Biotransformacija eufoboetirana A s gljivicom Sordaria tormento-alba s ciljem potrage za novim bioaktivnim molekulama

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UNIVERSITY OF SPLIT

FACULTY OF CHEMISTRY AND TECHNOLOGY

GRADUATE UNIVERSITY STUDY OF CHEMISTRY

ORGANIC CHEMISTRY AND BIOCHEMISTRY

BIOTRANSFORMATION OF EUPHOBOETIRANE A BY *Sordaria tomento-alba* AIMED AT THE SEARCH FOR NEW BIOACTIVE MOLECULES

DIPLOMA THESIS

MARIJA KIRIĆ

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BIOTRANSFORMACIJA EUFOBOETIRANA A S GLJIVICOM *Sordaria tomento-alba* S CILJEM POTRAGE ZA NOVIM BIOAKTIVNIM MOLEKULAMA

Marija Kirić, 338

Sažetak:
Latirani, koji se često nalaze u biljkama roda Euphorbia, pripadaju skupini biološki aktivnih policikličkih diterpenoida. Ova grupa spojeva i njihovi sintetski derivati dobili su značajnu pozornost zahvaljujući svojim biološkim aktivnostima i kliničkom potencijalu protiv različitih bolesti kao što je višestruka otpornost na lijekove (MDR), protuupalno djelovanje, citotoksično djelovanje protiv staničnih linija raka, antivirusna svojstva i njihova sposobnost induciranja proliferacije ili diferencijacije u neurone neuralnih progenitorskih stanica.
U ovom radu provedena je izolacija, pročišćavanje i biotransformacija eufoboetirana A (**4**) pomoću endofitne gljivice *Sordaria tomento-alba*. Cijele stanice kulture ove gljivice korištene su za biotransformaciju početnog supstrata (**4**) te su dobivena dva produkta biotransformacije od interesa, koji su odvojeni i pročišćeni kolonskom kromatografijom i HPLC-om. Strukture dobivenih produkata biotransformacije razjašnjene su na temelju opsežnih NMR i MS analiza podataka. Identificirani su kao Sordarianona (**23**) i 3,5,15-triacetoksi-12-hidroksilatira-6(17),13(20)-dien-14-on (**24**).
Ovo istraživanje pokazuje biotransformacijsku sposobnost endofitne gljivice *Sordaria tomento-alba* da vrši intramolekularne cikloadicije, hidroksilacije i izomerizacije dvostrukih ugljik-ugljik veza. Ove reakcije mogu dovesti do derivata latirana s pojačanim biološkim aktivnostima, što bi moglo pomoći u rasvjetljavanju strukturnih značajki po kojima ti spojevi pokazuju potencijal za liječenje neurodegenerativnih poremećaja.

Ključne riječi: latirani, eufoboetiran A, biotransformacija, endofitna gljivica, neurogeneza

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DIPLOMA THESIS

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BIOTRANSFORMATION OF EUPHOBOETIRANE A BY *Sordaria tomento-alba* AIMED AT THE SEARCH FOR NEW BIOACTIVE MOLECULES

Marija Kirić, 338

Abstract: Lathyranes, frequently found in plants of the genus *Euphorbia*, belong to the group of biologically active polycyclic diterpenoids. This family of compounds and their synthetic derivatives have received considerable attention owing to their interesting biological activities and clinical potential against different diseases like multi-drug resistance (MDR) reversal, anti-inflammatory activity, cytotoxic activity against cancer cell lines, antiviral properties and their capability to induce proliferation or differentiation into neurons of neural progenitor cells.
In this work, it has been carried out the isolation, purification and biotransformation of euphoboetirane A (**4**) by the endophytic fungus *Sordaria tomento-alba*. Whole cell culture of this fungus was used to biotransform the initial substrate (**4**) in two biotransformation products of interest, which were separated and purified by the column chromatography and HPLC. The structures of obtained biotransformation products were elucidated on the basis of extensive NMR and MS data analyses. They were identified as Sordarianona (**23**) and 3,5,15-triacetoxy-12-hydroxylathyra-6(17),13(20)-dien-14-on (**24**).
This research shows biotransformation ability of endophytic fungus *Sordaria tomento-alba* to perform intramolecular cycloadditions, hydroxylations and isomerisations of carbon-carbon double bonds. These reactions may lead to lathyrane derivatives with enhanced biological activities, which could help to clarify the structural features by which these compounds show potential for the treatment of neurodegenerative disorders.

Keywords: lathyrane, euphoboetirane A, biotransformation, endophytic fungus, neurogenesis

Thesis contains: 35 pages, 9 figures, 2 tables, 5 schemes, 4 supplements, 16 references

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Defence committee for evaluation and defense of diploma thesis:

1. Branka Andričić - PhD, Full Prof.	chair person
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3. Ani Radonić – Ph, Full Prof.	supervisor

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Diploma thesis was made in Department of Organic Chemistry, Faculty of Science, University of Cadiz, under mentorship of professor Ani Radonić and under the co-mentorship of professor Rosa María Durán Patrón, from March until July 2022.

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DIPLOMA THESIS TASK/ASSIGNMENT

- Isolation and purification of lathyrane euphoboetirane A (**4**) from *Euphorbia boetica*
- Biotransformation of euphoboetirane A (**4**) by the endophytic fungus *Sordaria tomento-alba*
- Separation and purification of biotransformation products by column chromatography and HPLC
- Identification of biotransformation products by NMR and mass spectroscopy

ABSTRACT

Lathyranes, frequently found in plants of the genus *Euphorbia*, belong to the group of biologically active polycyclic diterpenoids. This family of compounds and their synthetic derivatives have received considerable attention owning to their interesting biological activities and clinical potential against different diseases like multi-drug resistance (MDR) reversal, anti-inflammatory activity, cytotoxic activity against cancer cell lines, antiviral properties and their capability to induce proliferation or differentiation into neurons of neural progenitor cells.

In this work, it has been carried out the isolation, purification and biotransformation of euphoboetirane A (**4**) by the endophytic fungus *Sordaria tomento-alba*. Whole cell culture of this fungus was used to biotransform the initial substrate (**4**) in two biotransformation products of interest, which were separated and purified by the column chromatography and HPLC. The structures of obtained biotransformation products were elucidated on the basis of extensive NMR and MS data analyses. They were identified as Sordarianona (**23**) and 3,5,15-triacetoxy-12-hydroxylathyra-6(17),13(20)-dien-14-ona (**24**).

This research shows biotransformation ability of endophytic fungus *Sordaria tomento-alba* to perform intramolecular cycloadditions, hydroxylations and isomerisations of carbon-carbon double bonds. These reactions may lead to lathyrane derivatives with enhanced biological activities, which could help to clarify the structural features by which these compounds show potential for the treatment of neurodegenerative disorders.

Keywords: lathyrane, euphoboetirane A, biotransformation, endophytic fungus, neurogenesis

SAŽETAK

Latirani, koji se često nalaze u biljkama roda Euphorbia, pripadaju skupini biološki aktivnih policikličkih diterpenoida. Ova grupa spojeva i njihovi sintetski derivati dobili su značajnu pozornost zahvaljujući svojim biološkim aktivnostima i kliničkom potencijalu protiv različitih bolesti kao što je višestruka otpornost na lijekove (MDR), protuupalno djelovanje, citotoksično djelovanje protiv staničnih linija raka, antivirusna svojstva i njihova sposobnost induciranja proliferacije ili diferencijacije u neurone neuralnih progenitorskih stanica.

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Ključne riječi: latirani, eufoboetiran A, biotransformacija, endofitna gljivica, neurogeneza

CONTENTS

INTRODUCTION

Since ancient times plants have been a continuous source of beneficial and useful compounds. The use of plants from the genus *Euphorbia* in curative applications of herbal remedies dates back over thousands of years, particularly in traditional Chinese medicine. These plants are known as sources of biologically active poly- and macrocyclic diterpenes, such as lathyranes. As a result of their potential multidrug resistance (MDR) reversing effect and capability to induce neural progenitor cell (NPC) proliferation or differentiation into neurons, lathyrane diterpenoids have received noticeable attention.

Biocatalysis has become a valuable tool in obtaining new and effective products that are either impossible or challenging to obtain using conventional chemical procedures. The development of new biocatalytic methods is a constantly growing field of microbiology, chemistry, and genetic engineering since biocatalysts are environmentally friendly, easy-to-handle and selective. Microorganisms play a significant role in catalysing a wide range of reactions. Among them, fungi have garnered attention as a promising source of new biocatalysts, especially for chiral reactions. Regio-, chemo-, and stereoselective processes are of great importance in the synthesis of plenty pharmaceutical, agrochemical and chemical intermediates, food ingredients and active pharmaceuticals.

1. GENERAL PART

1.1. *Euphorbia boetica*

Euphorbia boetica (Figure 1) is an herb commonly encountered in the southern part of the Iberian Peninsula and endemic to Europe.[1] It belongs to a plant family Euphorbiaceae, which is recognized as one of the largest families of higher plants containing approximately 50 tribes, 300 genera and an extensive number of species, totaling around 7500 species. The genus *Euphorbia* (spurges) stands as one of the six largest genera of flowering plants, comprising nearly 2000 species. Production of a milky latex, which can cause skin irritation in humans upon contact, is characteristic by members of this genus.[2] Many species within the *Euphorbia* genus are used as medicinal plants for the treatment of migraine, gonorrhea, skin diseases, as wart cures and intestinal parasites.[3] Plants that belong to the family Euphorbiaceae are widely known as sources of biologically active polycyclic diterpenoids with a wide range of pharmacological properties including cytotoxic, anti-Hiv, antiviral, antimicrobial and modulation of multidrug resistance activity.[1,4] Diterpenoids with tigliane, myrsinol, ingenane, lathyrane, jatrophane and others skeletons are frequently found in plants of the genus *Euphorbia*.[3]

Figure 1. *Euphorbia boetica*

1.2. Lathyrane diterpenoids

Medicinal use of herbal remedies containing lathyranes dates back more than thousands of years, particularly in traditional Chinese medicine. They have found

therapeutic use for different medical disorders, like edema, migraine, intestinal parasites, gonorrhea and some skin diseases. These lathyranes and some of their synthetic derivatives have shown various interesting biological activities and clinical potential against different diseases like anti-inflammatory activity, multi-drug resistance (MDR) reversal, antiviral properties, cytotoxic activity against cancer cell lines and their capability to induce proliferation or differentiation into neurons of neural progenitor cells.[5]

Lathyranes are one of the largest groups of diterpenes, characterized by a 5/11/3-tricyclic ring system (Figure 2), frequently highly oxygenated and acylated. The fusion configuration of the A and B rings is most commonly *trans*, while that of the B and C rings is *cis*.[2]

The structural diversity of lathyrane diterpenoids is primarily the result of modifications of the 5/11/3-tricyclic ring system by esterification, etherification or oxidation. These compounds are mostly substituted with benzoyl, often with acyl groups, acetyl and phenylacetyl groups. Other groups that are also pretty common in lathyranes from natural sources are methoxy, tiglyl or cinnamoyl groups.

