brought to the linear total synthesis of this unusual β-amino acid initially developed by Pearson *et al.* to get rapidly synthetic intermediates (aldol **10** and ***N*-Boc-Adda**) useful for the preparation of targeted MC analogues through peptide coupling or *trans*-amidification reactions. Their use in a comprehensive structure/cross-reactivity relationship study toward mAb anti-MC MC159 binding ability has enabled us to conclude that the use of the greatly simplified macrocyclic heptapeptide of MC-LR is possible without suppressing a good recognition of the corresponding analogues by anti-MC mAbs. These results are valuable especially for selecting suitable analytes required for the development of immunoassays aimed at the non-selective (alert) or selective (toxin identification) detection of MCs and other cyanobacterial toxins such as nodularins, which also display the **Adda** β-amino acid unit. Interestingly, the two efficient synthetic strategies leading to the Adda conjugates **14, 15** and **17** are also promising methods that may prove useful in the near future for the rapid production of a new generation of specific phosphatase inhibitors. They constitute good alternatives to the general synthesis of Adda-containing peptides reported by Cundy *et al.* which relies on the ring opening of an Adda-derived β-lactam by various aminoesters.[30] Furthermore, the use of an "in house" heterotrifunctional cross-linking reagent has enabled us to prepare the first fluorescent conjugate of **Adda**. Interestingly, the presence of an additional reactive group (*i.e.*, aminooxy group), within the peptide scaffold of this conjugate allows the grafting of another reporter group (*e.g.*, a second fluorophore to create a FRET pair). Thus, this latter synthetic tool could be the starting point for the design of activity-based probes (ABPs)[31] to study and dissect serine/threonine phosphatase activity.

Experimental section

General

Column chromatography purifications were performed on silica gel (40–63 μm) from SdS. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. Compounds were visualised by one or more of the following methods: (1) illumination with a short wavelength UV lamp (*i.e.*, $\lambda = 254$ nm), (2) spray with a 0.2% (w/v) ninhydrin solution in absolute ethanol, (3) spray with a 3.5% (w/v) phosphomolybdic acid solution in absolute ethanol. All solvents were dried following standard procedures (CH_3CN: distillation over CaH_2, CH_2Cl_2: distillation over P_2O_5, DMF: distillation over BaO, NMP: distillation over BaO, THF: distillation over Na^0/benzophenone, toluene: distillation over Na^0). DIEA and TEA were distilled from CaH_2 and stored over BaO. Activated MnO_2 (tech., Mn 58% min) and protected amino acids were obtained from Alfa Aesar and Bachem respectively. The thiol-reactive water-soluble analogue of rhodamine 6G "FluoProbes® 532A maleimide" was purchased from Interchim. Chiral thiazolidinethiones **1** and **9** were synthesised using literature procedures.[20,32] The HPLC-gradient grade acetonitrile (CH_3CN) and methanol (CH_3OH) were obtained from Acros or Fisher Scientific. Buffers and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ cm). ^1H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) and relative to tetramethylsilane from CD_3CN ($\delta_H = 1.96$, $\delta_C = 1.79$ (CH_3), 118.26 (CN)), $CDCl_3$ ($\delta_H = 7.26$, $\delta_C = 77.36$) or CD_3OD ($\delta_H = 3.31$, $\delta_C = 49.00$).[33] J values are in Hz. Infrared (IR) spectra were recorded as thin-film on sodium chloride plates or KBr pellets using a Perkin Elmer FT-IR Paragon 500 spectrometer with frequencies given in reciprocal centimetres (cm^{-1}). Optical rotations were measured by using a Perkin Elmer 341 polarimeter (sodium D ray at $\lambda = 589$ nm). Elemental analyses were carried out on a Carlo Erba EA 1110 CHNS–O instrument. UV-visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Chromophore-containing compounds were quantified by UV-visible spectroscopy at the λ_{max} using the corresponding tabulated molar extinction coefficient. Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. Analytical HPLC was performed on a Thermo Electron Surveyor instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Finnigan SpectraSYSTEM liquid chromatography system equipped with UV-visible 2000 detector. Mass spectra were obtained with a Thermo Finnigan LCQ Advantage Max (ion-trap) apparatus equipped with an electrospray. The monoclonal antibodies anti-MC were obtained as previously described and the corresponding cross-reactivity experiments were performed by using standard conditions.[16]

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments and the purification steps. **System A:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and 0.1% aqueous trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.0) as eluents [20% CH_3CN (5 min), followed by linear gradient from 20 to 90% (35 min) of CH_3CN] at a flow rate of 1 mL min^{-1}. UV detection was achieved at 254 nm. **System B:** System A with the following gradient [0% CH_3CN (5 min), followed by linear gradient from 0 to 60% (30 min) of CH_3CN]. Visible detection was achieved at 550 nm. **System C:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and 0.1% aq. TFA as eluents [30% CH_3CN (5 min), followed by linear gradient from 30 to 60% (40 min) of CH_3CN] at a flow rate of 4 mL min^{-1}. UV detection was achieved at 260 nm. **System D:** System C with the following gradient [20% CH_3CN (5 min), followed by linear gradient from 20 to 30% (5 min) and

30 to 70% (40 min) of CH_3CN]. **System E:** RP-HPLC (Waters Xterra MS C_{18}, 5 µm, 7.8 × 100 mm) with CH_3CN and 0.1% aq. TFA as eluents [20% CH_3CN (5 min), followed by linear gradient from 20 to 30% (5 min) and 30 to 60% (30 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 250 nm. **System F:** System E with the following gradient [0% CH_3CN (5 min), followed by linear gradient from 0 to 40% (5 min) and 40 to 70% (30 min) of CH_3CN]. **System G:** System E with the following gradient [0% CH_3CN (5 min), followed by linear gradient from 0 to 30% (5 min) and 30 to 70% (35 min) of CH_3CN]. **System H:** System E with the following gradient [20% CH_3CN (5 min), followed by linear gradient from 20 to 30% (5 min) and 30 to 60% (30 min) of CH_3CN]. Visible detection was achieved at 550 nm.

