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Stereoselective Synthesis of Deuterium-Labeled (2*S*)-Cyclohexenyl Alanines, Biosynthetic Intermediates of Cinnabaramide

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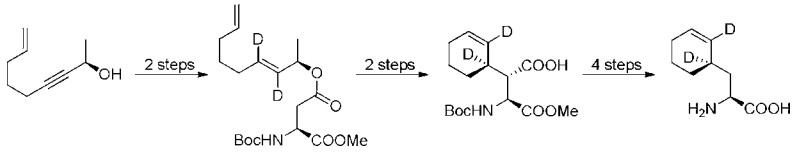
ABSTRACT



Dideuterated β-cyclohexenylalanines, proposed biosynthetic intermediates of the cinnabaramides, can be obtained from chiral alkynols *via* a sequence of Irland–Claisen rearrangement, ring closing metathesis, and radical decarboxylation. Feeding experiments indicate that both (2*S*)-β-cyclohexenylalanines can be incorporated into cinnabaramide, while the configuration at the cyclohexenyl ring does not restrict biosynthetic processing.

The proteasome is a key player in the regulation of a wide range of cellular processes by degradation of proteins, mediating processes such as amino acid recycling, cell differentiation, and apoptosis.[1] Therefore, proteasome inhibitors are interesting candidates as antitumor drugs.[2] Effective proteasome inhibitors are widespread in nature, produced by a wide range of microorganisms. Many of these inhibitors are peptide derived small molecules, such as the group of γ-lactam–β-lactones.[3]

In 1991, Omura et al. isolated a potent inhibitor from *Streptomyces lactacystineus*, called lactacystin.[4] But actually, the active compound was not lactacystin itself, but its *clasto*-form omuralide (Figure 1).[5] A closely related family of compounds are the salinosporamides,[6] isolated by Fenical from the marine actinomycete *Salinospora tropica*.[7] Almost the same structural motif was found in the cinnabaramides, a secondary metabolite of the terrestrial strain *Streptomyces JS 360*.[8]

The cinnabaramides and salinosporamides only differ in the side chain of the γ-lactam ring. Cinnabaramides and salinosporamide A inhibit the 20S-proteasome in the low nanomolar range.[8,9] The strained β-lactone ring of these

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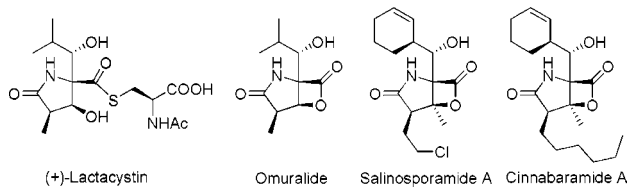


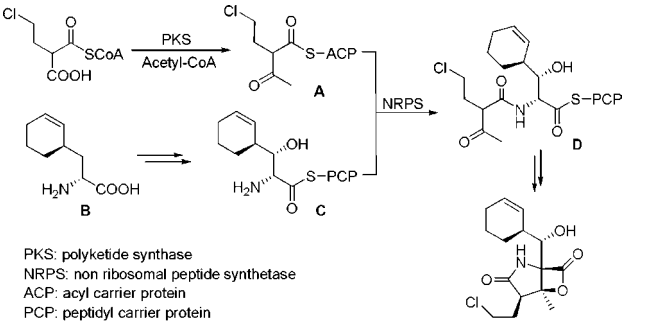
Figure 1. Naturally occurring γ-lactam–β-lactones.

compounds interacts with a threonine in the active center of the proteasome, inactivating the enzyme by ring opening and covalent blocking of the active center.[9] Salinosporamide is found to trigger apoptosis and is in phase 1 of clinical trials for the treatment of multiple myeloma.[10] Not surprising, these natural products arouse interest in the community of synthetic organic chemists, and a wide range of interesting syntheses have been developed in recent years for omuralide,[11] cinnabaramide,[12] and especially salinosporamide[13] and derivatives.[14] The recent developments in this field are nicely covered in reviews by Moore[3a] and Potts.[15]

Besides the development of straightforward protocols for the synthesis of these compounds, much effort has also been focused toward investigating their biosynthesis. While Moore et al. are involved in the biosynthesis of the salinosporamides,[3b,16] we are mainly interested in the cinnabaramides.[17]

In principle, the molecule can be divided into two major parts. In the case of the salinosporamides, the lower part of the molecule is the product of a polyketide synthase (PKS) leading to an activated β-ketothioester **A** (Scheme 1). The upper part, the unusual amino acid β-hydroxy cyclohexenylalanine, is formed *via* a shunt in the phenylalanine biosynthetic pathway, leading primarily to β-cyclohexenylalanine **B**.[16a] After coupling to the peptidyl carrier protein and oxidation by a cytochrome P450 hydroxylase (**C**), the two building blocks are coupled on a nonribosomal peptide synthetase (NRPS) to give **D**. Subsequent cyclization gives rise to salinosporamide.[16]

Scheme 1. Biosynthesis of Salinosporamide A according to Moore[16a]