The lathyrane skeleton possesses a privileged structure due to its capability to direct functional groups into a precisely defined space. This advantageous arrangement enables the potential interaction with multiple targets, offering increased versatility in its molecular interactions.[5] For example, as frequently found in bioactive diterpenes, the presence of the *gem*-dimethylcyclopropane subunit within the lathyrane skeleton plays a significant role for biological substrate-target interactions. On the other hand, the acylation pattern where aromatic moieties are of fundamental significance, is a critical factor in MDR reversal. Therefore, the activity of these compounds is dependent on the balance between multiple factors, rather than being solely influenced by a single factor.[5]

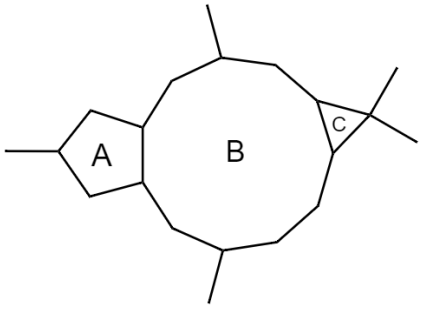


Figure 2. Lathyrane skeleton

1.3. Role of lathyranes in adult neurogenesis via activation protein kinase C

Aging processes and central nervous system insults, such as traumatic brain injury or stroke, are associated with irreversible neuronal loss that can lead to motor dysfunction, cognitive impairment and even personality changes.[6] Functional recovery after brain damage is often complicated by the rare occurrence of neuronal replacement in the damaged brain regions. In the adult brain, neural stem cells offer great promise for the development of neural repair strategies.[7]

Neurons are considered post-mitotic cells, meaning they are unable to undergo cell division. As a result, the generation of new neurons, a process called neurogenesis, primarily occurs through the differentiation of neural stem cells (NSCs). Adult neurogenesis takes place throughout life in two specific regions of the brain: the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ). In these specific brain regions, NSC undergo a process of asymmetrical division, resulting in the formation of undifferentiated multipotent neural progenitor cells (NPC), which, principally commit to the neuronal lineage under physiological conditions.[6] Proliferation, differentiation and survival of NSCs and NPCs is important because they can proliferate and differentiate into functional neural cells and that is of great significance for neuronal regeneration and functional recovery.[8]

During development, for NPC survival and maintenance, environmental factors are crucial. Grow factor (GF), like epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are significant for NSC and NPC proliferation, maintaining the undifferentiated state. Further differentiation is also dependent on the essential presence of these growth factors.[8]

Protein kinase C (PKC) is a group of serine/threonine kinases, characterized by a highly conserved catalytic domain, which is linked to a more divergent amino-terminal regulatory domain. A pseudosubstrate sequence located in the regulatory domain auto-inhibits PKCs when they are in an inactive state by preventing catalysis while occupying the substrate-binding pocket. When allosteric modulators and/or specific second messengers attach to the regulatory domain PKCs are activated. In order to enable substrate binding and PKC activation, they are temporarily pushing the pseudosubstrate region from the active site and bringing PKCs to the plasma membrane.[6]

PKC isozymes are classified as lipid-dependent kinases, as their activity relies on binding to phosphatidylserine. These isozymes can be categorized into three subfamilies based on their structural characteristics and need for calcium and additional co-factors.[9] First subfamily of PKCs is known as conventional or classical PKCs (PKCs α, β and γ). These PKCs are activated by the presence of diacylglycerol (DAG), Ca^{2+} and phosphatydilserine (PS). The second subfamily comprises the novel PKCs (nPKCs δ, ε, θ, and η) which are activated by DAG and PS. The third one is the atypical PKCs (aPKCs ζ and λ/ι) which require PS and protein-protein interactions.

As mentioned before, plants belonging to the genus *Euphorbia* are recognized for their exceptional chemical diversity of diterpenoids and wide range of pharmacological properties. Diterpenes possessing ingenane, tigliane, and lathyrane skeletons, commonly found in plants of this genus, are widely acknowledged as PKC activators. They are mimicking the effect of their physiological activator DAG by binding to their regulatory domain. A few lathyrane-type diterpenes possess the ability to mimic prostratin by reactivating HIV-1 latency trough a PKC-dependent pathway.[1]

It has been proven that lathyranes and 12-deoxyphorbols, like prostratin, promote proliferation of NPC by targeting and activating one or more PKC isozymes.[10] For instance, ingol-type lathyranes promote NPCs expansion (ELAC, **1**)[6] or NPCs differentiation into functional neurons (EOF2, **2**)[10] by PKC modulation (Figure 3). ELAC (**1**) induced subventricular zone (SVF) neural progenitor/stem cell proliferation, while its C-7 acetylated derivative did not induce any effect.[10] Therefore, presence of a free hydroxyl group at the C-7 position seems to be essential for the binding of the compound to the C-1 domain of PKC, analogously to what occurs with the C-20 position for phorbol esters.[11] On the other hand, EOF2 (**2**) promoted neuroblasts differentiation and survival in cultures of SVZ isolated cells,[6] and showed no effect in proliferation assays, whereas it was very effective in differentiating neuroblasts and facilitating their migration toward injuries in mouse models *in vivo*.[10] On the contrary, a similar molecule, diterpene EOF3 (**3**) did not show any effect on SVZ cell proliferation or differentiation.[10] Furthermore, euphoboetirane A (**4**) and epoxyboetirane A (**5**), two lathyranes isolated previously from *Euphorbia boetica*, showed a promising activity as promotors of NPC proliferation (Figure 3).[1] To elucidate the mechanism of action of these lathyrane derivatives and understand why structurally similar compounds can vary significantly in their ability to promote NPC proliferation, it is essential to conduct additional structure-activity

relationship studies. In turn, these studies would help to develop these compounds into components for the treatment of neurodegenerative disorders.

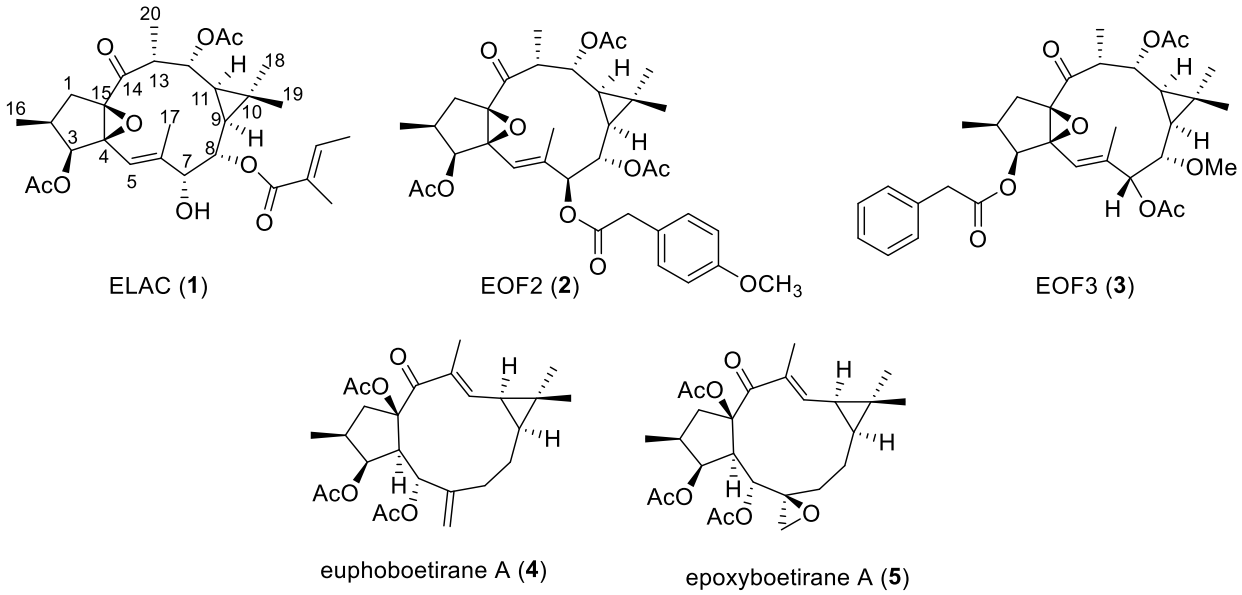


Figure 3. Lathyrane-type diterpenes tested on NPC proliferation or differentiation

Studies have also reported that the activation of conventional PKC supports the release of neurotrophic factors that promote proliferation of NPC such as the transforming growth factor alpha (TGFα). By interacting with and activating the epidermal growth factor receptor (EGFR), this neurotrophic factor (TGFα) stimulates proliferation of undifferentiated NPC. Thus, the TGFα release is used to evaluate the NPC proliferation.[11]

1.4. Biotransformation

Biocatalysis or biotransformation can be defined as the process where biological systems are used to catalyze the transformation of a compound that is not its natural substrate, into other derivatives. Whole cells, cell-free or isolated enzyme(s) can be used as biocatalysts.[12,13]

The use of biocatalysts has numerous advantages, among which we can highlight their ability to carry out more efficient and selective processes. Over the past few decades, biocatalysis has gained significant importance as a valuable tool for the chemical

synthesis of new agrochemicals, fragrances and drug derivatives with improved properties, or precursor/intermediate molecules involved in production processes. This is primarily attributed to the remarkable capability of biological systems to carry out chemo-, regio- and stereoselective reactions that are beyond the reach of traditional synthetic methods.

In general, the rates of enzyme-mediated processes are faster by a factor of 10^8-10^{10} compared to the corresponding non-catalysed reactions. In some cases, they even exceed a factor of 10^{17} and are far above the values that chemical catalysts can achieve, making enzymes very efficient catalysts. Moreover, unlike heavy metals, which are used as catalysts, biological systems, which are generally used in aqueous media, are environmentally safe reagents.