(2*R*,3*S*)-*N*,3-Dimethoxy-*N*,2-dimethyl-4-phenylbutanamide (3)

(*a*) *Crimmins aldolisation*: Chiral *N*-propionylthioazolidinethione **1** (26.2 g, 104 mmol) was dissolved in dry CH_2Cl_2 (250 mL) and the resulting solution was cooled to −78 °C with a bath of dry ice in acetone. $TiCl_4$ (12 mL, 109.4 mmol) was added dropwise and the resulting mixture allowed to stir for 15 min in order to form the titanium complex. Then, freshly distilled DIEA (18.9 mL, 114.4 mmol) was added and the mixture was stirred for 40 min under the same conditions to form the corresponding titanium enolate. Thereafter, freshly distilled NMP (20.0 mL, 208 mmol) was added. After 10 min, freshly distilled phenylacetaldehyde (13.4 mL, 114.4 mmol) was added. After keeping the solution at −78 °C for 30 min, the reaction mixture was allowed to warm to 4 °C and stirred at this temperature for a further 1 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 7 : 3, v/v) and quenched by the addition of aq. 50% NH_4Cl (300 mL). The organic layer was washed twice by aq. 1.0 N HCl (2 × 300 mL), aq. 10% Na_2CO_3 (300 mL) and finally brine (300 mL) before drying over Na_2SO_4. Aldol **2** was obtained as a yellow solid and used in the next step without further purification. R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.61; [α] −164 (*c* 0.6 $CHCl_3$); IR (KBr): ν_{max} 698, 772, 1047, 1156, 1221, 1258, 1316, 1453, 1496, 1643 (broad), 2359, 2926, 3416 (broad); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29 (d, J = 7.5 Hz, 3H, CH_3), 2.71–2.86 (m, 2H, CH_2), 3.04–3.09 (dd, J = 11.3 Hz, 1.9 Hz, 1H), 3.67–3.92 (m, 1H, CH), 4.18–4.23 (m, 1H, CH), 4.53–4.61 (qd, 6.8 Hz, 3.0 Hz, 1H, CH), 6.17 (d, 1H), 7.21–7.35 (m, 10H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.8, 27.0, 36.7, 40.7, 43.0, 70.1, 73.4, 125.4, 126.8, 128.7, 129.2, 129.5, 178.0; MS (ESI+): *m*/*z* 372.20 $[M + H]^+$, calcd for $C_{20}H_{21}NO_2S_2$: 371.52. (*b*) *Trans-amidification reaction*: Aldol **2** (37.1 g, 100 mmol) was dissolved in dry CH_2Cl_2 (150 mL) and *N*,*O*-dimethylhydroxylamine hydrochloride (14.6 g, 150 mmol) and imidazole (27.2 g, 400 mmol) were sequentially added. The resulting reaction mixture was stirred at room temperature for 12 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 7 : 3, v/v). The solution was then washed with deionised water (3 × 300 mL), the organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (600 g) with a step gradient of AcOEt (30–50%) in cyclohexane as the mobile phase, to give the intermediate Weinreb amide as a yellow oil (15.1 g, 64 mmol). R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.12; [α] −25.7 (*c* 1.3 in CH_3OH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25 (d, J = 7.5 Hz, 3H), 2.66–2.90 (m, 3H, CH_2 & CH), 3.13 (s, 3H, CH_3), 3.45 (s, 3H, CH_3), 3.63 (s, 1H, OH), 4.06–4.13 (m, 1H, CH), 7.19–7.22 (m, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.2, 31.8, 37.2, 40.0, 61.2, 72.9, 126.3, 128.4, 129.1, 138.3, 177.9. (*c*) *Alkylation reaction*: Iodomethane (40.1 mL, 636.4 mmol) was dissolved in dry THF (140 mL) and the resulting solution was stirred with solid Na_2CO_3 (1.38 g, 13 mmol) for 10 min. The mixture was cooled to 4 °C, filtered through cotton and added to the Weinreb amide previously obtained (15.1 g, 64 mmol). Thereafter, 2.49 g (63.6 mmol) of NaH (60% dispersion in mineral oil) was added at 4 °C. The reaction mixture was stirred at room temperature for 4 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 6 : 4, v/v). Further amount of NaH (60% oil, 1.24 g, 31.8 mmol) was added and the reaction mixture was stirred at room temperature for further 2 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 6 : 4, v/v). After cooling to 4 °C, the excess of NaH was destroyed by addition of aq. sat. NH_4Cl (150 mL). The mixture was extracted twice by AcOEt (3 × 200 mL). The organic layer was washed with brine (250 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by chromatography on a silica gel column (300 g) with a mixture of cyclohexane–AcOEt (6 : 4, v/v) as the mobile phase to give **3** as a yellow oil (10.85 g, 43.2 mmol, overall yield for the three steps 43%). Spectroscopic data are identical to those reported by Pearson *et al.*

(4*S*,5*S*,2*E*)-2,4-Dimethyl-5-methoxy-6-phenylhex-2-en-1-ol (5)

(*a*) *Reduction*: Weinreb amide **3** (10.71 g, 42.6 mmol) was dissolved in dry CH_2Cl_2 (100 mL) and the resulting solution cooled to −78 °C with a bath of dry ice in acetone. A 1.0 M solution of DIBAL-H in dry toluene (100 mL, 100 mmol) was added dropwise over a period of 80 min. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 7 : 3, v/v) and quenched by sequential addition of methanol (40 mL) and aq. sat. NH_4Cl (40 mL). The resulting solution was allowed to warm to room temperature for 30 min. The mixture was then filtered trough a Celite® 545 pad (to remove aluminium salts) and washed with a large amount of Et_2O (300 mL). The combined organic layers were washed with aq. 2.0 N HCl (200 mL), dried over $MgSO_4$, filtered and concentrated under vacuum to give intermediate aldehyde as an orange oil that was used in the next step without further purification. R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.63; [α] −3.6 (*c* 1.1 in CH_3OH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.16 (d, J = 7.2 Hz, 3H, CH_3), 2.66–2.99 (m, 2H, CH_2), 3.28 (s, 3H, CH_3), 3.84–3.90 (m, 1H, CH), 7.14–7.31 (m, 5H, Ph), 9.66 (s, 1H, C(O)H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 7.6, 37.5, 49.0, 58.1, 81.7, 126.6, 129.3, 138.1. (*b*) *Wittig reaction*: The freshly prepared aldehyde (8.48 g) was dissolved in dry toluene (275 mL), stabilised phosphorus ylide (1-ethoxycarbonylethylidene)triphenylphosphorane (18.52 g, 51.1 mmol) was added and the resulting mixture was stirred under reflux for 12 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 8 : 2, v/v). The reaction mixture was concentrated under reduced pressure and the resulting residue was purified by chromatography on a silica gel column (250 g) with a step gradient of AcOEt (0–10%) in cyclohexane as the mobile phase, to give the targeted alkene **4** as a yellow oil. R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.59; [α] −6.78 (*c* 0.6 in CH_3OH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.07 (d, J = 6.8 Hz,

