A Fast and Efficient MN-Approach for Reactivity of Natural Product Exploration in Plant Extract: Application to Diterpene Esters from *Euphorbia dendroides*

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ABSTRACT

Natural products represent a rich source of bioactive compounds covering a large chemical space. Even if challenging, this diversity can be extended by applying chemical modifications. However, these studies require generally multigram amounts of isolated natural products and face frequent testing failures. To overcome this limitation, we propose a rapid and efficient approach that uses molecular networking (MN) to visualize new chemical diversity generated by simple chemical modifications of natural extract. Moreover, the strategy deployed enables the most appropriate reagents to be defined quickly upstream a reaction on a pure compound, in order to maximize chemical diversity. This methodology was applied to the latex extract of *Euphorbia dendroides* to follow the reactivity towards a series of acids and Lewis acids of three class of diterpene esters identified in this species: jatrophane, terracinolide, and phorbol. Through the molecular networking interpretation, in aim to illustrate our approach, two Lewis acids were selected for chemical modification on previously isolated jatrophane

esters. Three rearranged compounds (**3**−**5**) were obtained when exposed to $BF_3.OEt_2$, showing that the most appropriate reagents can be selected by MN interpretation.

Medicinal chemists face a challenge in addressing novel therapeutic targets and developing effective new drugs from potential candidates typically identified through extensive compound screening. One constraint within these compound libraries lies in the inherent characteristics of the molecules, with a majority being planar compounds (2D) constructed around sp2-rich aromatic or heteroaromatic cores.[1] As a result, these libraries only address a small part of the chemical space, a concept encompassing all potential small organic molecules.[2] With higher Fractional sp3 character and three-dimensional (3D) shape, Natural Products (NPs) cover a larger chemical space.[3] Their structural diversity offers a wide range of biological activities and mechanisms of action, fostering the development of new drugs.[4–7] Throughout history, roughly one-third of authorized small-molecule drugs have drawn inspiration from NPs.[8] Chemical modifications of NPs can generate diverse derivatives, further increasing the chemical space covered and allowing the study of their structure/activity relationships. However, these studies require generally multigram amounts of isolated NPs and face frequent testing failures. Indeed, finding selective reactions on NPs that are highly functionalized remains a challenge, and many of them are not accessible with organic synthesis.[7] In addition, it is often difficult to isolate NP in sufficient quantity and purity from raw material.

With the recent developments in metabolomic sciences dedicated to NPs,[9–11] it is possible to visualize and identify hundreds of compounds present in complex mixtures. An elegant way of exploring the reactivity of NPs towards various reagents is to carry out chemical reactions directly on the extracts and analyze the chemical modifications generated using a molecular network approach. Molecular Networking (MN) is based on tandem mass spectrometry coupled with high performance liquid chromatography (LC-MS) data, enabling structurally related molecules to be visualized and annotated in a spectral map.[10] This approach is available on an open-access platform, Global Natural Products Social Molecular Networking (GNPS; http://gnps.ucsd.edu), which enables the organization and the sharing of LC-MS/MS

data among researchers.[10] Molecular networking has been successfully applied for metabolomics, drug discovery, and medical studies.[12]

In this study, we focus our attention on diterpene esters isolated from *Euphorbia* plants.[13,14] They have a wide range of biological activities and their highly diverse chemical structures make them particularly attractive for drug discovery and development.[14,15] They possess different types of skeletons, functional groups, and stereochemical features. Some of them have unique or rare structures.[13,14,16] Diterpene esters are often isolated from natural sources in small quantities, making them scarce and expensive. However, they are worth studying for their potential uses in medicine.[14] Synthesis of diterpene esters and analogues to explore structure-activity relationships, is a real challenge. To overcome this limitation and expand the natural product pool, Appendino and co-workers undertook chemical modification of lathyrane esters.[17] A series of new functionalized diterpenoids have been obtained by transannular Diels-Alder reaction. In particular, they showed that mild Lewis acid gave better results than stronger Lewis acid or protic acid. In another study, Wang and colleagues showed that the use of Lewis acid $BF_3.OEt_2$ induced a transformation of the lathyrane-type diterpene ester skeleton.[18] We propose here to increase the structural diversity of diterpene esters by carrying out simple chemical modifications directly on plant extracts, using mass spectrometry based molecular networking to evaluate mixtures composition. We selected 17 reactants among Brønsted and Lewis acids to generate new derivatives from an acetonitrile (MeCN) extract of *Euphorbia dendroides*. Careful analysis of the molecular network generated from reaction media has yielded useful information about the reactivity of three class of diterpenoids, jatrophane, terracinolide, and phorbol ester, toward each reactant. When the best conditions were found, we applied the transformation on pure jatrophane esters isolated in multigram amounts, euphodendroidins E and F, to characterize the compounds formed.[19]

