ORGANIC LETTERS

2013
Vol. 15, No. 15
3898–3901

First Total Synthesis and Stereochemical Revision of Laxaphycin B and Its Extension to Lyngbyacyclamide A

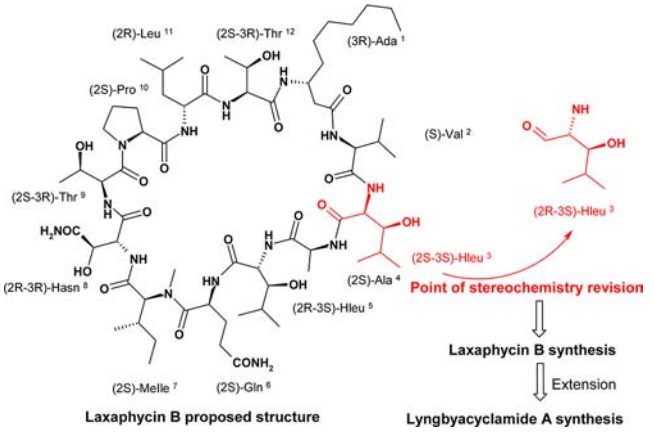
France Boyaud,[†] Zahia Mahiout,[†] Christine Lenoir,[‡] Shoubin Tang,[§] Joanna Wdzieczak-Bakala,[‡] Anne Witczak,[†] Isabelle Bonnard,[†] Bernard Banaigs,[†] Tao Ye,[∥] and Nicolas Inguimbert*[,†]

Laboratoire des Biomolécules et de l'Environnement (LCBE), Université de Perpignan Via Domitia, centre de phytopharmacie, 58 avenue P. Alduy, 66860 Perpignan, France, Institut de Chimie des Substances Naturelles, UPR 2301, CNRS avenue de la terrasse, 91198 Gif-sur-Yvette Cedex, France, School of Chemical Biology & Biotechnology, Peking University, Shenzhen Graduate School, University Town of Shenzhen, Xili, Shenzhen, 518055, China, and Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China

Nicolas.inguimbert@univ-perp.fr

Received June 12, 2013

ABSTRACT



The first total synthesis of laxaphycin B was accomplished through stepwise automated Solid Phase Peptide Synthesis (SPPS), leading to the structural revision of its stereochemistry especially with regard to the configuration of one of the two 3-hydroxyleucines of this cyclic dodecapeptide of marine origin. The analogous Lyngbyacyclamide A was obtained by an extension of this synthesis.

Laxaphycins and their relatives are cyclic lipopeptides isolated from cyanobacteria, outstanding producers of marine secondary metabolites.[1,2] They can be subdivided into two main classes: one made up of undecapeptides, and the second, of dodecapeptides corresponding respectively to laxaphycin A (**1**) and B (**2**) derivatives (Figure 1). Intriguingly, both compounds act synergistically on cancer cells or fungi, but when tested separately only laxaphycin B endorses the activity albeit with consequent reduction. Nevertheless, Laxaphycin B showed cytotoxicity activity against a wide range of cancer cell lines with IC_{50} ranging from 0.2 to 6.0 μM.[3] Unfortunately how it acts is not known.

As part of our program on the study of marine secondary metabolites, we were interested in gaining some insight

[†] LCBE Université de Perpignan.
[‡] Institut des Substances Naturelles ICSN Gif Sur Yvette.
[§] Peking University Shenzhen.
[∥] Hong Kong Polytechnic University.

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10.1021/ol401645m
Published on Web 07/15/2013