3H, CH_3), 1.28 (d, $J = 6.1$ Hz, 3H, CH_3), 1.74 (s, 3H, CH_3), 2.57–2.85 (m, 2H, CH_2), 3.26 (s, 3H, CH_3), 4.16–4.23 (q, $J = 7.2$ Hz, 2H), 6.68 (d, $J = 10.1$ Hz, 1H, CH), 7.14–7.74 (m, 5H, Ph). (*c*) *Reduction*: After its purification, alkene **4** (7.59 g, 27.5 mmol) was dissolved in dry THF (55 mL) and the resulting solution was cooled to −78 °C with a bath of dry ice in acetone. A 1.0 M solution of DIBAL-H in dry toluene (68 mL, 68 mmol) was added dropwise over a period of 1 h. The resulting reaction mixture was stirred at this temperature for 90 min and checked for completion by TLC (cyclohexane–AcOEt, 8 : 2, v/v). Thereafter, the reaction was quenched by sequential addition of methanol (30 mL) and aq. sat. NH_4Cl (30 mL). The resulting solution was allowed to warm to room temperature for 30 min. The mixture was then filtered trough a Celite® 545 pad (to remove aluminium salts) and washed with CH_2Cl_2 (150 mL). The organic layer was washed with aq. 1.0 N HCl (200 mL); re-extraction of this latter aq. layer with Et_2O (280 mL) was needed for a complete recovery of the desired alcohol. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under vacuum. The resulting brown oily residue was purified by chromatography on a silica gel column (400 g) with a step gradient of AcOEt (0–20%) in cyclohexane as the mobile phase, to give allylic alcohol **5** as a pale yellow oil (4.76 g, 20.3 mmol, overall yield for the three steps 48%). R_f (cyclohexane–AcOEt, 8 : 2, v/v) 0.19; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.02 (d, $J =$ 6.8 Hz, 3H, CH_3), 1.57 (s, 3H, CH_3), 2.37 (bs, 1H, OH), 2.37–2.84 (m, 3H, CH_2 & CH), 3.19 (s, 4H, CH_3 & CH), 3.97 (s, 2H, CH_2), 5.32 (d, $J = 9.8$ Hz, 1H, CH), 7.18–7.26 (m, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.9, 16.4, 35.9, 38.0, 58.1, 68.6, 87.1, 126.2, 128.3, 128.4, 129.3, 135.0, 139.6. All other spectroscopic data are identical to those reported by Pearson *et al.*

(6*S*,7*S*,2*E*,4*E*)-Ethyl 4,6-dimethyl-7-methoxy-8-phenylocta-2,4-dienoate (7)

(*a*) *Oxidation*: Allylic alcohol **5** (3.4 g, 14.5 mmol) was dissolved in dry toluene (100 mL) and the resulting solution was cooled to 0 °C. Activated MnO_2 (tech., Mn 58% min, 13.6 g, 156 mmol) was added and the resulting suspension vigorously stirred at 55 °C for 2 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 8 : 2, v/v). The resulting suspension was filtered on a Celite® 545 pad and washed with a large amount of CH_2Cl_2. The resulting filtrate was concentrated under reduced pressure to give aldehyde **6** as a yellow oil (3.39 g, 14.5 mmol, quantitative yield). R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.68; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.13 (d, $J = 6.8$ Hz, 3H, CH_3), 1.62 (s, 3H, CH_3), 2.80 (bm, 3H, CH_3), 3.29–3.24 (m, 4H, CH_3 & CH), 6.41 (d, $J = 9.8$ Hz, 1H, CH), 7.16–7.31 (m, 5H, Ph), 9.38 (s, 1H, C(O)H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 9.5, 14.8, 37.1, 38.0, 58.7, 85.8, 126.5, 128.4, 128.5, 129.5, 138.0, 138.6, 138.9, 156.7, 195.6. (*b*) *Wittig reaction*: The freshly prepared aldehyde **6** (3.39 g, 14.5 mmol) was dissolved in dry toluene (80 mL), stabilised phosphorus ylide (1-ethoxycarbonylmethylidene)triphenylphosphorane (6.06 g, 17.4 mmol) was added and the resulting reaction mixture was stirred under reflux overnight. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 8 : 2, v/v). Thereafter, the reaction mixture was filtered on a Celite® 545 pad, washed with CH_2Cl_2 and concentrated under reduced pressure. The resulting oily residue was purified by chromatography on a silica gel column with a step gradient of AcOEt (0-20%) in cyclohexane as the mobile phase to give the ester **7** as a yellow oil (3.14 g, 10.4 mmol, yield 72%). R_f (cyclohexane–AcOEt, 8 : 2, v/v) 0.76; [α] −45.2 (*c* 0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.07 (d, $J =$ 6.4 Hz, 3H, CH_3), 1.29 (t, $J = 3$ Hz, 3H, CH_3), 1.66 (s, 3H, CH_3), 2.60–2.84 (m, 3H, CH_2 & CH), 3.20–3.25 (m, 4H, CH_3 & CH), 4.18–4.25 (q, $J = 7.1$ Hz, 2H, CH_2), 5.78–5.83 (m, 2H, 2 × CH), 7.17–7.27 (m, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.8, 14.8, 16.2, 37.5, 38.6, 59.1, 60.7, 87.0, 116.7, 126.6, 128.7, 129.9, 132.9, 139.4, 144.8, 150.0, 167.9.