RESULTS AND DISCUSSION

From the MeCN diterpenoid ester-enriched extract of *Euphorbia dendroides*, 23 compounds were previously isolated and identified (Figure S1, Supporting Information)[19–21]. The compounds possess differently functionalized carbon skeletons of jatrophane, terracinolide and deoxyphorbol types. They are characterized by structural features such as an endocyclic double bond, a 14-keto group in jatrophane and terracinolide, and an enolate in phorbol. To explore the chemical reactivity of these molecules, we selected five Brønsted acids and 11 Lewis acids, some of which have already shown interesting rearrangements on lathyrane esters.[17,18]

The crude and chemically modified extracts obtained from each reactant were analyzed by mass spectrometry analysis in positive mode on a Q-TOF instrument, and classical molecular networking was generated with GNPS using the "analogs search mode". In order to check the presence of newly formed derivatives, molecular networks were generated and visualized using Cytoscape.[22] A color mapping, assigning a specific color according to the reactant used and white color for the crude extract, made it possible to visualize the conditions under which the compounds were formed. We also circled jatrophane-, terracinolide-, and phorbol-type derivatives in green, blue and pink, respectively. This enabled us to easily assess the reactivity of each diterpene class under various chemical conditions.

We first evaluate chemical modifications with Brønsted acids. The acetonitrile diterpenoid ester-enriched extract was treated separately with acetic acid, trifluoroacetic acid, hydrochloric acid, sulfuric acid, or formic acid for 16 h. A molecular network generated from the enriched extract and the five modified extracts in acidic conditions and a diagram of a qualitative analysis are shown in Figure 1A and 1B, respectively. This diagram shows the percentage of shared nodes with the enriched extract (in orange), shared nodes with at least one other acidic condition (in yellow), and non-shared nodes (in green) that indicate potential new derivatives generated with a specific acid. The diagram highlights the high potential of sulfuric

acid (H_2SO_4) compared with other acids for generating new derivatives, of which 62% are probable new compounds, and 29% specific to this acid. Trifluoroacetic acid (TFA) allows generating 51% of new derivatives, but only 9% are specific to this acid. When looking at the different types of diterpene present (Figure 1A), formic acid (HCOOH, in pink) and acetic acid (AcOH, in red) appear to generate more newly formed derivatives in the phorbol family (nodes surrounded in pink) than the other acids, while H_2SO_4 (in orange) and hydrochloric acid (HCl, in blue) appear to play a more important role in modifying terracinolide (nodes surrounded in blue). To modify jatrophane type compounds (nodes surrounded in green) and expand their structural diversity, H_2SO_4 appears to be the most appropriate acid among those tested. In a previous work by Appendino and co-authors, an acid-catalyzed rearrangement of a lathyrane was observed, suggesting a similar type of rearrangement for jatrophane compounds.[17]