Biocatalysts work within a temperature range of 20-40 °C (preferably at around 30 °C) and a range of pH 5-8, which reduces problems of isomerisation, racemization, decomposition and rearrangement. As these are mild conditions, they are easy to establish, which facilitates their use in biotransformation processes. Moreover, they are not limited to natural substrates, but exhibit a high tolerance by accepting a large number of synthetic substances.[14]

Biocatalysts have proven to be an invaluable asset for synthetic chemists over a significant period. The development of new biocatalytic methods is a continually expanding field, with ongoing efforts in the intensive screening of new microorganisms and/or their enzymes. Many reactions are catalysed by microorganisms and fungi are a promising source of new enzymes, mostly for chiral reactions. Biotransformation has proved to be an alternative approach with high potential for the development of sustainable technologies for the synthesis of medicines and chemicals, i.e., green chemistry. Furthermore, this technology is considered environmentally friendly and economically competitive in the search for new molecules useful for the pharmaceutical and chemical industries.[13]

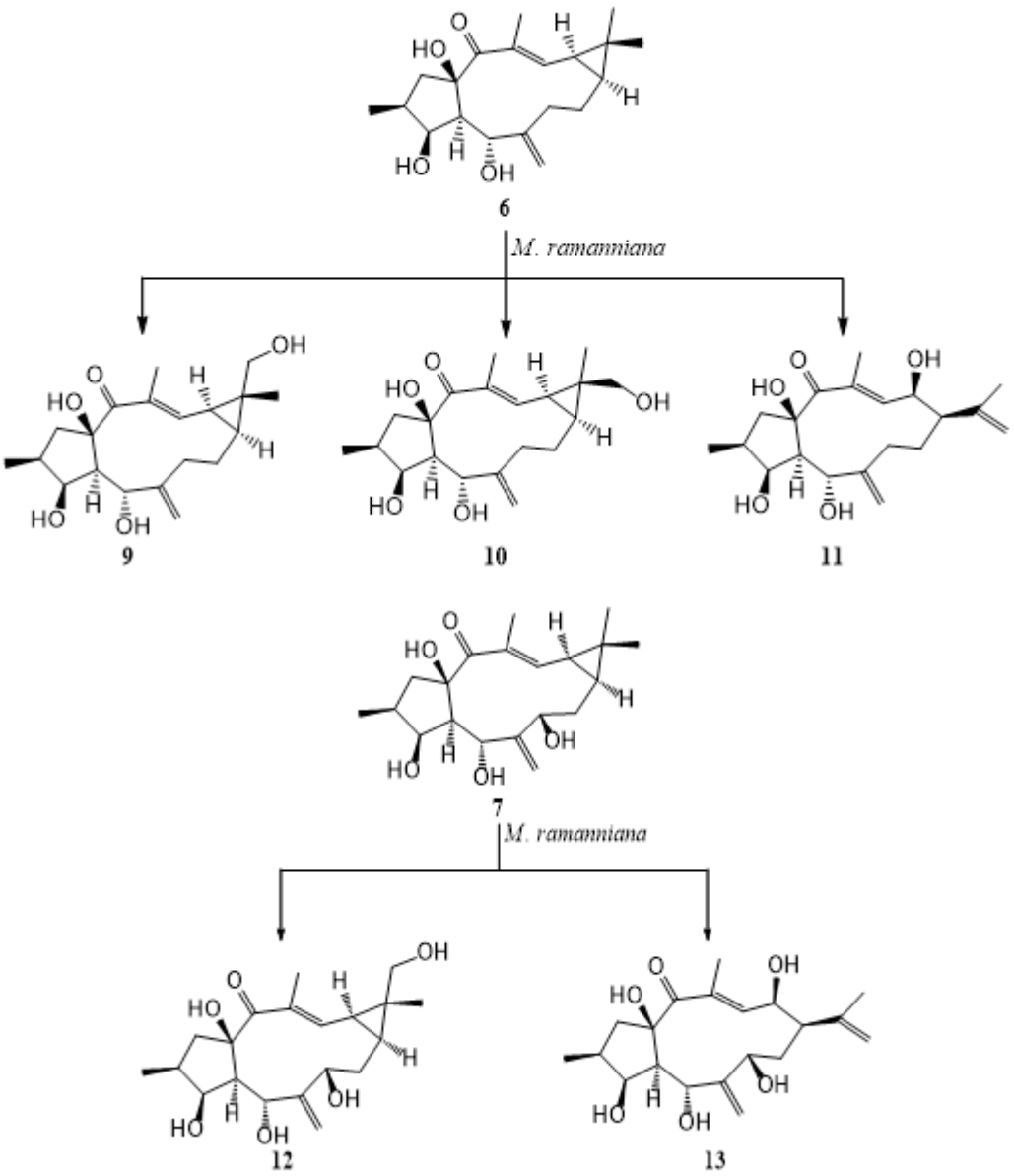
1.5. Fungi as biocatalysts

Fungi can catalyse a wide range of chemical reactions with large substrate tolerance. They have a high metabolism and fast growth, and are easy and inexpensive to

cultivate. That is why fungi have been one of the most extensively studied whole-cell systems for biotransformation processes.[13] Although the published literature on chemical biotransformations is extensive, that on the biotransformation of lathyranes is scarce.

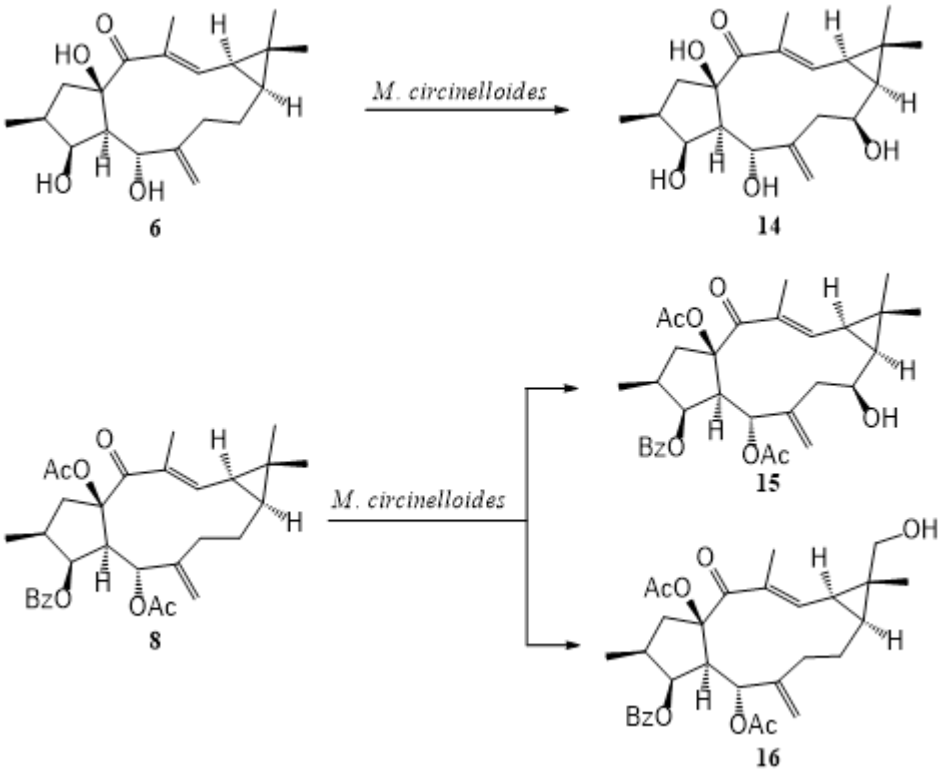
Yiqing Wu et al.[15] studied the biotransformation of three lathyrane diterpenoids (**6**-**8**) by the fungi *Nocardia iowensis* sp. nov. NRRL 5646, *Mucor circinelloides* CICC 40242 and *Mortierella ramanniana* CGMCC 3.03413 (Schemes 1, 2 and 3). The aim was to obtain lathyrane derivatives with hydroxyl groups at non-activated carbons and to increase the structural diversity of lathyrane diterpenoids. Ten new metabolites were isolated (**9**-**18**), identified and, together with all substrates, examined for the MDR reversing activities against adriamycin (ADM)-resistant human MCF-7 breast cancer cells (MCF-7/ADM) and their cytotoxicity against three human cancer cell lines (MCF-7, MCF7/ADM and Caco-2).

The biotransformation of lathyrol (**6**) by *M. ramanniana* led to three new metabolites (**9**-**11**), two isomeric monohydroxylated lathyrol derivatives (**9**-**10**) and an unpublished cyclopropane rearrangement product (**11**). In contrast, biotransformation of 7β-hydroxylathyrol (**7**) by this fungus led to only one of the two isomeric monohydroxylated derivatives at the geminal methyls (**12**), together with the 10,11-seco-lathyrane obtained by cyclopropane ring-opened (**13**) (Scheme 1).[15]



Scheme 1. Biotransformation of lathyrol (**6**) and 7β-hydroxylathyrol (**7**) by *Mortierella ramanniana*

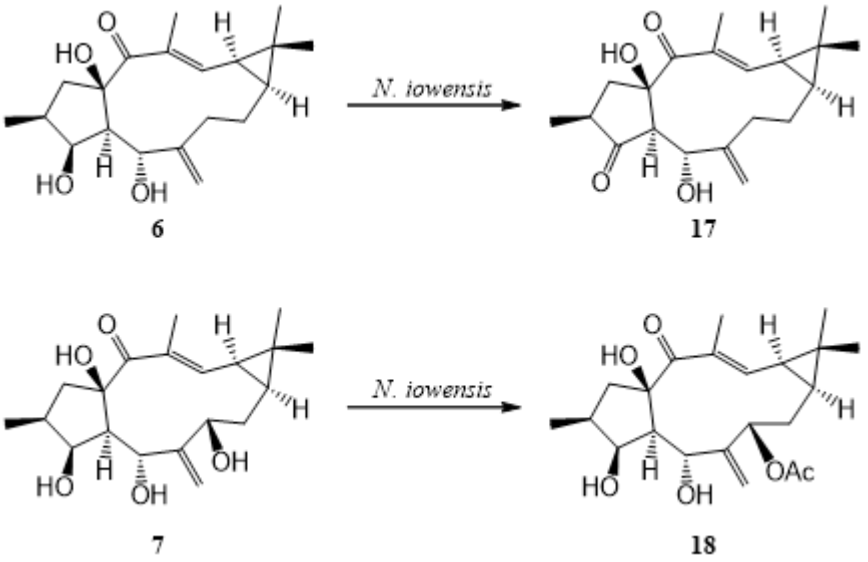
On the other hand, the biotransformation of lathyrol (**6**) by *M. circinelloides* resulted in a new regioselectively hydroxylated metabolite at C-8 (**14**) and that of Euphorbia factor L3 (**8**) in two regioselectively hydroxylated metabolites at C-8 (**15**) and C-18 (**16**) (Scheme 2).[15]



Scheme 2. Biotransformation of lathyrol (**6**) and Euphorbia factor L3 (**8**) by *Mucor circinelloides*

These results show the hydroxylation capacity of enzymatic systems present in the *M. ramanniana* and *M. circinelloides* fungi on the lathyrane skeleton.