(2*S*,3*R*,4*E*,6*E*,8*S*,9*S*)-1-*O*-(*tert*-Butoxycarbamoyl)-3-hydroxy-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoate (11)

(*a*) *Reduction*: Ester **7** (1.0 g, 3.3 mmol) was dissolved in dry THF (12 mL), and the resulting solution was cooled to −78 °C with a bath of dry ice in acetone. A 1.0 M solution of DIBAL-H in dry toluene (12 mL, 12 mmol) was added dropwise over 15 min. The resulting reaction mixture was stirred at −78 °C for 90 min and checked for completion by TLC (cyclohexane–AcOEt, 8 : 2, v/v). Thereafter, the reaction was quenched at −78 °C by slow addition of methanol (5 mL) and aq. sat. NH_4Cl (5 mL). Then the solution was allowed to warm to room temperature for 30 min. The organic compounds were extracted by AcOEt (3 × 50 mL) after the addition of 1.0 N aq. HCl (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was then purified by chromatography on a silica gel column (30 g) with a step gradient of AcOEt (0–20%) in cyclohexane as the mobile phase, giving the intermediate alcohol as a yellow oil that was used in the next step without further purification. R_f (cyclohexane–AcOEt, 8 : 2, v/v) 0.26; [α] −20.2 (*c* 1.7 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.04 (d, $J = 6.8$ Hz, 3H), 1.66 (s, 3H), 2.19 (bs, 1H, OH), 2.56–2.85 (m, 3H, CH_2 & CH), 3.16–3.23 (s, 4H, CH_3 & CH), 4.18 (d, $J = 6.0$ Hz, 2H, CH_2), 5.40 (d, $J = 9.8$ Hz, 1H), 5.68–5.77 (dt, 1H, $J = 15$ Hz, 6.3 Hz, CH), 7.16–7.20 (m, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.3, 15.9, 36.3, 37.8, 58.3, 63.3, 86.6, 125.5, 125.6, 127.8, 129.0, 132.3, 135.4, 136.1, 138.9. (*b*) *Oxidation*: The alcohol was dissolved in dry toluene (35 mL) and the resulting solution was cooled to 4 °C. Activated MnO_2 (tech., Mn 58% min, 4.0 g, 26.8 mmol) was added and the reaction mixture was stirred at 50 °C for 1 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 7 : 3, v/v) and the suspension was filtered on a Celite® 545 pad and washed with a large amount of CH_2Cl_2. The filtrate was concentrated under reduced pressure to give aldehyde **8** as a yellow oil that was used in the next step without further purification. R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.74; [α] −6.8 (*c* 0.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.10 (d, $J = 6.8$ Hz, 3H, CH_3), 1.70 (s, 3H, CH_3), 2.76–2.80 (m, 3H, CH_3), 3.28 (s, 4H, CH_3 & CH), 5.95 (d, $J = 9.8$ Hz, 1H, CH), 6.07–6.15 (dd, $J = 15.5$ Hz, 7.5 Hz, 1 H, CH), 7.09–7.31 (m, 6H, Ph & CH), 9.57 (d, $J = 7.5$ Hz, 1H, C(O)H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.6, 15.5, 37.2, 38.1, 58.7, 86.4, 126.4, 127.4, 128.4, 129.2, 133.1, 138.9, 146.8, 158.0. (*c*) *Crimmins aldolisation*: Chiral *N*-propylthiazolidinethione **9** (1.3 g, 4.95 mmol) was dissolved in dry CH_2Cl_2 (35 mL) and the resulting solution was cooled to −78 °C with a bath of dry ice in acetone. $TiCl_4$ (592 µL, 5.4 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 20 min in order to form the titanium complex. Then, freshly distilled DIEA (891 µL, 5.4 mmol) was added at −78 °C and the mixture

was stirred for a further 40 min at room temperature to form the titanium enolate. Thereafter, freshly distilled NMP (962 µL, 10 mmol) was added at −78 °C. 10 min later, a solution of freshly prepared aldehyde **8** in dry CH_2Cl_2 (5 mL) was added dropwise. The reaction mixture was stirred at −78 °C for 30 min, then allowed to warm at 4 °C and stirred for a further 1 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 7 : 3, v/v) and quenched by the sequential addition of aq. sat. NH_4Cl (15 mL) and aq. 1.0 N HCl (15 mL). This mixture was then washed twice by aq. 1.0 N HCl (2 × 20 mL) and brine (20 mL) before drying over Na_2SO_4. The organic layer was filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (60 g) with a step gradient of AcOEt (0–20%) in cyclohexane as the mobile phase, to give the aldol **10** as a yellow oil. R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.49; [α] +121.7 (*c* 0.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.97 (d, J = 6.6 Hz, 3H, CH_3), 1.32 (d, J = 5.1 Hz, 3H, CH_3), 1.64 (s, 3H, CH_3), 2.49–3.33 (m, 12H, CH_3, 3 × CH_2, CH_2, CH_2 & 3 × CH), 4.43–4.46 (t, J = 5.9 Hz, 1H, CH), 4.59 (t, J = 5.3 Hz, 1H, CH), 5.17–5.24 (m, 1H, CH), 5.42 (d, J = 9.6 Hz, 1H, CH), 5.52–5.60 (dd, J = 6.7 Hz, 15.6 Hz, 1H, CH), 6.32 (d, J = 15.8 Hz, 1H, CH), 7.17–7.25 (m, 10H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.8, 12.8, 16.3, 32.4, 36.7, 36.8, 38.3, 44.9, 58.8, 69.2, 74.5, 86.9, 126.1, 126.3, 127.4, 128.3, 129.0, 129.5, 132.6, 136.5, 136.8, 137.0, 139.4, 177.6, 201.4; MS (ESI+): *m/z* 506.53 [M − H_2O + H]$^+$, dehydration reaction occurred within the ESI probe under our ionisation conditions, calcd for $C_{30}H_{37}NO_3S_2$: 523.76; elemental analysis: calcd (%) for $C_{30}H_{37}NO_3S_2$: C, 68.53; H, 7.48; N, 2.66; found: C, 68.78; H, 7.22; N, 2.75. (*d*) *Trans-esterification reaction*: Aldol **10** was dissolved in dry THF (20 mL), *tert*-butyl-*N*-hydroxycarbamate (1.31 g, 3.9 mmol) and imidazole (674 mg, 9.9 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 12 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 8 : 2, v/v) and volatiles were removed by evaporation under vacuum. The resulting residue was taken up with AcOEt (50 mL) and sequentially washed by aq. 10% citric acid (50 mL), aq. sat. $NaHCO_3$ (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a mixture of cyclohexane–AcOEt (9 : 1, v/v) as the mobile phase, to give the compound **11** as an opaque white oil (1.03 g, 2.3 mmol, overall yield for the four steps 70%). R_f (cyclohexane–AcOEt, 8 : 2, v/v) 0.32; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.03 (d, J = 6.7 Hz, 3H, CH_3), 1.24 (d, J = 6.7 Hz, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.64 (s, 3H, CH_3), 2.56–2.99 (m, 4H, 4 × CH), 3.18–3.24 (m, 4H, CH_3 & CH), 4.58 (t, J = 4.6 Hz, 1H, CH), 5.43 (d, J = 9.7 Hz, 1H, CH), 5.51–5.58 (dd, J = 6.8 Hz, 8.8 Hz, 1H, CH), 6.32 (d, J = 15.6 Hz, 1H, CH), 7.17–7.30 (m, 5H), 7.89 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.0, 12.8, 16.3, 28.1, 29.5, 29.8, 30.4, 32.1, 36.9, 38.4, 44.2, 58.8, 73.5, 83.9, 87.0, 125.2, 126.1, 128.3, 129.4, 129.6, 132.7, 136.7, 137.4, 139.5, 156.2, 174.5.