In principle, one might expect a very similar biosynthetic pathway also for the cinnabaramides, which should differ mainly in the PKS-subunit. To study the cinnabaramide biosynthesis, we were interested in obtaining deuterium-labeled building blocks which can be used for feeding experiments. Recently, we described the stereoselective synthesis of dideuterated (2*R*,3*S*,4*S*)-β-cyclohexenylserine,[18] a postulated intermediate in an early biosynthetic proposal.[16a] Later, the configuration of the amino acid was determined to be (2*S*).[16b] Therefore, it was not so surprising that no incorporation of this amino acid itself, or in the activated form, was observed in feeding experiments. Recent biosynthetic studies by the Müller group indicate that, in the case of the cinnabaramides, **B** is probably coupled to the corresponding β-ketoester and that the cyclochrome P-450 oxidation at the β-position proceeds later on in the biosynthesis.[19]

To prove this proposal, we developed a stereoselective synthesis of the two isomeric dideuterated (2*S*)-β-cyclohexenylalanines. So far, the absolute configuration of the cinnabaramide intermediates is not yet determined, but the (2*S*) configuration seems reasonable, based on the analogy to the salinosporamide biosynthesis. In the natural product, the (4*S*)-configuration is found, and therefore the (2*S*,4*R*)-β-cyclohexenylalanine **1** should be the correct

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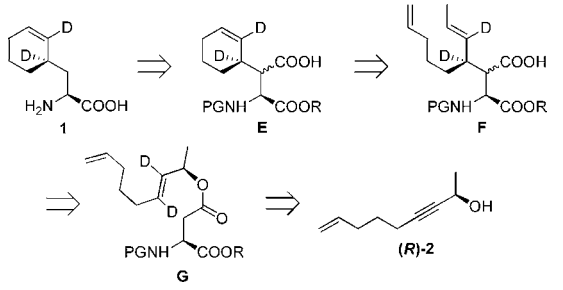
intermediate. Interestingly, the biosynthetic enzyme responsible for the activation of the β-cyclohexenylalanine seems to have a rather broad substrate spectrum, allowing also the incorporation of other amino acids.[16b,20] Therefore, we were interested to see if the configuration of the 4-position does play any role.

Our retrosynthetic plan toward the (2*S*,4*R*)-**1** is shown in Scheme 2. D_2-labeled β-cyclohexenylalanine **1** should be accessible *via* radical decarboxylation of the corresponding aspartate derivative **E**, while the cyclohexenyl ring should be the result of a ring closing metathesis (RCM). The key step of the synthesis is a stereoselective Claisen rearrangement providing the diene **F** from the allyl ester **G**. In this step the stereogenic center at C-4 is incorporated. Depending on the configuration of the allyl alcohol used, both isomers can be obtained stereoselectively. The configuration at C-3 of **F** does not play any role, because this stereogenic center is removed later on. The required dideuterated *trans* allyl alcohol should be available from the corresponding propargyl alcohol **(*R*)-2** *via* reduction using $LiAlD_4$.

Scheme 2. Retrosynthesis of (2*S*,4*R*)-Dideuterated Cyclohexenylalanine **1**



We began our synthesis with the coupling of 5-bromopentene with butyn-3-ol (Scheme 3). Although this reaction is described in the literature,[21] we were able to improve the yield of **2** significantly by using $LiNH_2$ as a base (instead of BuLi). The racemic alkynol **2** was subsequently subjected to an enzymatic kinetic resolution using Novozym 435. The reaction stops after 50% conversion, and both enantiomeric compounds were obtained with high yield and *ee*. Unfortunately, we were not able to determine the *ee*-value of the unreacted alcohol directly by GC (no separation) and therefore, for analytical purposes, the remaining alcohol **(*S*)-2** was also converted into the acetate **(*S*)-3**. Upon saponification, both enantiomers of alkynol **2** were obtained.

For the synthesis of the deuterated (2*S*,4*R*)-cyclohexenylalanine **1**, **(*R*)-2** was reduced with $LiAlD_4$ (Scheme 3). The *in situ* formed vinylalumoxane was quenched by the addition of D_2O giving rise to the *trans*-configured dideuterated allyl alcohol **(*R*)-4**. Coupling to semiprotected aspartate[22] using the Steglich protocol provided the required allylester **5**, the substrate for the subsequent Claisen rearrangement. Our group was investigating Claisen rearrangements of amino acids for a long time.[23] While the best results are obtained for α-amino acid esters with chelated enolates,[24] this protocol is not really suitable for β-amino acid esters, such as **5**. Here the Ireland−Claisen rearrangement[25] is the method of choice,[26] providing **6** in high yield and diastereoselectivity. While the stereogenic center at C-2 remains unchanged, the configuration at C-4 is determined by the stereogenic center of the allyl alcohol (chirality transfer almost perfect). The configuration at C-3 is the result of the enolate formation in the deprotonation step. This center is formed as a 9:1 mixture, according to NMR. But this center is negligible, because it is removed later on. The diastereomeric rearrangement products could be separated by flash chromatography. Diastereomerically pure **6** was subjected to ring closing metathesis providing cyclohexenyl aspartate **7** in high yield. In case the diastereomeric mixture was subjected to RCM also at this stage, a separation of the isomers was possible.