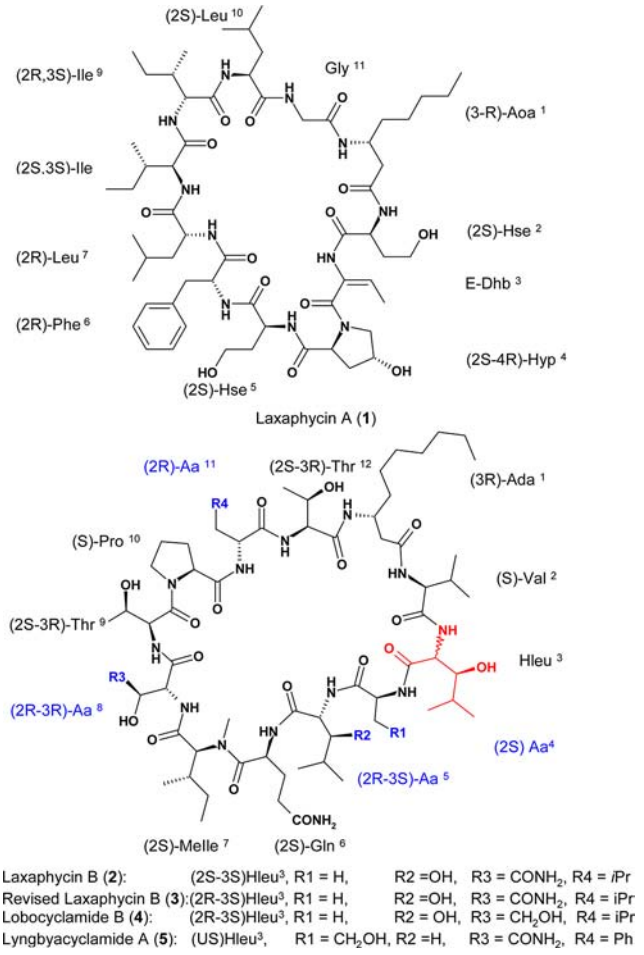

Figure 1. Structure residues numbering and stereochemistry of the reported laxaphycins A (**1**) and B (**2**) and of the parents lobocyclamide B (**4**), lyngbyacyclamide A (**5**), and of revised laxaphycin B (**3**). (US: unknown stereochemistry, Aa: amino acid).

into not only the ecological role of laxaphycin B but also its use as a template for the discovery of new anticancer drugs through the study of its structure activity relationships (SAR).[4] In order to engage in SAR studies, we needed first to confirm its structure and second to develop a synthesis offering some flexibility in terms of amino acids mutations. Automated stepwise Solid phase Peptide Synthesis (SPPS), offering both adaptability and efficiency, was chosen for the development of the synthesis. We report herein the first solid-phase based total synthesis of laxaphycin B and its stereochemical revision.

The first Laxaphycin B gross structure was determined by extensive NMR and mass spectroscopy studies.[5] After which, the absolute configurations of the amino acids were determined by an advanced Marfey method.[6] Laxaphycin B is characterized by an alternation of hydrophobic and hydrophilic residues having opposite configurations at the α or β carbons constituting the 36-membered macrolactam ring. Thus from a structural point of view, Laxaphycin B is a complex lipopeptide composed of a rare (3*R*)-β-aminodecanoic acid (Ada) together with the nonstandard amino acids: (2*R*,3*R*)-hydroxyasparagine (Hasn), (2*R*,3*S*)-hydroxyleucine (Hleu), and its (2*S*,3*S*) diastereoisomer. The laxaphycin B family has been extended over the years with the isolation of other analogues. The lobocyclamides B, C and the lyngbyacyclamides A, B differentiate not only in their amino acid content but also by subtle variation of their configurations that remain unknown for the nonribosomal amino acids contained in lyngbyacyclamide A (Figure 1).[7,8] As none of the laxaphycin-type peptides had been synthesized so far, we synthesized laxaphycin B in order to confirm its structure. The main challenges in this synthesis were to obtain the nonnatural amino acids bearing suitable protective groups for the Fmoc SPPS strategy and the formation of the macrocycle.