N-Boc-Adda

This *N*-protected unusual β-amino acid was finally synthesized in 50% from compound **11** by a Mitsunobu cyclisation followed by sodium naphthalide reduction in a "one-pot" process, under the same experimental conditions as reported by Pearson *et al.* All spectroscopic data of this compound are identical to that previously reported in the literature.

Dipeptide H-D-Glu(MeAla-OMe)-OMe (12)

(*a*) *Peptide coupling*: Boc-D-Glu-OMe (386 mg, 1.47 mmol), H-MeAla-OMe (250 mg, 1.63 mmol) and PyBrOP reagent (685.3 mg, 1.47 mmol) were dissolved in dry CH_3CN (2 mL) and the resulting mixture was cooled to 4 °C. Dry DIEA (768 µL, 4.41 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 12 h. The reaction was checked for completion by TLC (CH_2Cl_2–CH_3OH, 9 : 1, v/v). The mixture was evaporated to dryness and the resulting residue was taken up with AcOEt (25 mL), washed by aq. 10% citric acid (25 mL), aq. sat. $NaHCO_3$ (25 mL) and brine (25 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a step gradient of CH_3OH (0–4%) in CH_2Cl_2 as the mobile phase, giving Boc-D-Glu(MeAla-OMe)-OMe as a yellow oil (530 mg, 1.47 mmol, quantitative yield). R_f (CH_2Cl_2–CH_3OH, 9 : 1, v/v) 0.65; IR (KBr): ν_{max} 756, 1028, 1052, 1084, 1168, 1214, 1366, 1408, 1455, 1519, 1644, 1714, 1743, 2979, 3340 (broad); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.39 (d, J = 7.3 Hz, 3H, CH_3), 1.44 (s, 9H, tBu), 1.94–2.52 (m, 4H, CH_2 β Glu, CH_2 γ Glu), 2.92 (s, 3H, CH_3), 3.71 (s, 3H, CH_3), 3.74 (s, 3H, CH_3), 4.29 (bs, 1H, CH α Glu), 5.22 (q, J = 7.2 Hz, 1H, CH α MeAla); MS (ESI+): *m/z* 383.07 [M + Na]$^+$, 399.00 [M + K]$^+$, calcd for $C_{16}H_{28}N_2O_7$: 360.41. (*b*) *Boc removal*: Dipeptide Boc-D-Glu(MeAla-OMe)-OMe (404 mg, 1.12 mmol) was dissolved in dry THF (12 mL). TFA (6.7 mL, 89.7 mmol) was added dropwise and the resulting reaction mixture was heated under reflux for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2–CH_3OH, 9 : 1, v/v) and the reaction mixture was evaporated to dryness. The resulting oily residue was purified by chromatography on a silica gel column with a step gradient of CH_3OH (0–10%) in CH_2Cl_2 as the mobile phase, to give the TFA salt of H-Glu(MeAla-OMe)-OMe **12** as a yellow oil (244 mg, 0.65 mmol, yield 58%). R_f (CH_2Cl_2–CH_3OH, 9 : 1, v/v) 0.24; ^{1}H NMR (300 MHz, CD_3OD): δ 1.37 (d, J = 7.1 Hz, 3H, CH_3), 1.85–2.03 (m, 2H, CH_2 β Glu), 2.49–2.55 (m, 2H, CH_2 γ Glu), 3.01 (s, 3H, CH_3), 3.62 (t, J = 6.7 Hz, 1H, CH α Glu), 3.70 (s, 3H, CH_3), 3.73 (s, 3H, CH_3), 4.69 (q, J = 7.3 Hz, 1H, CH α MeAla); MS (ESI+): *m/z* 261.07 [M + H]$^+$, calcd for $C_{11}H_{20}N_2O_5$: 260.29.

Dipeptide H-D-Glu(Ala-OMe)-OMe (13)

(*a*) *Peptide coupling*: Boc-D-Glu-OMe (261 mg, 1.0 mmol), H-Ala-OMe (HCl salt, 139 mg, 1.0 mmol) and BOP reagent (530 mg, 1.2 mmol) were dissolved in dry CH_3CN (2 mL) and the resulting solution was cooled to 4 °C. Dry DIEA (628 µL, 3.6 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2–CH_3OH, 9 : 1, v/v). Thereafter, the mixture was evaporated to dryness and the resulting residue was taken up with AcOEt (25 mL), sequentially washed with aq. 10% citric acid (25 mL), aq. sat. $NaHCO_3$ (25 mL) and brine (25 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a step gradient of CH_3OH (0–3%) in

CH_2Cl_2 as the mobile phase, to give Boc-D-Glu(Ala-OMe)-OMe as a yellow oil (310 mg, 0.9 mmol, yield 90%). R_f (CH_2Cl_2–CH_3OH, 9 : 1, v/v) 0.39; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41 (s, 9H, tBu), 1.87–2.35 (m, 4H, CH_2 β Glu, CH_2 γ Glu), 3.72 (s, 6H, 2 × CH_3), 4.34–4.40 (m, 1H, CH α Glu), 4.50–4.60 (m, 1H, CH α Ala), 5.34 (d, J = 7.9 Hz, 1H, NH), 6.78 (d, J = 6.0 Hz, 1 H, NH). (*b*) *Boc removal*: Dipeptide Boc-D-Glu(Ala-OMe)-OMe (310 mg, 0.9 mmol) was dissolved in THF–H_2O (10 mL, 95 : 5, v/v). TFA (5.3 mL, 71 mmol) was added dropwise and the resulting reaction mixture was stirred at reflux for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2–CH_3OH, 9 : 1, v/v) and the mixture was evaporated to dryness. The resulting oily residue was dissolved in deionised water and lyophilised to give H-D-Glu(Ala-OMe)-OMe **13** as a yellow oil (320 mg, 0.89 mmol, quantitative yield). This compound was used in the next reaction step without further purification.