Finally, the biotransformation of lathyrol (**6**) and 7β-hydroxylathyrol (**7**) by *N. iowensis* led to two new derivatives obtained by regioselective oxidation and acetylation of the hydroxyl groups at C-3 (**17**) and C-7 (**18**), respectively (Scheme 3).[15]



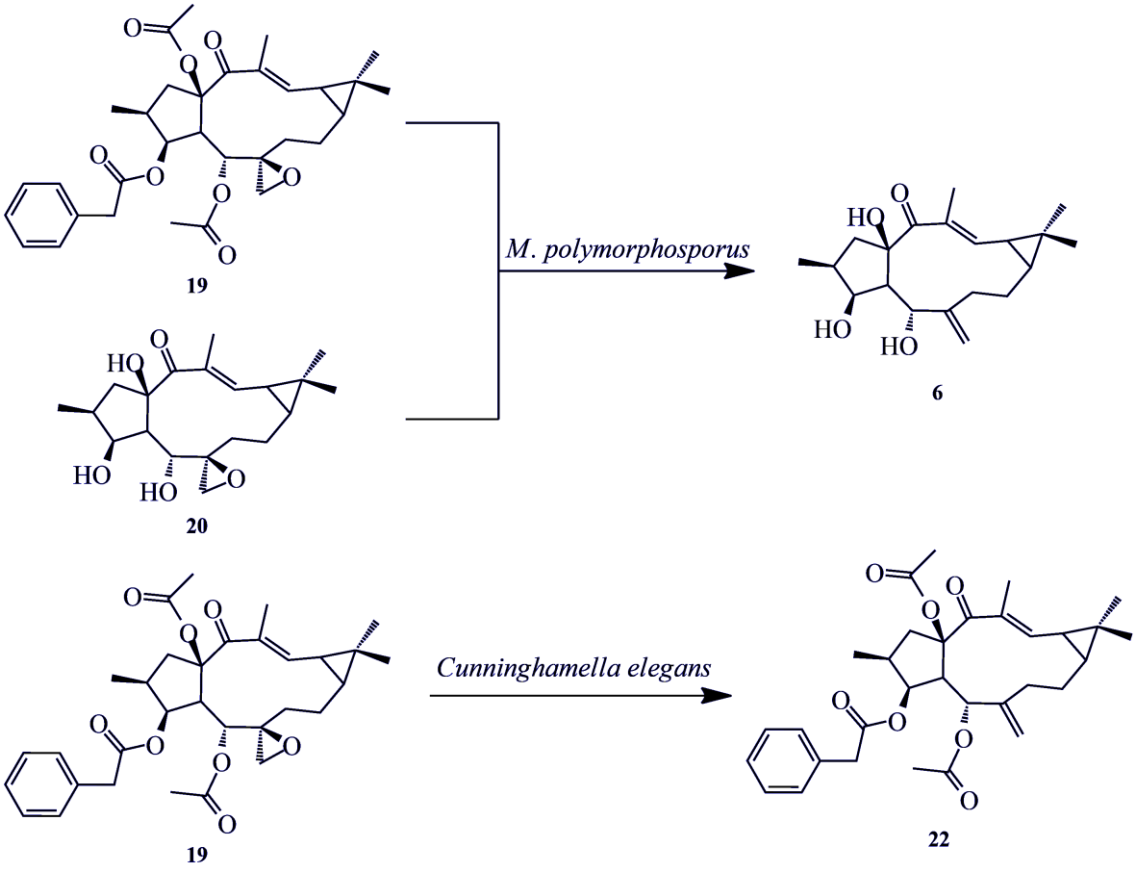
Scheme 3. Biotransformation of lathyrol (**6**) and 7β-hydroxylathyrol (**7**) by *Nocardia iowensis*

The cytotoxic activities of compounds **6**-**18** were evaluated against the human colon cancer cell line Coca-2, the breast cancer cell line MCF-7, and the Adriamycin-resistant cell line MCF-7/ADM using the MTT colorimetric assay. Metabolite **13** exhibited weak inhibitory activity against MCF-7 and moderate inhibitory activity against Coca-2 while metabolite **11** exhibited only low cytotoxicity against Coca-2. Among all the tested compounds, only 3-oxo-lathyrol (**17**) demonstrated weak cytotoxic activity against all the selected cancer cell lines, indicating that presence of the 3-oxo group plays a crucial role in enhancing the cytotoxic activity of lathyrol (**6**). Selective activity against sensitive cell lines studies was showed by two new rearranged 10,11-seco-lathyrane derivatives (**11** and **13**), suggesting that the opening of the three-membered ring could be a crucial factor for achieving optimal cytotoxic activity in lathyrane diterpenoids.[15]

The study also included an evaluation of the multidrug resistance (MDR) reversing activities of all lathyrane derivatives (6-18) using the rhodamine 123 accumulation assay. Active compounds (**6**-**9**, **15**-**16**) showed a dose-depended MDR reversing effect on cancer cells.[15]

On the other hand, Liu et al.[16] reported the biotransformation of euphorbia factor L1 (**19**) and its hydrolysed derivative **20** by the fungi *Mucor polymorphosporus* and *Cunninghamella elegans*. Two biotransformation products were obtained (**6, 22**). Compound **6** was obtained from the culture of *M. polymorphosporus* when co-incubated

with both substrate **19** and **20**, while the compound **22** was only obtained from the culture of *C. elegans* when incubated with substrate **19**. The reduction of the epoxy ring to an exocyclic double bond was observed in both substrates when they were subjected to the action of *M. polymorphosporus*. Metabolite **6** also experienced a further tri-deesterification by this fungus (Scheme 4).



Scheme 4. Biotransformation of **19** and **20** by *Mucor polymorphosporus* and **19** by *Cunninghamella elegans*

The ability of the biotransformation products (**6, 22**) to inhibit P-glycoprotein activity was evaluated. Compound **22** was the most active with an IC_{50} value of 15.50 μM, through down-regulating P-glycoprotein expression at the protein level rather than at the MDR1 mRNA level.[16]

2. EXPERIMENTAL PART

2.1. Materials and methods

Plant material

The whole plants of *Euphorbia boetica* were collected at El Pinar del Hierro (Chiclana de la Frontera), Cadiz, Spain, in March 2020.

Microorganism

Sordaria tomento-alba is an endophytic fungus isolated from maize plants from Colombia. It was identified by the Spanish Type Culture Collection (CECT). The methodology used consisted of a morphological study by microscopic observation, obtaining growth data, colony morphology and sclerotia production. For this purpose, the strain was inoculated on Potato Dextrose Agar (PDA), Corn Meal Agar (CMA) and Malt Extract Agar (MEA) media at 26 °C and 37 °C. In addition, a molecular study was carried out in which the DNA region of the intergenic space (ITS1 and ITS2) of the 5,8 S rRNA, 28S rRNA and β-Tubulin genes were amplified and sequenced.

S. tomento-alba was preserved on PDA plugs of 1 cm diameter in H_2O at 4 °C. This culture is deposited in the Mycological Herbarium Collection of the University of Cadiz.

Culture medium

PDA was used for the growth of the fungus *Sordaria tomento-alba* and PDB (Potato Dextrose Broth) for the biotransformation experiment.

Sterilization

The culture media and materials used in the biotransformation experiment were sterilized in a autoclave model Raypa, Series AE-DRY under standard conditions of 121 °C and 1 atmosphere overpressure for 20 min.

2.1.1. Chromatographic techniques

Thin layer chromatography

Thin layer chromatography (TLC) was performed on Merck Kiesegel 60 F_{254} plates, with a layer thickness of 0,25 mm and fluorescent indicator. Hexane/ethyl acetate mixtures of different polarity were used as the mobile phase.

The visualization of the products was carried out with two different stains:

- UV light, using a UVLS-24 EL Series UV Lamp at λ=254 nm and 365 nm.
- Vanillin stain (9 g vanillin, 5 mL sulphuric acid, 150 mL 96% ethanol and 40 mL water). The TLC plate was briefly immersed in the stain and heated with a heat gun at high power until the coloured spots appeared.

Column chromatography

Column chromatography (CC) was carried out on silica gel 60-200 micron Merck, using hexane, ethyl acetate, methanol or mixtures of increasing polarity. The eluent was passed through the column by the application of air pressure. The samples to be separated were introduced dissolved in the first eluent mixture.

High performance liquid chromatography

Purification by High Performance Liquid Chromatography (HPLC) was performed with a Merck-Hitachi LaChrom liquid chromatograph, equipped with a UV-vis detector (L-7490) and a differential refractometer detector (RI-7490), using Piramyde System software, version 1.0. LiChroCART LiChrospher Si 60 (5 μm, 250 mm × 4 mm) and LiChroCART LiChrospher Si 60 (10 μm, 250 mm × 10 mm) columns were used.

Sample preparation was carried out with a small column chromatography packed with silica gel to remove the baseline and subsequent filtering through 0,22 μm pore size Teflon filters supplied by VWR.

The eluents used were ethyl acetate, hexane and a mixture of them at different percentages, which were degassed with helium for 10 minutes or by ultrasound for 20 minutes.

2.1.2. Instrumental techniques

Nuclear magnetic resonance (NMR)

^{1}H and ^{13}C NMR measurements were recorded on a Bruker 700 MHz Nuclear Magnetic Resonance (NMR) spectrometer with $SiMe_4$ as the internal reference. Spectra were assigned using a combination of 1D and 2D techniques.

Chemical shifts are expressed on the δ scale in ppm and coupling constants (*J*) in hertz (Hz). The δ values are referenced to the $CDCl_3$ (Merck, Darmstadt, Germany) residual peak for ^{1}H at 7.25 ppm and for ^{13}C at 77.0 ppm.

The multiplicity of signals is indicated by the following abbreviations: *s* (singlet), *d* (doublet), *t* (triplet), *c* (quartet), *m* (multiplet), *dd* (doublet of doublet), *ddd* (doublet of doublet of doublet) etc. Multiplets are defined as an interval of δ.

Mass spectrometry

High-resolution mass spectrometry (HRMS) was performed in a QTOF mass spectrometer in the positive-ion ESI mode.

2.2. Isolation and purification of latirane euphoboetirane A (4)

The aerial parts of the fresh plant of *Euphorbia boetica* (3.0 kg) were frozen with liquid nitrogen, powdered, and extracted with methanol (MeOH, 2.5 L ×3) at room temperature for 24 h. The MeOH extract was evaporated under reduced pressure and suspended in water (1 L) and then sequentially partitioned with hexane (1.5 L ×3) and dichloromethane (1.5 L ×3). After removing the solvent, hexane and dichloromethane extracts were chromatographed in CC on silica gel in a gradient mixture of *n*-hexane/ethyl acetate of increasing polarity. The obtained fractions were analyzed by ^{1}H-NMR.

The fractions that showed the presence of euphoboetirane A (**4**) were further purified by CC and HPLC. The fractions purified in this diploma thesis yielded 313.6 mg of **4**.

2.3. Biotransformation of euphoboetirane A (4)

Sordaria tomento-alba was cultured in Petri dishes on PDA for 4 days at 25 °C in the presence of white light (day light lamp). The medium was sterilized in autoclave at 121 °C for 20 minutes and then cooled down at room temperature before use. Mycelium from this culture was transferred to eighteen 500 mL Erlenmeyer flasks containing 200 mL of sterilized PDB medium. Each flask was inoculated with 6 mycelium plugs of 1 cm diameter and then incubated at 24-26 °C and 200 rpm under white light. After 3 days, 300 µL of a dimethyl sulfoxide (DMSO) solution of euphoboetirane A (**4**) was added to each flask at a final concentration of 85 ppm. The flasks were incubated under the conditions described above for 13 days after inoculation. Culture controls consisted of fermentation flasks containing 300 µl of DMSO. Substrate controls contained the sterile medium with the same concentration of **4**.

Extraction of the fermentation broth

After the biotransformation process was completed, the contents of the flasks were filtered through a Büchner funnel under vacuum using a 200 µm pore size "Nytal" filter.

The culture medium was extracted three times with ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure at a rotary evaporator to a crude extract (Figure 4).