The next step, the radical decarboxylation, was found to be the most critical one of the whole sequence. We decided to apply the Barton protocol using the corresponding *N*-hydroxythiopyridone esters.[27] Unfortunately, this ester was found to be very sensitive, undergoing fast decomposition. Attempts to isolate and purify it were unsuccessful. Therefore, we had to carry out the activation and decarboxylation in a one-pot protocol. We investigated a wide range of coupling reagents for the initial step, the formation of the active ester. No ester formation was observed by using chloroformates, carbonyldiimidazole, or T3P as coupling reagents. DCC at −20 °C provided the required ester, but in impure form, and therefore, the decarboxylated product was very difficult to separate from the byproducts formed. The best results were obtained using $PhPOCl_2/NEt_3$ in the activation step, and the very mild BEt_3/O_2 protocol[28] for the radical formation. *t*-BuSH was used as a H-source. Under these optimized conditions, the required product **8** could be obtained in 49% yield. The final steps toward **1** proceeded quantitatively. The isomeric (2*S*,4*S*)-derivative was obtained from **(*S*)-2** in an analogous fashion and comparable yield.

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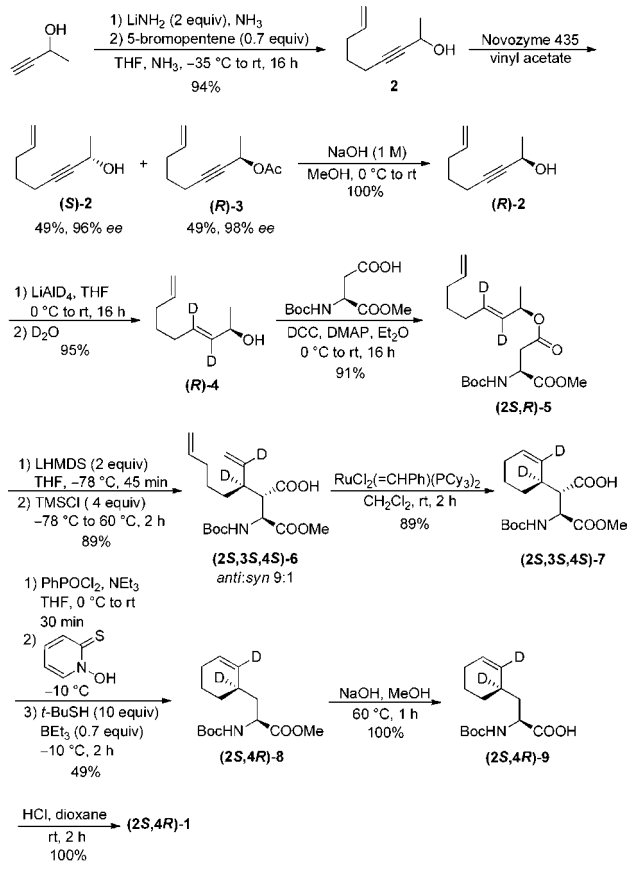
Scheme 3. Synthesis of d_2-**(2*S*,4*R*)-1**



Subsequent feeding experiments were conducted using compound **1**, which was stepwise administrated to the culture broth of *Streptomyces* sp. JS360 (see Supporting Information). Organic extracts of the culture supernatants were analyzed using HPLC-MS, which unambiguously demonstrated incorporation of **1** into cinnabaramide resulting in the production of dideuterated cinnabaramides in a yield comparable to that of native cinnabaramides. Based on this efficient incorporation we can conclude that (2*S*,4*R*)-β-cyclohexenylalanine **1** most likely serves as the native substrate of the cinnabaramide peptide synthetase. Interestingly, while the salinosporamide pathway shows a broad substrate tolerance allowing for the incorporation of alternative amino acids, no cinnabaramides derived from incorporation of proteinogenic amino acids have been detected in extracts of the wild type producer. But interestingly, the diastereomeric (2*S*,4*S*)-cyclohexenylalanine was incorporated as well. This may indicate that either (1) the free amino acids cannot be activated for further incorporation or (2) *in vivo* insufficient quantities of amino acids are available for secondary metabolite biosynthesis. As the latter scenario is unlikely we assume that it should be possible to incorporate alternative amino acids in the respective position of cinnabaramides. Ongoing work in our laboratories is addressing this question. After conducting a bioactivity screening, we expect to discover more cytotoxic novel cinnabaramides.

In conclusion, we have shown that the Irland−Claisen rearrangement of β-amino acid allylic esters is a highly suitable tool for the stereoselective synthesis of β-substituted aspartates. Subsequent ring closing metatheses and decarboxylations give rise to deuterated β-cyclohexenylalanine derivatives. Feeding experiments indicate that (2*S*)-β-cyclohexenylalanine is an intermediate in the biosynthesis of cinnabaramide. The configuration at the cyclohexenyl ring does not play any role, as obviously both stereoisomers are accepted by the peptidyl carrier protein. Further biosynthetic studies are currently under investigation.

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Supporting Information Available. Experimental procedures as well as analytical and spectroscopic data of all new compounds, details of the feeding experiments, and copies of NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.