Acyclic microcystin analogue (14)

(*a*) *Trans-amidification*: Aldol **10** (206 mg, 0.55 mmol) and TFA salt of dipeptide H-D-Glu(MeAla-OMe)-OMe **12** (145 mg, 0.39 mmol) were dissolved in dry THF (12 mL). Imidazole (68 mg, 0.86 mmol) and DMAP (4.8 mg, 40 µmol) were sequentially added. The resulting reaction mixture was stirred at room temperature for 12 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 6 : 4, v/v). Thereafter, the solvent was evaporated under reduced pressure. The resulting residue was dissolved in a mixture CH_2Cl_2–Et_2O (20 mL, 1 : 1, v/v). This organic layer was washed with aq. 10% citric acid (25 mL) and brine (25 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a mixture of cyclohexane–AcOEt (6 : 4, v/v), then CH_2Cl_2–CH_3OH (96 : 4, v/v) as the mobile phase to give the methyl diester of the targeted MC analogue (165 mg, 0.29 mmol, yield 52%) as a white amorphous powder. R_f (cyclohexane–AcOEt, 6 : 4, v/v) 0.15; IR (KBr): ν_{max} 666, 701, 752, 968, 1030, 1083, 1214, 1374, 1455, 1495, 1539, 1645, 1742, 2932, 3389 (broad); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.03 (d, J = 6.8 Hz, 3H), 1.15 (d, J = 7.0 Hz, 3H), 1.39 (d, J = 7.3 Hz, 3H), 1.64 (s, 3 H), 1.83–1.88 (m, 2H), 2.08–2.84 (m, 6H), 2.93 (s, 3H), 3.17–3.23 (m, 4H), 3.71 (s, 3H), 3.77 (s, 3H), 4.41–4.54 (m, 2H), 5.14 (q, J = 7.3 Hz, 1 H), 5.41 (d, J = 9.9 Hz, 1H), 5.50–5.58 (dd, J = 6.6 Hz, 9.0 Hz, 1H), 6.29 (d, J = 15.5 Hz, 1H), 7.17–7.29 (m, 5H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.8, 13.2, 15.0, 16.7, 26.1, 26.7, 30.6, 32.2, 37.2, 38.8, 45.9, 52.8, 52.9, 53.0, 53.1, 59.2, 74.2, 87.4, 126.4, 126.5, 128.7, 130.0, 133.2, 136.6, 137.3, 139.9, 172.6, 172.9, 173.3, 176.6; MS (ESI+): m/z 575.00 $[M + H]^+$, calcd for $C_{31}H_{46}N_2O_8$: 574.72. (*b*) *Saponification*: Methyl diester (50 mg, 87 µmol) was dissolved in CH_3OH (3 mL). Aq. 1.0 M LiOH (1.3 mL, 1.3 mmol) was added dropwise. The resulting reaction mixture was stirred at room temperature for 30 min. The reaction was checked for completion by RP-HPLC (system A). The reaction was quenched by addition of acetic acid (0.6 mL, 696 µmol). Thereafter, the mixture was evaporated to dryness. The resulting residue was dissolved with a mixture of 0.1% aq. TFA and CH_3CN (1 : 1, v/v, 4 mL) and purified by semi-preparative RP-HPLC (system C, 3 injections, t_R = 18.6–21.2 min). The product-containing fractions were lyophilised to give microcystin analogue **14** as a white amorphous powder (39 mg, 71.6 µmol, yield 82%, a 1 : 1 mixture of two diastereomers). ^{1}H NMR (300 MHz, CD_3OD): δ 0.82–0.86 (3H), 1.00–1.07 (3H), 1.35–1.39 (3H), 1.45–1.47 (3H), 1.80–2.90 (8H), 2.90–2.92 (3H), 3.19–3.26 (4H), 4.11–4.16 (1H), 4.25–4.39 (2H), 4.90 (1H, partially masked by water peak), 5.34–5.47 (1H), 5.54–5.72 (1H), 6.19–6.25 (1H), 7.13–7.24 (5H). The complexity of the spectrum for this mixture of two diastereomers makes a complete assignment impossible. MS (ESI+): m/z 546.87 $[M + H]^+$, 569.00 $[M + Na]^+$, calcd for $C_{29}H_{42}N_2O_8$: 546.67; HPLC (system A): t_R = 13.7 min and 14.0 min (two diastereomers), purity >95%.

Acyclic microcystin analogue (15)

(*a*) *Trans-amidification*: Aldol **10** (50 mg, 0.95 mmol) and TFA salt of dipeptide H-Glu(Ala-OMe)-OMe **13** (38 mg, 0.7 mmol) were dissolved in dry THF (3 mL). Imidazole (15 mg, 0.2 mmol) and DMAP (6.1 mg, 50 µmol) were sequentially added. The resulting mixture was stirred at room temperature for 12 h. The reaction was checked for completion by TLC (cyclohexane–AcOEt, 6 : 4, v/v). Thereafter, the solvent was evaporated under reduced pressure. The resulting residue was dissolved in a mixture of CH_2Cl_2–Et_2O (20 mL, 1 : 1, v/v) and washed with aq. 10% citric acid (25 mL) and brine (25 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a mixture of cyclohexane–AcOEt (6 : 4, v/v), then CH_2Cl_2–CH_3OH (96 : 4, v/v) as the mobile phase to give the methyl diester of the targeted MC analogue as a white amorphous powder (21 mg, 0.37 mmol, yield 39%). R_f (cyclohexane–AcOEt, 6 : 4, v/v) 0.15. (*b*) *Saponification*: Methyl diester (21 mg, 37 µmol) was dissolved in CH_3OH (2 mL). Aq. 1.0 M LiOH (0.55 mL, 550 µmol) was added dropwise. The resulting reaction mixture was stirred at room temperature for 12 h. The reaction was checked for completion by RP-HPLC (system A) and quenched by addition of acetic acid (0.6 mL, 696 µmol). Thereafter, the reaction mixture was evaporated to dryness. The resulting residue was dissolved with a mixture of 0.1% aq. TFA and CH_3CN (1 : 1, v/v, 4 mL) and purified by semi-preparative RP-HPLC (system C, 2 injections, t_R = 16.6-20.4 min). The product-containing fractions were lyophilised to give microcystin analogue **15** as a white amorphous powder (15.7 mg, 29.5 µmol, yield 80%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.01 (d, J = 6.6 Hz, 3H), 1.20 (d, J = 7.0 Hz, 3H), 1.38 (d, J = 7.3 Hz, 3H), 1.63 (s, 3H), 1.82–2.88 (m, 8H), 3.20–3.25 (m, 4H), 4.16 (t, J = 7.5 Hz, 1H, CH), 4.30–4.44 (m, 2H), 4.87 (1H, partially masked by water peak), 5.41 (d, J = 9.9 Hz, 1H), 5.57–5.64 (dd, J = 7.3 Hz, 8.3 Hz, 1H), 6.24 (d, J = 15.7 Hz, 1H), 7.17–7.25 (m, 5H); MS (ESI-): m/z 531.47 $[M - H]^-$, 1063.20 $[2M - H]^-$, calcd for $C_{28}H_{40}N_2O_8$: 532.64; HPLC (system A): t_R = 17.6 min, purity 94%.