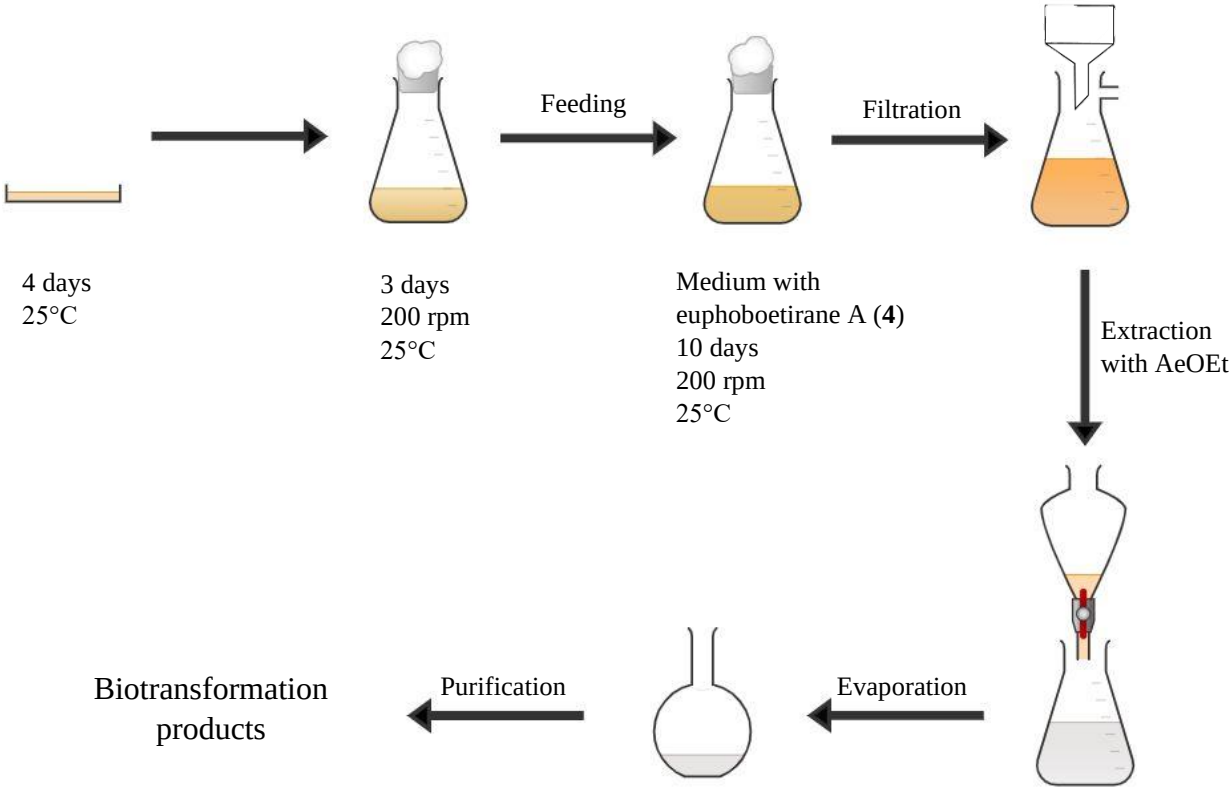


Figure 4. Diagram of the biotransformation of euphoboetirane A (**4**) by *Sordaria tomento-alba*

Purification of crude extract

The crude extract, together with the culture and substrate controls, were subjected to thin layer chromatography. TLC plates were developed and visualized under UV light and by heating after dipping into vanillin. The presence of biotransformation products was observed (Figure 5).

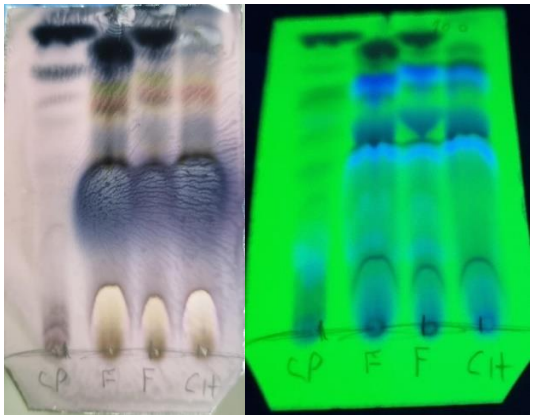
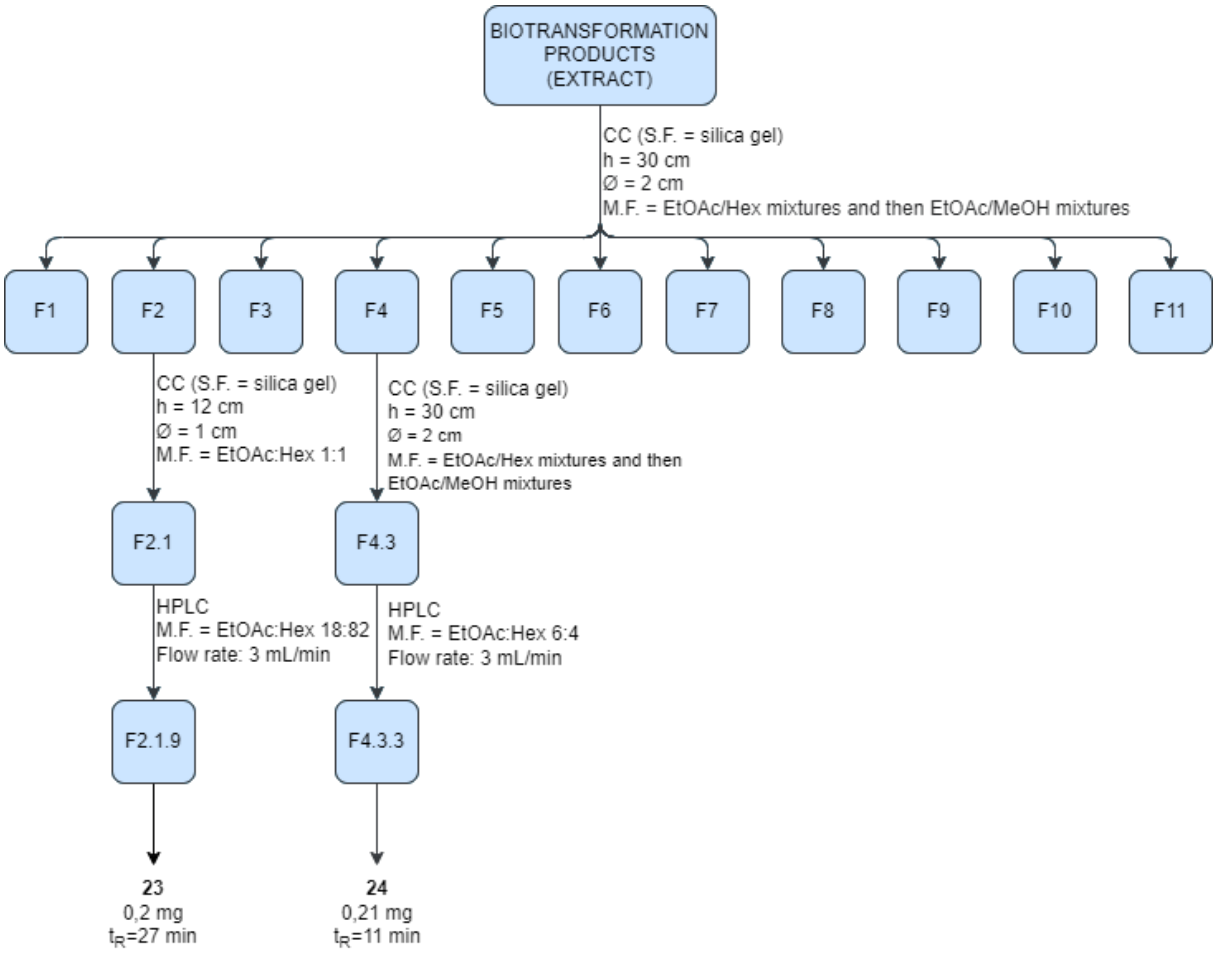


Figure 5. TLC plates showing biotransformation products together with substrate control and culture control

The resulting residue containing biotransformation products was then subjected to CC on silica gel, using a gradient mixture of *n*-hexane/ethyl acetate (10-100%) of increasing polarity and ethyl acetate/methanol at the end as eluents to afford eleven fractions, according to TLC analysis.

All fractions obtained were analyzed by ^{1}H-NMR. Fractions showing signals of latirane-type sesquiterpenes in their ^{1}H-NMR spectrum, Figures S1 and S3 (Supplementary), were further purified by HPLC according to the conditions described in the Scheme 5. Fractions that did not contain compounds of interest were discarded.



Scheme 5. Purification of the biotransformation products

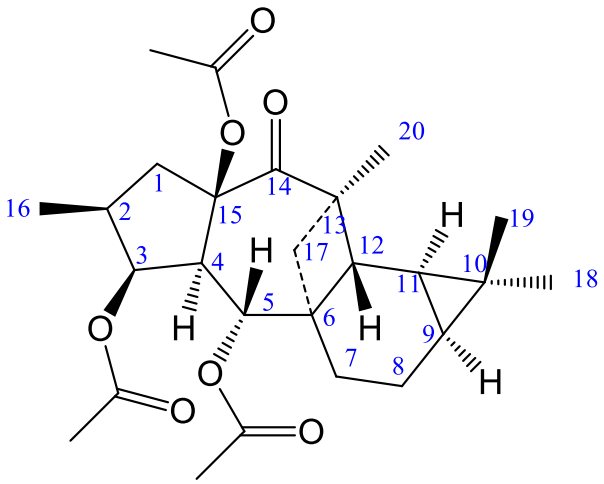
Fraction 2 (53.32 mg) was purified by CC on silica gel, using *n*-hexane:ethyl acetate 1:1 and then semi-preparative HPLC with flow rate of 3 mL/min, and *n*-hexane:ethyl acetate 18:82 (v/v) as mobile phase, to give 0.20 mg of **23** (t_R = 27 min).

Similarly, fraction 4 (57.21 mg) was purified by CC on silica gel, using a gradient mixture of *n*-hexane/ethyl acetate (10-100%) of increasing polarity, and ethyl acetate/methanol at the end as eluents and then by semi-preparative HPLC with 3 mL/min flow rate, and *n*-hexane/ethyl acetate 6:4 (v/v) as mobile phase, to give 0.21 mg of **24** (t_R = 11 min) (Scheme 5).

3. RESULTS

Structures of two biotransformation products of interest (**23** and **24**), after isolation and purification from the crude extract, have been elucidated by extensive spectroscopic analysis using one- (Tables 1 and 2) and two-dimensional NMR spectroscopy experiments and high-resolution mass spectrometry. Obtained results for each product separately are shown.