In order to dispose of a versatile synthetic method to generate a library of laxaphycin B analogues to establish SAR we chose to develop an on-resin "head-to-tail" cyclization of a linear precursor of laxaphycin B. This strategy allows the isolation and purification of the linear intermediate to be avoided, minimizing dimerization side reactions and resulting in the increased overall yield of the final product. Subsequent application of this concept used the side chain of Asp/Asn and Glu/Gln in conjunction with temporary allyl protection for the C-terminal α-carboxyl group.[9]

In our retrosynthetic analysis of laxaphycin B, we identified β-hydroxyaspartic acid or glutamic acid as one of the possible residues to be anchored *via* their side chains onto the resin and as the final point of the cyclization. However, glutamic acid was discarded because the cyclization would have been impaired by the coupling of the *N*-methylisoleucine onto its α-carboxylic function. Therefore, we used the (2*R*,3*R*)-Fmoc-β-hydroxyaspartic α-allyl ester developed for this purpose.[10] The (2*R*,3*S*)-Fmoc-Hleu-(OTBDMS)-OH, (2*S*,3*S*)-Fmoc-Hleu-(OTBDMS)-OH, and (3*R*)-Fmoc-β-aminodecanoic acid necessary for the laxaphycin B synthesis were obtained after production of the unprotected amino acids generated from known procedures.[11,12]

After immobilization of the N-Fmoc-3-hydroxyaspartic acid α-allyl ester onto a low loading rink amide MBHA

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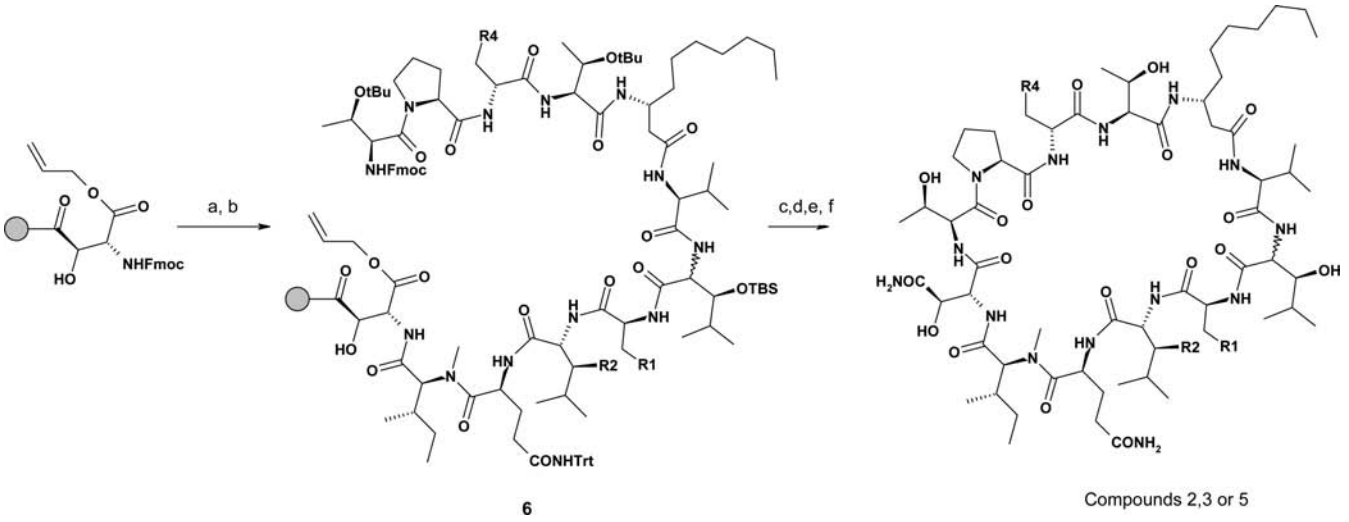

Figure 2. Syntheses of laxaphycin B (**2**, **3**) and lyngbyacyclamide **5**. Reagents and conditions: (a) (i) 20% v/v piperidine/DMF (ii) Fmoc-NMeIle, HATU, DMF, MW 75 W, 5 min. (b) Repeat conditions (a) for: Fmoc-Gln-(Trt) followed by capping with 0.5 M acetic anhydride in DMF for 3 min, 60 °C, 40 W, (2*R*,3*S*)-Fmoc-Hleu-(OTBDMS)-OH or (2*R*)-Fmoc-Leu for **5**, 15 min, Fmoc-Ala-OH or Fmoc-Hse(*t*Bu)OH for **5**, (2*S*,3*S*)-Fmoc-Hleu-(OTBDMS)-OH for **2**, (2*R*,3*S*)-Fmoc-Hleu-(OTBMS)-OH for **3** and **5** for 15 min, Fmoc-Val-OH, (3*R*)-Fmoc-Ada for 15 min, Fmoc-Thr(tBu)-OH, (2*R*)-Fmoc-Leu-OH or (2*R*)-Fmoc-Phe for **5**, Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH; (c) $Pd(PPh_3)_4$, $CHCl_3$/AcOH/NMM: 3.7/0.2/0.1; (d) 20% v/v piperidine, DMF, rt, 2 × 2 min; (e) DIC, oxyma, DMF, 70 °C, 25 W, 3 × 15 min; (f) TFA/TIS/H_2O 95/2.5/2.5, v/v/v, rt, 3 h.