Acyclic microcystin analogue (16)

Aldol **10** (17 mg, 33 µmol) was dissolved in CH_3OH (2 mL). Aq. 1.0 M LiOH (0.36 mL, 360 µmol) was added and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system A) and acidified to pH ~3 by addition of acetic acid. Thereafter, volatiles were removed by evaporation under reduced pressure. The resulting residue was dissolved with a mixture of 0.1% aq. TFA and CH_3CN (1 : 1, v/v, 4 mL) and purified by semi-preparative

RP-HPLC (system D, 2 injections, t_R = 32.9-33.7 min). The product-containing fractions were lyophilised to give microcystin analogue **16** as a white amorphous powder (7.6 mg, 23.1 μmol, yield 70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.04 (d, J = 6.8 Hz, 3H), 1.20 (d, J = 7.1 Hz, 3H), 1.64 (s, 3H), 2.59–2.84 (m, 4H), 3.18–3.24 (m, 4H), 4.47–4.51 (m, 1H), 5.45 (d, J = 9.8 Hz, 1H), 5.51–5.58 (dd, J = 15.7 Hz, 7.1 Hz, 1H), 6.31 (d, J = 15.5 Hz, 1H), 7.18–7.30 (m, 5 H); MS (ESI-): m/z 331.47 $[M - H]^-$, 663.27 $[2M - H]^-$, calcd for $C_{20}H_{28}O_4$: 332.44; HPLC (system A): t_R = 21.6 min, purity >95%.

TFA salt of tripeptide H-Adda-*iso*-D-Glu(Ala)-OH (17)

(*a*) *Peptide coupling*: ***N*-Boc-Adda** (9.0 mg, 21 μmol) was dissolved in dry DMF (800 μL). Uronium salt-based coupling reagent HATU (9.5 mg, 25 μmol) and 2,4,6-collidine (5 μL, 63 μmol) were sequentially added and the resulting reaction mixture was stirred at room temperature. The complete conversion of ***N*-Boc-Adda** into the corresponding HOAt active esters was checked by RP-HPLC (system A). Then, TFA salt of H-Glu(Ala-OMe)-OMe **13** (9.1 mg, 63 μmol) was added and the reaction mixture was stirred at room temperature. The reaction was checked for completion by RP-HPLC (system A), the disappearance of HOAt active esters was observed but the coupling product seemed to remain adsorbed on the C_{18} stationary phase. Thereafter, AcOEt (5 mL) was added and the crude reaction mixture was sequentially washed with aq. 10% citric acid (5 mL), aq. sat. $NaHCO_3$ (25 mL) and brine (5 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The crude product was used in the next step without further purification. (*b*) *Boc removal*: The crude fully-protected tripeptide Boc-Adda-*iso*-D-Glu(Ala-OMe)-OMe was dissolved in CH_2Cl_2 (1 mL). TFA (74 μL, 100 μmol) was added dropwise and the resulting reaction mixture was stirred at reflux for 2 h. The reaction was checked for completion by ESI mass spectrometry and the mixture was evaporated to dryness. The resulting oily residue was dissolved in deionised water and lyophilised to give TFA salt of H-Adda-D-Glu(Ala-OMe)-OMe. This product was used in the next step without further purification. MS (ESI+): m/z 560.07 $[M + H]^+$, calcd for $C_{35}H_{53}N_3O_9$: 559.71. (*c*) *Saponification*: Crude tripeptide H-Adda-*iso*-D-Glu(Ala-OMe)-OMe was dissolved in CH_3OH (1 mL). Aq. 1.0 M LiOH (200 μL) was added and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by ESI mass spectrometry and acidified to pH ~3 by addition of acetic acid. Thereafter, volatiles were removed by evaporation under reduced pressure. The resulting residue was dissolved with a mixture of CH_3CN and 0.1% aq. TFA (1 : 2, v/v, 3 mL) and purified by semi-preparative RP-HPLC (system E, 2 injections, t_R = 19.8–20.9 min). The product-containing fractions were lyophilised to give the microcystin analogue **17** as a white amorphous powder (3.3 mg, 5 μmol, overall yield for the three steps 24%). ^{1}H NMR (300 MHz, CD_3OD): δ 0.93 (d, J = 6.8 Hz, 3H), 1.11 (d, J = 7.0 Hz, 3H), 1.19–1.29 (m, 9H), 1.57 (s, 3H), 1.89–1.96 (m, 2H), 2.20–2.27 (m, 2H), 2.51–2.75 (m, 4H), 3.15 (s, 3H), 3.79 (t, J = 8.9 Hz, 1H), 4.16–4.25 (m, 2H), 5.36–5.52 (dd, J = 15.6 Hz, 8.9 Hz, 1H), 5.50 (d, J = 9.8 Hz, 1H), 6.38 (d, J = 15.6 Hz, 1H), 7.08-7.10 (m, 5H); MS (ESI+): m/z 532.13 $[M + H]^+$, calcd for $C_{28}H_{41}N_3O_7$: 531.65; HPLC (system A): t_R = 15.3 min, purity 100%.