Sordarianona (23)



23

Molecular formula: $C_{26}H_{36}O_8$

Colorless oil

HRMS (ESI): *m/z* 483.2374 $[M+Na]^+$ (calcd. for $C_{26}H_{36}O_7Na$, 483.2359)

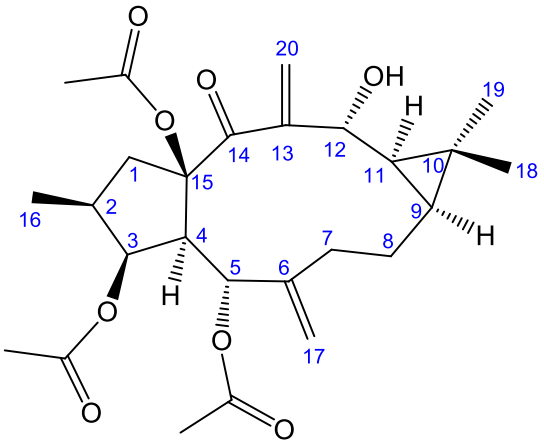
***g*HMBC (selected correlations):** H-20 → C-12, C-13, C-14; H-5 → C-12; H-3 → C-1, C-15, OCO-3; H-12 → C-14, C-5, C-13, C-6, C-11, C-10; H-4 → C-5, C-14, C-6; H-1α → C-2, C-3; H-1β → C-16, C-2, C-4, C-15; OCOMe-5 → OCO-5; OCOMe-3 → OCO-3; OCOMe-15 → OCO-15;H-17→C-14, C-5, C-13, C-6, C-12, C-7, C-20; H-7 → C-8, C-9; H-19 → C-10, C-18, C-9; H-18 → C-19, C-10, C-9, C-11; H-16 → C-2, C-1, C-3; H-11 → C-18, C-13, C-6

Table 1. NMR ($CDCl_3$) spectroscopic data for biotransformation product **23**

Position	δ_H (700 MHz)	δ_C (175 MHz)
1α	3.31, dd (15.6, 11.2)	41.2 CH_2
1β	1.54, dd (15,6, 6.6)	
2	2.41-2.38, m	34.7, CH
3	5.46, t (5.5)	76.2 CH
4	2.38-2.34, m	52.7, CH
5	5.42, d (10.5)	72.7, CH
6	-	37.6, C
7a	1.24-1.19, m	24.3, CH_2
7b	1.0-1.96, m	
8a	1.89-1.83, m	16.6, CH_2
8b	1.79-1.74, m	
9	0.73-0.70, m	18.9, CH
10	-	16.0, C
11	0.61 d (9.0)	19.5, CH
12	2.92, d (4.4)	34.3, CH
13	-	50.5, C
14	-	207.4, C
15	-	92.2, C
16	0.95, d (6.9)	15.5 CH_3
17a	2.10-2.06, m)	37.1, CH_2
17b	1.92, dd (13.6, 4.8)	
18[a]	1.03,[a] s	30.10,[b] CH_3
19[a]	0.99,[a] s	16.6,[b] CH_3
20	1.34, s	20.0, CH_3
OCO-3	-	170.9, C
OCOMe-3	2.09, s	20.9, CH_3
OCO-5	-	170.2, C
OCOMe-5	2.10, s	29.9,[c] CH_3
OCO-15	-	169.8, C
OCOMe-15	1.96, s	20.8,[c] CH_3

[a, b, c] Interchangeable assignments

(2*S*,3*S*,4*R*,5*R*,9*S*,11*R*,12*S*,15*R*)-3,5,15-Triacetoxy-12-hydroxylathyra-6(17),13(20)-dien-14-ona (24)



24

Molecular formula: $C_{26}H_{37}O_8$

Colorless oil

HRMS (ESI): *m/z* 477.2505 $[M+H]^+$ (calcd. for $C_{26}H_{37}O_8$, 477.2488)

gHMBC (selected correlations): H-20a, H-20b → C-12, C-13, C-14; H-5 → C-7, C-4, C-3, C-17, C-6, OCO-5; H-3 → C-1, C-15, OCO-3; H-17a, H-17b → C-7, C-5; H-12 → C-11, C-20, C-13, C-14; H-4 → C-5, C-15, C-6, C-14; H-1α → C-2, C-4, C-3, C-14; H-1β → C-16, C-2, C-15; OCOMe-5 → OCO-5; OCOMe-3 → OCO-3; OCOMe-15 → OCO-15; H-7 → C-8, C-9, C-5, C-17, C-6; H-19 → C-10, C-18, C-11; H-18 → C-19, C-10, C-9, C-11; H-16 → C-2, C-1, C-3; H-11 → C-18, C-12

Table 2. NMR ($CDCl_3$) spectroscopic data for biotransformation product **24**

Position	δ_H (700 MHz)	δ_C (175 MHz)
1α	3.09, dd (16.2, 8.6)	46.9, CH_2
1β	2.39, dd (16.2, 11.9)	
2	2.55-2.49, m	37.7, CH
3	5.58, t (4.0)	76.5, CH
4	3.14, dd (10.5, 3.9)	53.7, CH
5	5.64, d (10.5)	74.2, CH
6	-	142.7, C
7a	2.19-2.12, m	29.1, CH_2
7b	1.60, dd (18.1, 10.7)	
8a	1.91-1.84, m	22.1, CH_2
8b	0.72-0.66, m	
9	0.63-0.58, m	29.7, CH
10	-	19.5, C
11	0.85, dd (10.1, 9.0)	34.2, CH
12	4.76, d (10.1)	65.4, CH
13	-	148.8, C
14	-	192.4, C
15	-	92.5, C
16	1.03, d (6.6)	14.0, CH_3
17a	5.19, d (2.9)	116.8, CH_2
17b	4.81, s	
18[a]	1.10,[a] s	28.9,[b] CH_3
19[a]	1.15,[a] s	14.9,[b] CH_3
20a	6.40, s	125.6, CH_2
20b	6.17, s	
OCO-3	-	170.5, C
OCOMe-3	2.09, s	21.1, CH_3
OCO-5	-	169.5, C
OCOMe-5	2.12, s	21.5,[c] CH_3
OCO-15	-	169.5, C
OCOMe-15	1.95, s	20.7,[c] CH_3

[a, b, c] Interchangeable assignments

4. DISCUSSION

In order to deepen the study of the relationship between the functionalization presented by a lathyrane diterpenes and its neurogenesis-promoting activity, in this work we have subjected euphoboetirane A (**4**) to biotransformation by the endophytic fungus *Sordaria tomento-alba* trying to functionalize unactivated carbons.

The biotransformation of euphoboetirane A (**4**), isolated from the plant *Euphorbia boetica*, by *Sordaria tomento-alba* was carried out using whole cells which allow us use of the whole arsenal of fungal enzymes to obtain different lathyrane derivates. This environmentally friendly strategy often provides high enantioselectivity, low toxicity and it does not require cofactor recycling or enzyme purification.

Sordaria tomento-alba was grown in PDB medium for three days at 200 rpm. Then, a DMSO solution of euphoboetirane A (**4**) was fed at a concentration of 85 ppm and incubated for 10 more days under daylight condition, as indicated in the procedure described in the Experimental section (Section 2.3.). The culture broth was subjected to extraction with ethyl acetate and the crude extract purified by CC and HPLC to yield two biotransformation products (**23** and **24**). Their structures have been elucidated by extensive spectroscopic analysis using one- and two-dimensional NMR spectroscopy experiments and high-resolution mass spectrometry.

Compound **23** was obtained as a colorless oil and its molecular formula was determined as $C_{26}H_{36}O_7$ based in its HRESIMS, which showed a $[M+Na]^+$ ion at *m/z* 483.2374 (calcd. for $C_{26}H_{36}O_7$ Na, 483.2359), demonstrating equal degree of unsaturation than the starting compound euphoboetirane A (**4**). However, the characteristic signals for double bond carbons and protons in the NMR spectra had disappeared, indicating that two new cycles may have formed. Careful inspection of the ^{1}H and ^{13}C NMR spectra (Table 1) revealed that **23** retained all the main rings A and C features of **4.** The main difference was the above mentioned absence of the 6,17- and 12,13-double bonds and the shielding of the H-5, H-9 and H-11 signals in the ^{1}H-NMR spectrum. Moreover, an additional ^{1}H-NMR doublet at δ_H 2.92 (4.4 *Hz*), together with an aliphatic methylene group [δ_H 2.10-2.06 (m) and 1.92 (dd, 13.6 and 4.8 *Hz*), δ_C 37.1] were present (Table 1). In the HMBC spectrum, the correlation between this methylene group and C-14, C-5, C-13, C-6, C-12, C-7 and C-20 (Figure 6) indicated that an intramolecular [2+2] cycloaddition has occurred

inducing the formation of new bonds between the carbons 6(12) and 13(17). This cycloaddition led to a new skeleton, giving compound **23** named Sordarianone (**23**). The stereochemistry of **23** was determined by the NOE effect between H-12 and H-5 and H_3-19; H-5 and H_3-19, and between H-11 and H_3-20 and H-12 (Figure 7).

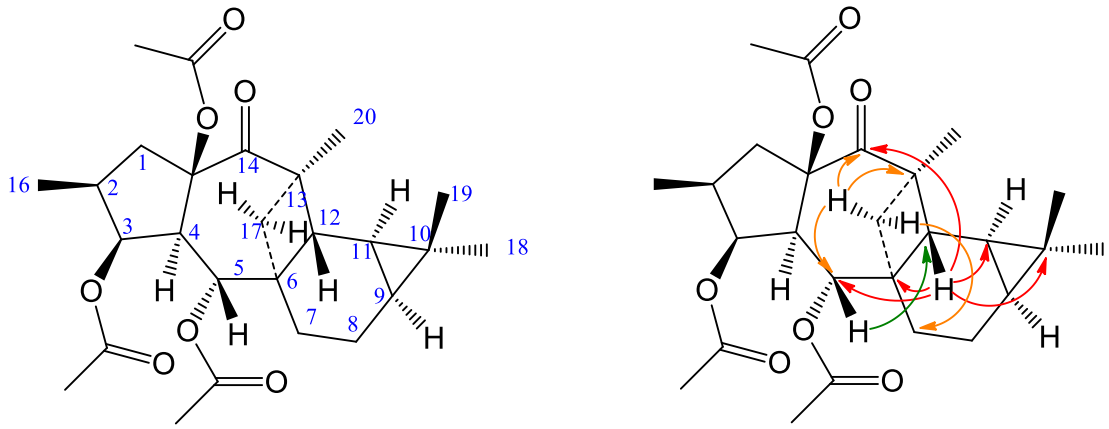


Figure 6. Selected HBMC correlations observed for compound **23**

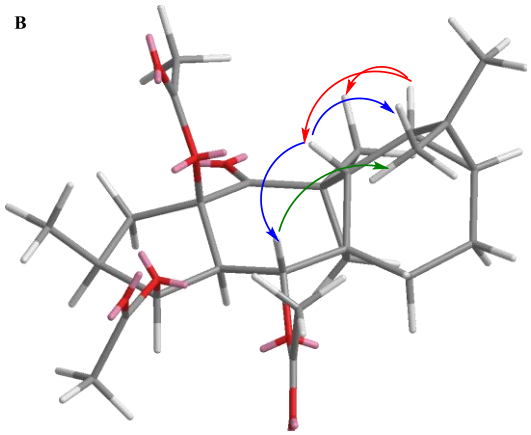


Figure 7. Selected NOE observed for compound **23**

Compound **24** was isolated as a colorless oil and its molecular formula was determined as $C_{26}H_{37}O_8$ by HRMS (ESI), which showed $[M+H]^+$ ion at *m/z* 477.2505 (calcd. for 477.2488). Its NMR spectroscopic data (Table 2) show the presence of two terminal double bonds (δ_H 5.19 and 4.81 ppm, δ_C 116.8 ppm and δ_H 6.40 and 6.17 ppm, δ_C 125.6 ppm assigned to C-20 and C-17 respectively) and an additional hydroxyl group (δ_H 4.76 ppm, δ_C 65.4 ppm, assigned to C-12) as the main difference from euphoboetirane A (**4**), indicating that hydroxylation and isomerization of the C12(13) double bond occurred. HMBC correlations between H_2-20 with C-12, C-13 and C-14 indicated that the second exocyclic double bond was located between C-13 and C-20, while the

correlation between the new hydroxyl proton signal (H-12) with C-11, C-20, C-13 and C-14 located it at C-12 (Figure 8). The stereochemistry of the hydroxyl group was at C-12 was assigned as α-configuration based on the NOESY-1D correlation of H-12 with H-7b, H-8b and H_3-19 (δ_H 1.15, s) and between H-11 and- H_3-18 (δ_H 1.10, s), H-17b and H-20a (Figure 9). Therefore, the structure of **24** was established as (2*S*,3*S*,4*R*,5*R*,9*S*,11*R*,12*S*,15*R*)-3,5,15-triacetoxy-12-hydroxylathyra-6(17),13(20)-dien-14-ona (**24**).