resin (0.36 mmol/g), followed by removal of the Fmoc protecting group, the linear precursor **6** was assembled by a standard SPPS protocol, using the effective 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate HATU as a coupling reagent and a 5-fold excess of standard amino acids with regard to the resin capacity (Figure 2). The choice of this relatively expensive reagent was dictated by preliminary results which revealed the presence of two difficult coupling sites. The first one was located at the Gln and N-Me-Ile junction, and the second, between Ada and Thr, with both of them requiring a triple coupling although their insertion still remained incomplete, thus necessitating a capping step of the resin with acetic anhydride after the Fmoc-N-Me-Ile coupling. In an effort to spare the precious and nonproteinogenic amino acids, only 2.5 equiv were used and the coupling time was consequently extended to 15 min to allow for completion of the reaction. After synthesis of the linear precursor **6**, the α-carbonyl allyl protecting group of Asn was removed using $Pd(PPh_3)_4$. Head to tail cyclization of the resin bound peptide was accomplished after Fmoc removal using DIC/oxyma (3 × 15 min), a base-free condition known to reduce epimerization.[13]

The peptide was removed from the resin with simultaneous side chain deprotection using standard TFA mediated acidolysis affording 40 mg of crude cyclic peptide with a yield of 28% based on manufacturer's resin loading and a purity around 60%. The crude peptide was purified by reversed-phase high performance liquid chromatography (HPLC) yielding 3 mg of pure compound **2** with a recorded molecular mass of 1395 $(M+H)^+$ and 1417 $(M+Na)^+$ from LC/MS ESI+ analysis corresponding to the one expected for laxaphycin B. Furthermore, MS^2 and MS^3 experiments confirmed the cyclic structure of the peptide **2**. Indeed, only water molecules were lost, as observed when the natural laxaphycin B underwent the same experiment. Additional HPLC analyses were performed in order to confirm that the synthetic peptide **2** was identical to the natural one.

Due to poor UV absorption of laxaphycin B, Evaporative Light Scattering Detection (ELSD) affording enhanced sensitivity was used for the sample analysis. Compound **2** (100% purity) showed a definite peak at 16.3 min, but when coinjected with natural laxaphycin B, an additional peak separated by 0.5 min appeared thus highlighting that despite their identical mass the two peptides differ (Figure 3). These differences were further confirmed after comparison of the two NMR spectra that showed significant differences in the chemical shifts of the amide and CHα protons (Figure S11).Therefore, we could conclude from this first set of experiments that compound **2** is certainly a diastereoisomer of the natural compound.

After having carefully scrutinized the reported literature concerning the analogs of laxaphycin B, we have hypothesized that the two 3-hydroxyleucines of laxaphycin B have the same configuration namely 2*R*,3*S* as is the case for the lobocyclamide B (**4**) (Figure 1).[7] And, from a more general point of view, the stereochemistries at the α-carbon are conserved for all the laxaphycin B relatives.[3] Furthermore by reconsidering the Marfey experiments, we noticed that