Adda

***N*-Boc-Adda** (18 mg, 42 μmol) was dissolved in CH_2Cl_2 (2 mL). TFA (124 μL, 1.7 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system A) and the reaction mixture was evaporated to dryness. The resulting oily residue was purified by semi-preparative RP-HPLC (system G, 2 injections, t_R = 18.0-23.9 min). The product-containing fractions were lyophilised to give **Adda** as a TFA salt (15.6 mg, 35 μmol, yield 84%). ^{1}H NMR (300 MHz, CD_3CN): δ 1.00 (d, J = 6.8 Hz, 3H), 1.18 (d, J = 7.1 Hz, 3H), 1.63 (s, 3H), 2.64–2.83 (m, 4H), 2.51–2.75 (m, 4H), 3.20 (s, 3H), 3.90 (t, J = 9.0 Hz, 1H), 5.53–5.56 (m, 2H), 6.40 (d, J = 15.6 Hz, 1H), 7.19–7.27 (m, 5H); ^{13}C NMR (75.5 MHz, CD_3CN): δ 12.4, 14.5, 15.1, 16.0, 29.6, 36.7, 38.2, 58.6, 86.9, 126.2, 128.4, 129.5, 132.2, 139.3, 139.7, 145.4; MS (ESI+): m/z 332.00 $[M + H]^+$, calcd for $C_{20}H_{29}NO_3$: 331.46; HPLC (system A): t_R = 17.2 min, purity 89%.

Adda-tripod conjugate (19)

N-Hydroxysuccinimidyl carbamate cross-linking reagent **18** (5.0 mg, 5.4 μmol) was dissolved in dry NMP (200 μL). A solution of **Adda** (TFA salt, 3.1 mg, 7 μmol) and dry DIEA (1.8 μL, 11 μmol) was prepared in dry NMP (400 μL) and added to the NMP solution containing active carbamate. The resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system A). A mixture of 0.1% aq. TFA and CH_3CN (9 : 3, v/v, 2 mL) was added and the resulting solution was purified by semi-preparative RP-HPLC (system E, 2 injections) The product-containing fractions were lyophilised to give peptide conjugate **19** as a white amorphous powder (3.0 mg, 2.6 μmol, yield 48%). MS (ESI+): m/z 1193.13 $[M + O + H_2O + H]^+$, calcd for $C_{54}H_{78}N_8O_{16}S_2$ 1158.49. Under our ionisation conditions, both oxidation of one double bond of Adda moiety (C4–C5) or (C6–C7) and hydration of phthalimide moiety were observed. HPLC (system A): t_R = 22.1 min, purity 97%.

FluoProbes® 532A labelled Adda-tripod conjugate (20)

(*a*) *Removal of the phthaloyl group*: Peptide conjugate **19** (3 mg, 2.6 μmol) was dissolved in CH_3OH (500 μL) and a solution of hydrazine monohydrate (3.9 μL) in CH_3OH (45 μL) was added. The reaction was stirred at room temperature for 2 h and checked for completion by RP-HPLC (system A). The reaction flask was stored at −20 °C overnight. Thereafter, 0.1% aq. TFA (pH 2.2, 2 mL) was added and the resulting solution was purified by semi-preparative RP-HPLC (system F, 2 injections). The product-containing fractions were lyophilised to give the free aminooxy tripod as a white amorphous powder. MS: see ESI†. (*b*) *Removal of the SEt group*: Free aminooxy tripod (2.3 mg, 2 μmol) was dissolved in aq. 0.1 M $NaHCO_3$ buffer (pH 8.5, 300 μL). A solution of DTT (12 mg, 78 μmol) in aq. 0.1 M $NaHCO_3$ buffer (pH 8.5, 200 μL) was added and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system A). 0.1% aq. TFA (pH 2.2, 2 mL) was added and the resulting aq. solution was purified by semi-preparative RP-HPLC (system H, 1 injection). The product-containing fractions were lyophilised to give the free sulfhydryl

tripod as a white amorphous powder. MS: see ESI†. (*c*) *Fluorescent labeling*: Free sulfhydryl tripod (0.5 mg, 0.6 μmol) was dissolved in aq. 0.1 M $NaHCO_3$ buffer (pH 8.5, 200 μL). A solution of FluoProbes 532A® maleimide labeling reagent (0.5 mg, 0.7 μmol) in dry CH_3CN (200 μL) was added and the resulting reaction mixture was protected from light and stirred at room temperature for 1 h. The labeling reaction was checked for completion by RP-HPLC (system B). Further amount of aq. 0.1 M $NaHCO_3$ buffer (pH 8.5, 200 μL) was added. Finally, 0.1% aq. TFA (pH 2.2, 2 mL) was added and the resulting aq. solution was purified by semi-preprative RP-HPLC (system H, 1 injection). The product-containing fractions were lyophilised to give fluorescent tripod **20** as a pink amorphous powder. Quantification was achieved by UV-visible measurements at $\lambda_{max} = 532$ nm of FluoProbes 532A® by using the ε value 115 000 M^{-1} cm^{-1} (overall yield for the three steps estimated after RP-HPLC purification 5%). MS (ESI+): *m/z* 1769.40 $[M + O + H_2O + H]^+$. Expected molecular ion $[M + H]^+$ of **20** at *m/z* 1737.49 was not observed. Under our ionisation conditions, both oxidation of one double bond of Adda moiety (C4–C5) or (C6–C7) and hydration of maleimide moiety were observed. HPLC (system B): $t_R = 29.4$ min, purity 81%.

Functionalisation and peptide immobilisation on silica surfaces

(*a*) *Preparation of the aldehyde-functionalised silica surface*: This was achieved from a silicon substrate (75 × 25 × 1 mm) doped with a thick SiO_2 layer (thickness 500 nm) by using experimental conditions already reported by us.[13] (*b*) *Immobilisation of fluorescent labelled "microcystin-tripod"* **20**: This fluorescent aminooxy pseudopeptide was prepared as a 10 μM solution in immobilisation buffer (potassium phosphate 0.1 M, pH 7.4) and 1 μL of the resulting solution was put over the freshly prepared aldehydic surface. After 4 h of incubation in the dark and in a humid atmosphere at room temperature, the reaction solution was carefully removed and the surface was washed for 10 min with phosphate buffer (10 mM + 0.05% Tween, pH 7.4). Finally, slide was stored with EIA buffer (0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.1% BSA and 0.01% sodium azide) overnight before observation. Fluorescence scanning was performed with an Olympus inverted microscope model IX71 (4X objective) equipped with a camera PCO 1600, and the resulting images were analysed using ImageJ software (http://rsbweb.nih.gov/ij/).

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