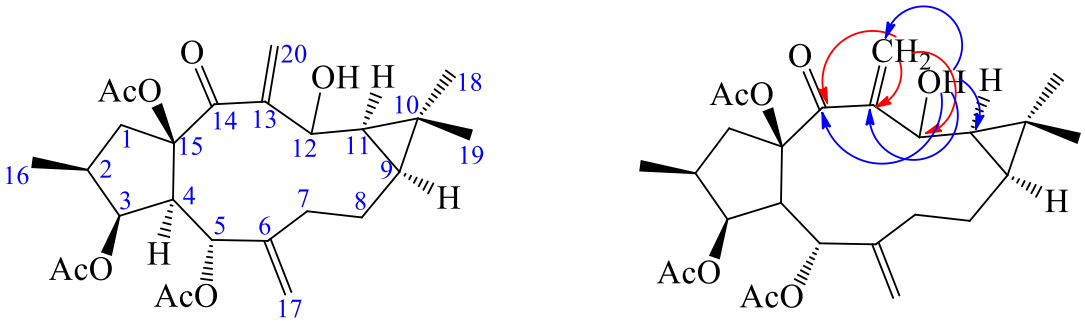


Figure 8. Selected correlations HBMC in compound **24**

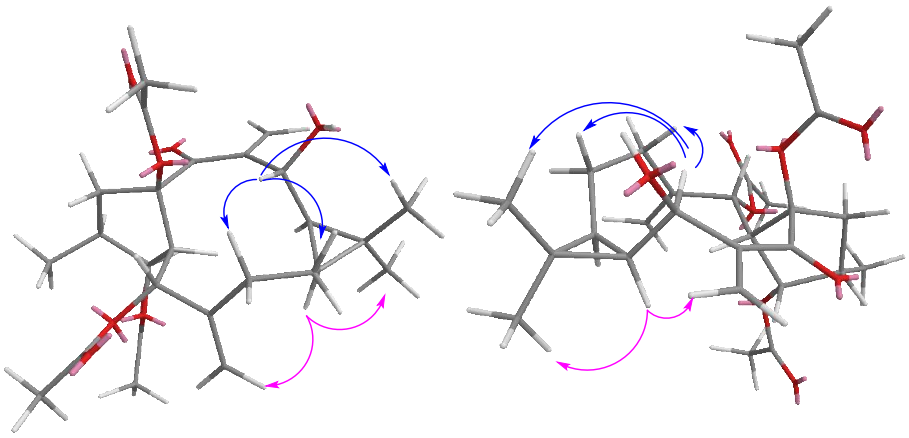


Figure 9. Selected NOE observed for compound **24**

4.1. Future prospects

Future perspectives include optimization of biotransformation conditions to increase the yield of biotransformation products. Some of the parameters to be optimized could be the timing of addition of the xenobiotic (**4**) to the fungus *Sordaria tomento-alba* as well as the feeding time.

Furthermore, it is proposed to perform bioassays on the biotransformation products (**23** and **24**) to determinate their biological activities. The most interesting bioassays for this research would be the ability to induce proliferation or differentiation into neurons of neural progenitor cells. However, there are other interesting biological activities that could be investigated as they are common among the lathyrane diterpenoids, such as antiviral properties, cytotoxic activity against cancer cell lines, anti-inflammatory activity and multi-drug resistance reversal.

Once, the potential applications of both lathyrane diterpenoids have been established, a study of their toxicity should be carried out.

5. CONCLUSIONS

- Biotransformation of euphoboetirane A (**4**) by *Sordaria tomento-alba* using whole cells resulted in two biotransformation products, compounds **23** and **24**, which are described here for the first time.
- The enzymatic system of the fungus *Sordaria tomento-alba* is capable of intramolecular [2+2] cycloadditions, hydroxylations and isomerisations of carbon-carbon double bonds.
- Whole-cell microbial biocatalysis can be used as an alternative approach to obtain new lathyrane derivatives with potential applications for the treatment of neurodegenerative disorders.

6. ABBREVIATIONS

MDR - Multidrug resistance

NPC - Neural progenitor cell

NSCs - Neural stem cells

SVZ - Subventricular zone

DG - Gyrus of the hippocampus

GF - Grow factor

EGF - Epidermal growth factor

bFGF - Basic fibroblast growth factor

PKC - Protein kinase C

DAG - Diacylglycerol

PS - Phosphatydilserine

HIV-1 - Human Immunodeficiency Virus 1

ELAC - 3,12-di-O-acetyl-8-O-tigloilingol

TGFα - Transforming growth factor alpha

EGFR - Epidermal growth factor receptor

C - Celzius

ADM - Adriamycin

mRNA - Messenger ribonucleic acid

CECT - Spanish Type Culture Collection

PDA - Potato Dextrose Agar

CMA - Corn Meal Agar

MEA - Malt Extract Agar

DNA - Deoxyribonucleic acid

TLC - Thin layer chromatography

CC - Column chromatography

HPLC - High Performance Liquid Chromatography

NMR - Nuclear magnetic resonance

$SiMe_4$ - Silicon tetramethane

$CDCl_3$ - Deuterated chloroform

ppm - Parts per million

J - Coupling constant

Hz - Hertz

s - Singlet

d - Doublet

t - Triplet

c - Quartet

m - Multiplet

dd - Doublet of doublet

ddd - Doublet of doublet of doublet

HRMS - High-resolution mass spectrometry

MeOH- Methanol

DMSO - Dimethyl sulfoxide

rpm - Revolutions per minute

Na_2SO_4 - Sodium Sulfate

AeOEt - Ethyl acetate

UV - Ultraviolet

Hex - Hexane

EtOAc -

t_R – Retention time

NOE - Nuclear Overhauser effect

NOESY - Nuclear Overhauser Effect Spectroscopy

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8. SUPPLEMENTARY

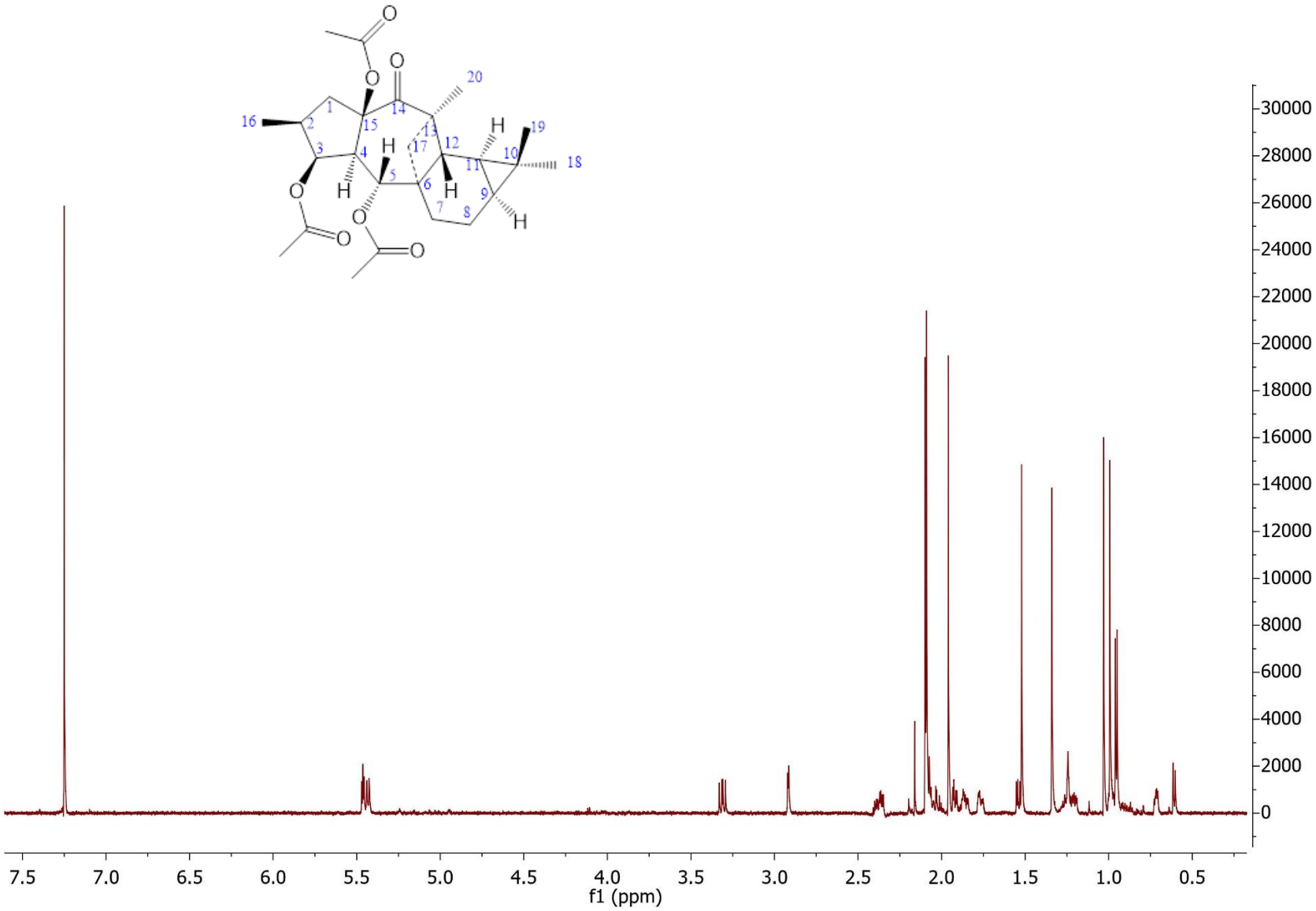

Figure S1. ^{1}H NMR of Sordarianone (**23**)