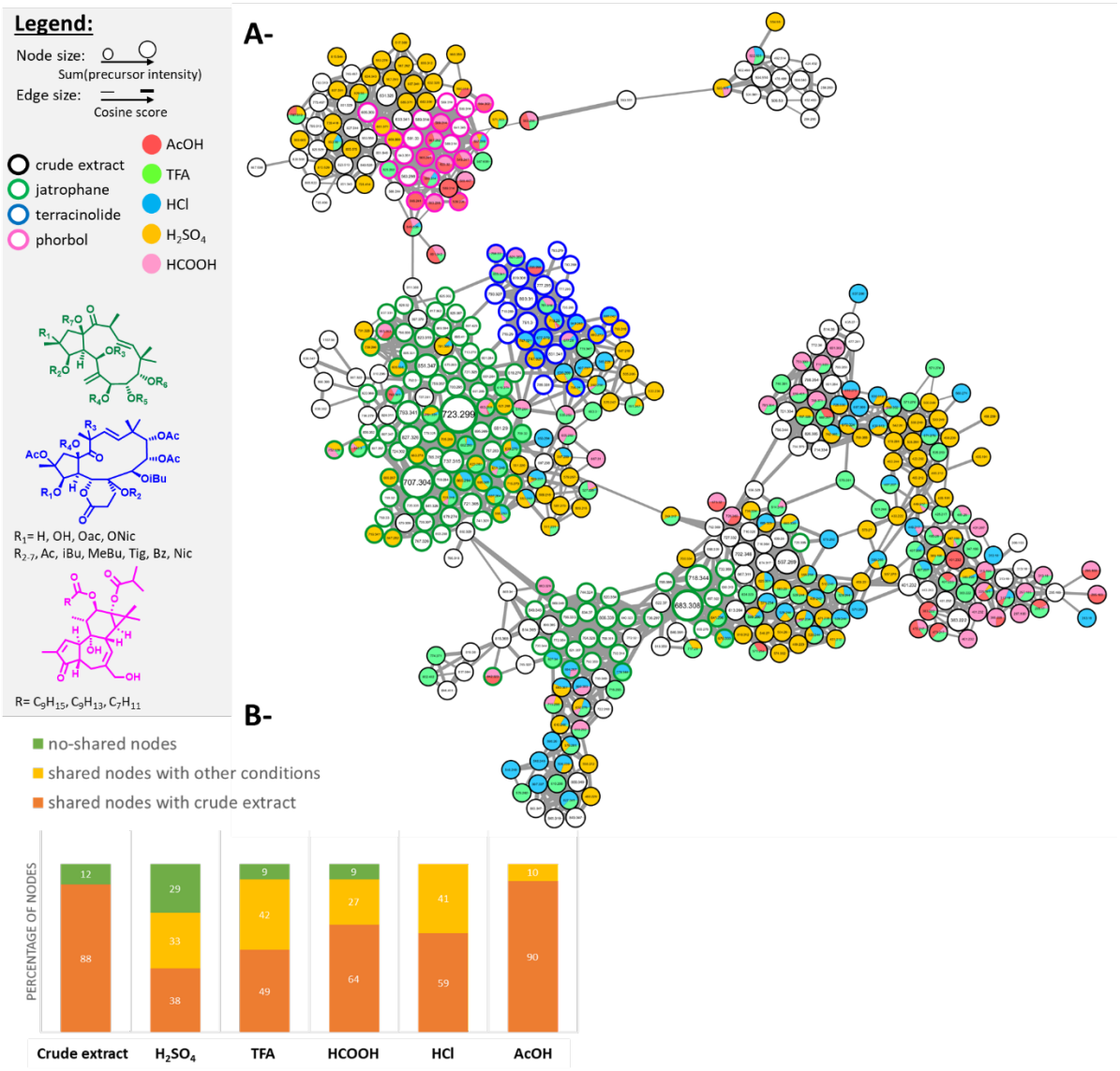


Figure 1. A- Diterpene esters molecular network from *E. dendroides* extract and modified extracts by Bronsted acids. Node size depending on the sum of precursor intensity, and edge size are consistent with the cosine score. Reference compounds and analogs mode allowed to annotate jatrophane (green), terracinolide (blue), and phorbol (pink) groups. **B-** Diagram of the percentage of nodes shared with the crude extract (orange), with at least one of the other conditions (yellow), and not shared (green), for each modified extract by acids.

We then evaluate 11 Lewis acids for their reactivity on diterpene esters in mixture. The extract solubilized in dichloromethane was treated for 16 h at r.t. with 10 eq of Lewis acid. Molecular networking was generated from the enriched extract and 11 modified extracts (Figure 2). The three diterpene families annotated in the diterpene cluster suggest that Montmorillonite K10, ytterbium triflate, trimethylaluminium, zinc(II) chloride, and lithium tetrafluoroborate gave low modification rates. In contrast, aluminum chloride ($AlCl_3$, in

orange), boron trifluoride etherate ($BF_3.OEt_2$, in blue), and ferric chloride ($FeCl_3$, in pink) are the most efficient reactants to induce chemical modification on jatrophane and terracinolide compounds. In the phorbol cluster, new derivatives appeared to be generated by the diethyl aluminum chloride (Et_2ClAl). Lewis acids are known to activate unsaturated C-heteroatom bonds (adducts formation or σ-coordination) without changing its oxidation state. As described in the literature, this type of reactant could result in the cleavage of acetate groups[23], or in Diels-Alder reactions for exemple.[24,25] Lewis acids have also been used to convert lathyrane-type diterpene through an intramolecular tandem ring-opening/cyclization process[18] or offer transannular reactions via C-C and C-O bond reorganizations[17,26]. Due to the presence of a carbonyl group in the jatrophane-, terracinolide-, and phorbol-types, the σ-coordination of Lewis acid could activate its electrophilic position and lead to further rearrangement.