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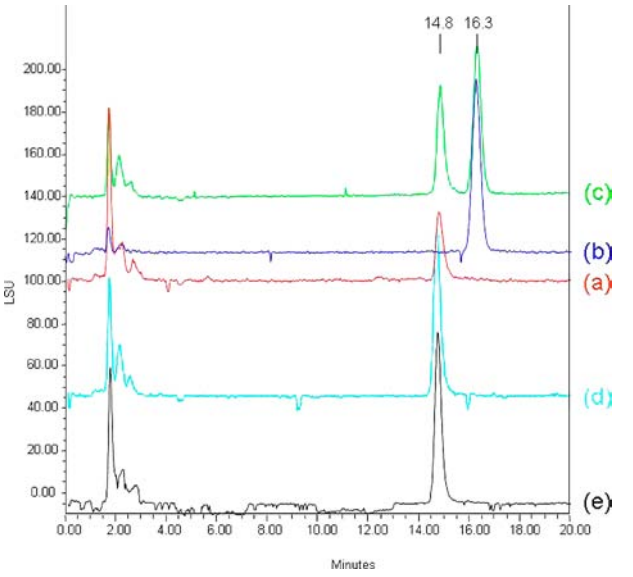

Figure 3. HPLC chromatogram with ELSD detection of (a) natural laxaphycin B, (b) synthetic laxaphycin B **2**, (c) coinjection of natural and synthetic laxaphycin B **2**, (d) synthetic laxaphycin B **3**, (c) coinjection of natural and synthetic laxaphycin B **3**.

(2*R*,3*S*)-Hleu was always detected while the (2*S*,3*S*) diastereoisomer appeared only after long hydrolysis times. Hence we have suspected that this later isomer resulted from racemization of the (2*R*,3*S*) isomer (Figures S13−14). The corresponding compound **3** was obtained using the procedure described above leading to 3 mg of pure compound coeluting with natural laxaphycin B (Figure 3) and presenting the same fragmentation profile during MS^n experiments as its natural counterpart. In addition, comparison of the ^1H NMR spectra of both compounds revealed a perfect overlap of the amide and C-α regions (Figure S17).

Thus, the original assignment of the hydroxyleucines as two diastereoisomers could be discarded as this was unambiguously proven by this total synthesis. Indeed the absolute stereochemistry at the hydroxyleucine in position 3 was reassigned as 2*R*,3*S* by correlating the ^1H NMR data, HPLC retention time, and LCMS analysis with those of the natural product. Furthermore, compound **3** showed the same cytotoxicity as that of laxaphycin B in a panel of cancerous cell lines. Considering that laxaphycin B, lobocyclamide B, and lyngbyacyclamide A are all produced by cyanobacteria, we could hypothesize that they originated from a similar biosynthetic pathway. Consequently the nonribosomal amino acids in position 1, 3, 5, and 7 contained in lyngbyacyclamide must have the same configuration as those found in lobocyclamide B and laxaphycin B as this was presently demonstrated. Therefore, we applied the former synthesis to lyngbyacyclamide A (**5**) that was obtained in pure form (Supporting Information Figures S19−21). Furthermore the ^1H NMR spectra do not differ significantly from the ones in DMSO reported by Maru et al. (Table S2).[8]

In conclusion, we have described the first total synthesis of laxaphycin B and this synthesis could be extended to the other members of the laxaphycin B family, thus suggesting that this class of compounds has a similar biosynthetic pathway. Furthermore, this result has opened the way for the realization of structure−activity studies on this family, as well as the synthesis of laxaphycin B relatives.

Acknowledgment. This work was supported by grants from ANR (ANR-2010-BLAN-1533-02), and from Perpignan University (BQR). The spectroscopic experiments were performed using the Bio2Mar facilities at the University of Perpignan (http://bio2mar.obs-banyuls.fr/fr/index.html). We also thank Dr. B. Legrand (Institut des Biomolécules Max Mousseron, UMR 5247, CNRS Universités Montpellier I et II, UFR des Sciences Pharmaceutiques et Biologiques, 15 Avenue Charles Flahault, 34093 Montpellier Cedex 5) for the recording of CD spectra and R. Hargraves (Université de Perpignan) for improvement of the manuscript.

Supporting Information Available. Synthesis protocols, NMR spectra, LC/MS analysis and HPLC chromatograms of (2*R*,3*S*)-Fmoc-Hleu(3-OTBDMS)-OH, (2*S*,3*S*)-Fmoc-Hleu(3-OTBDMS)-OH, (3*R*)-Fmoc-Ade, compounds **2**, **3**, and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.