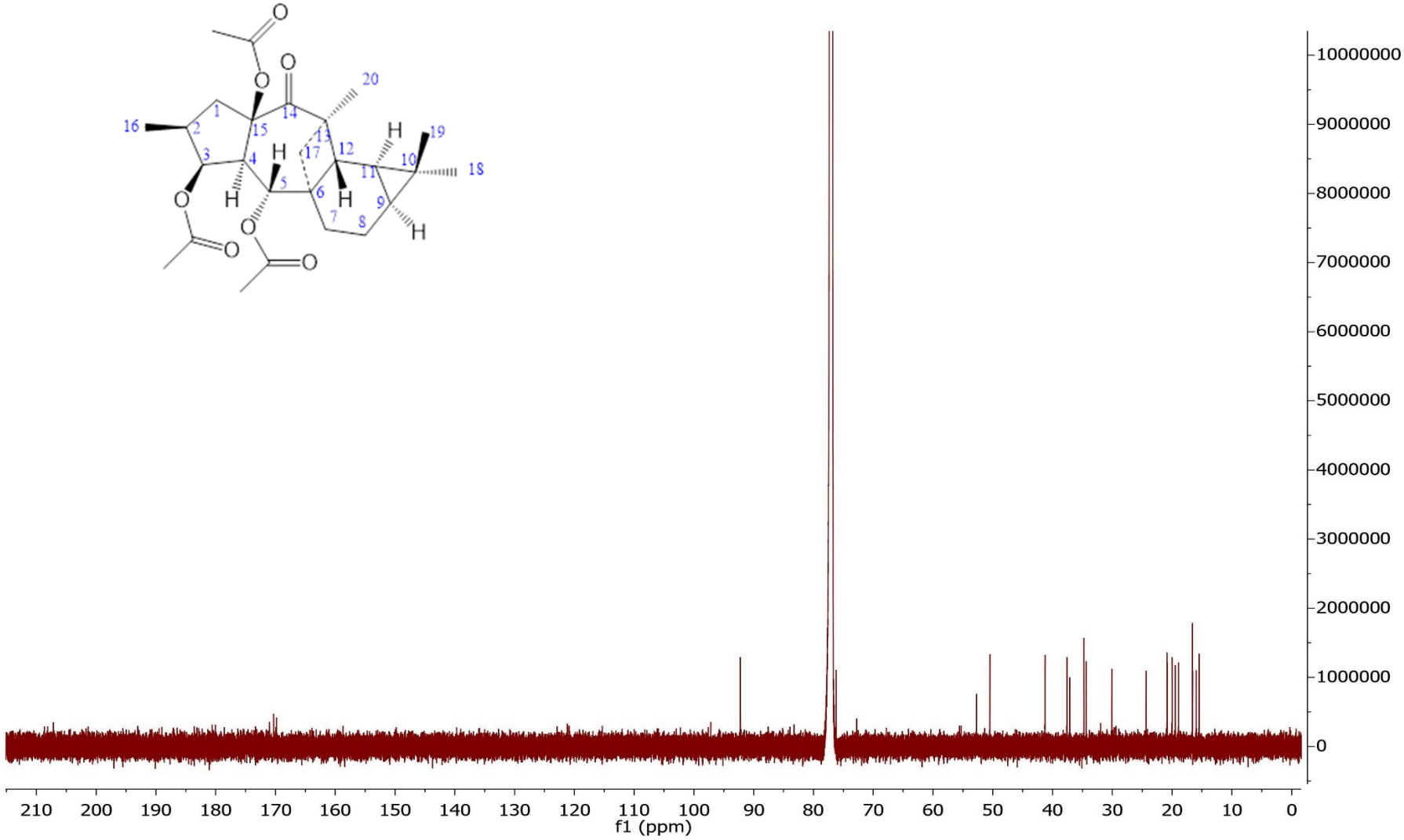

Figure S2. ^{13}C NMR of Sordarianone (**23**)

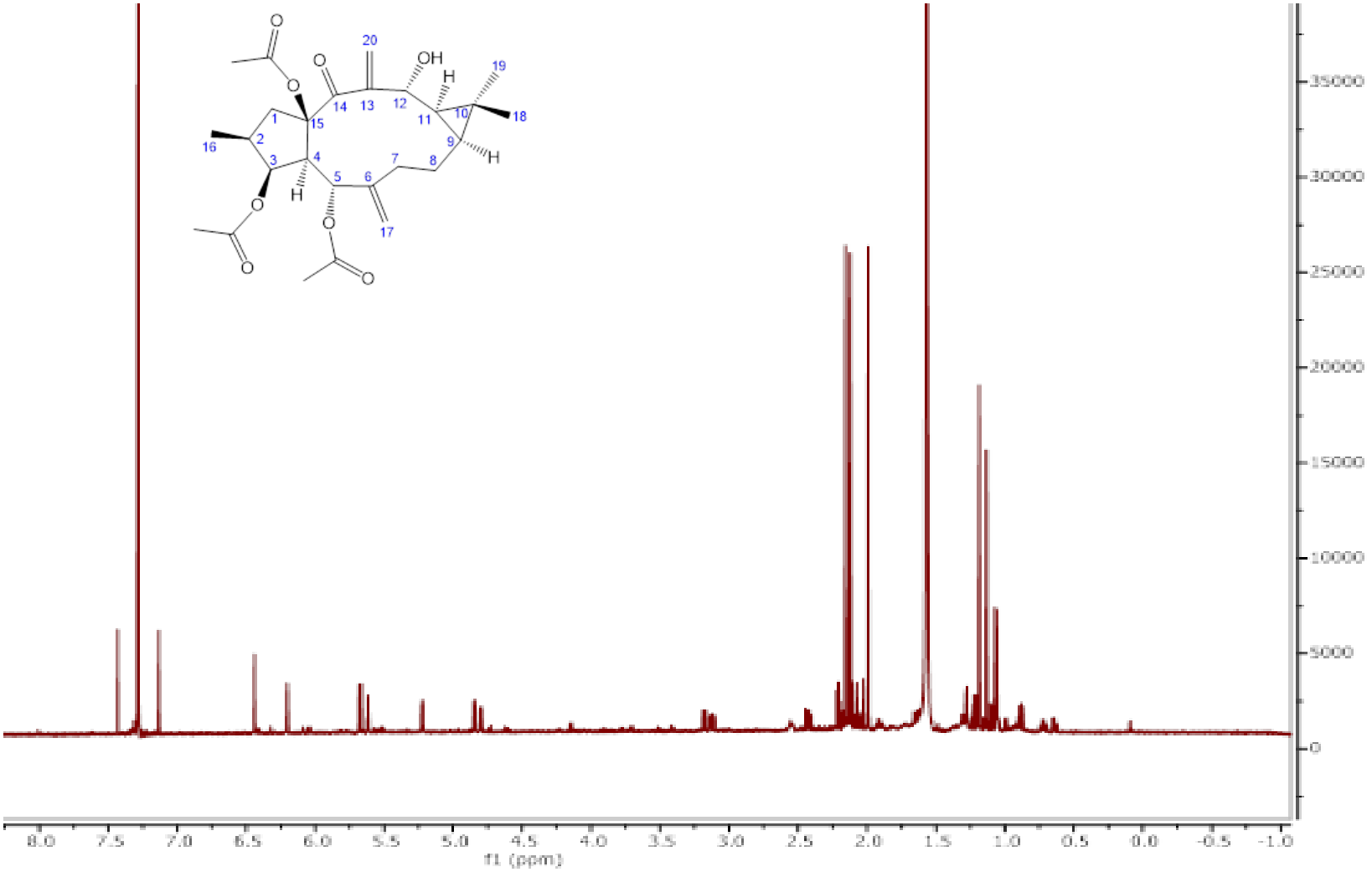

Figure S3. ^{1}H NMR of (2*S*,3*S*,4*R*,5*R*,9*S*,11*R*,12*S*,15*R*)-3,5,15-Triacetoxy-12-hydroxylathyra-6(17),13(20)-dien-14-ona (**24**)

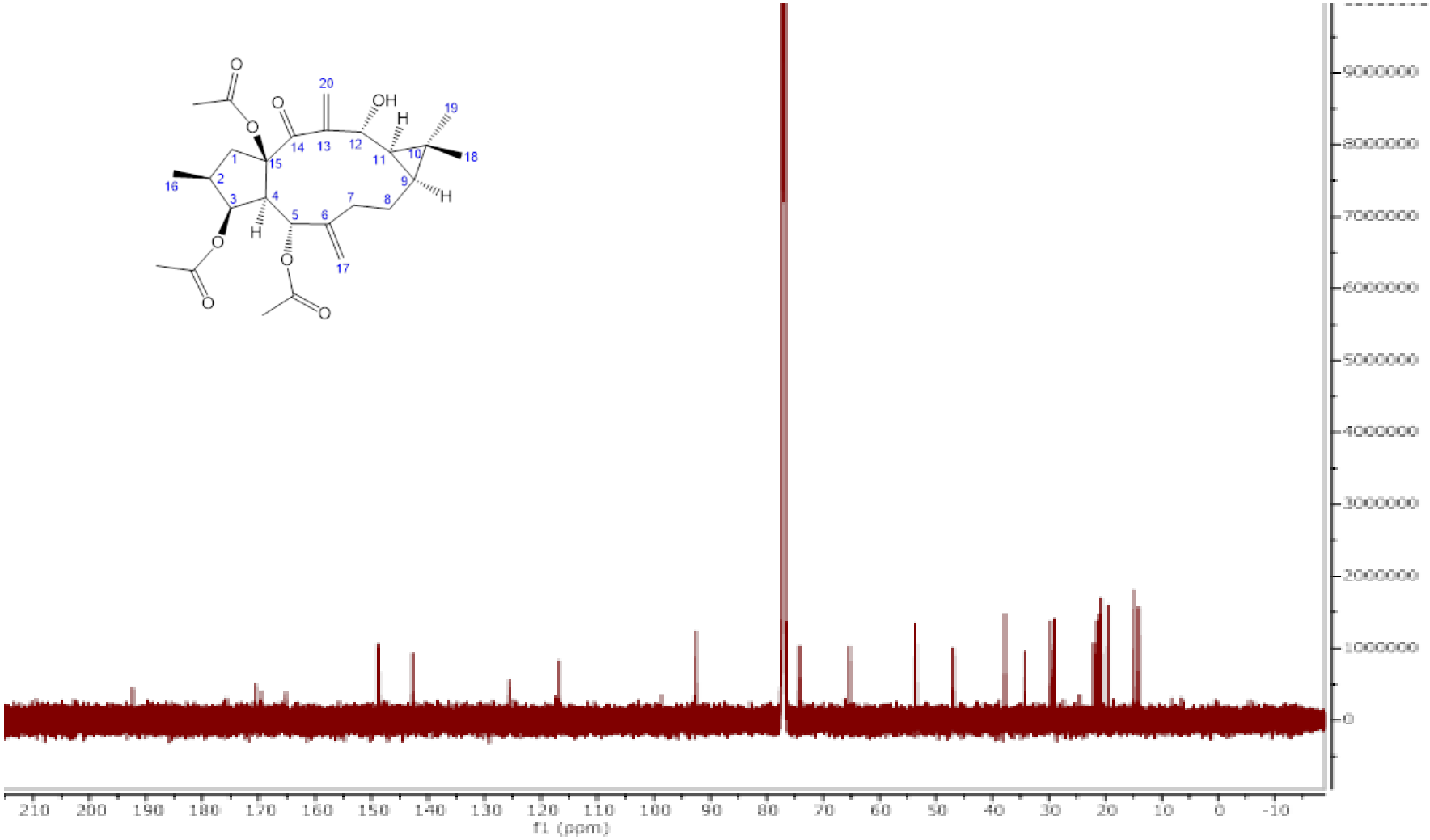

Figure S4. ^{13}C NMR of (2*S*,3*S*,4*R*,5*R*,9*S*,11*R*,12*S*,15*R*)-3,5,15-Triacetoxy-12-hydroxylathyra-6(17),13(20)-dien-14-ona (**24**)