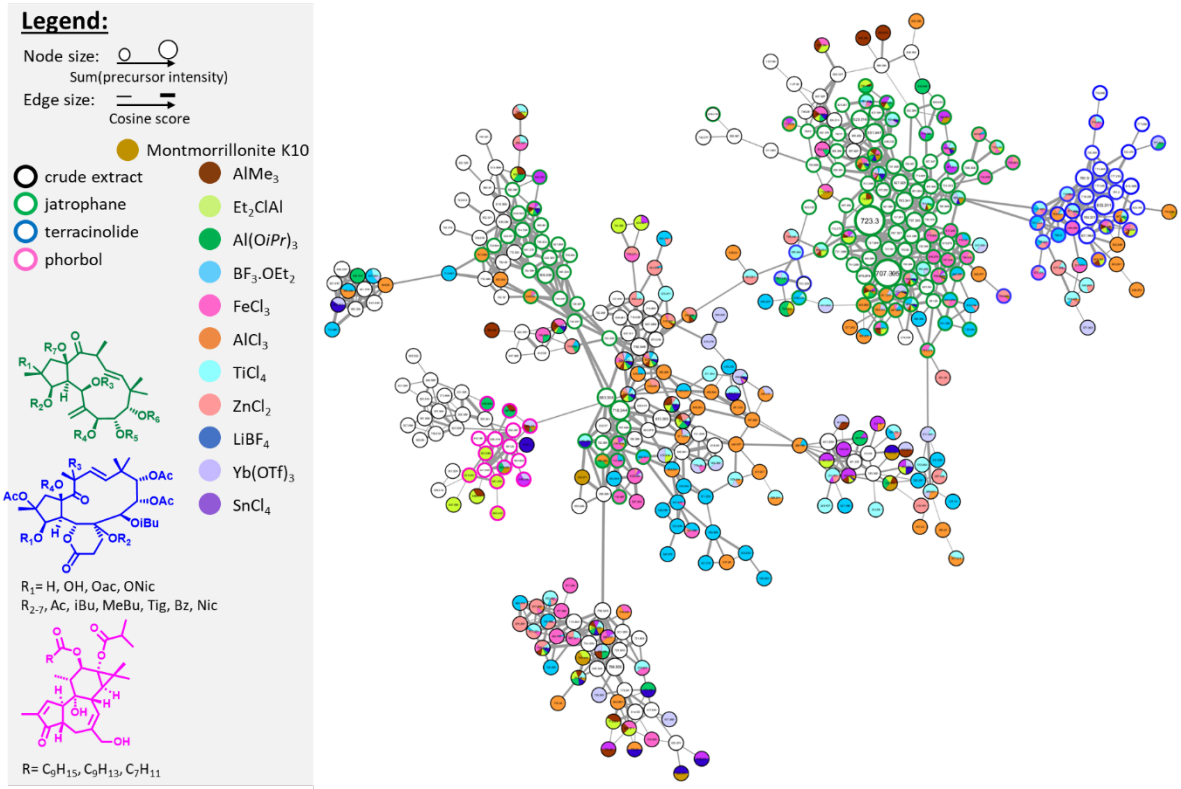
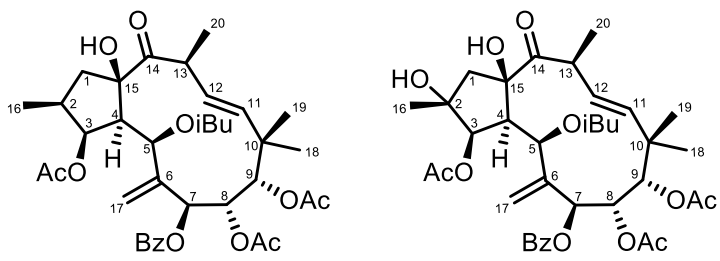


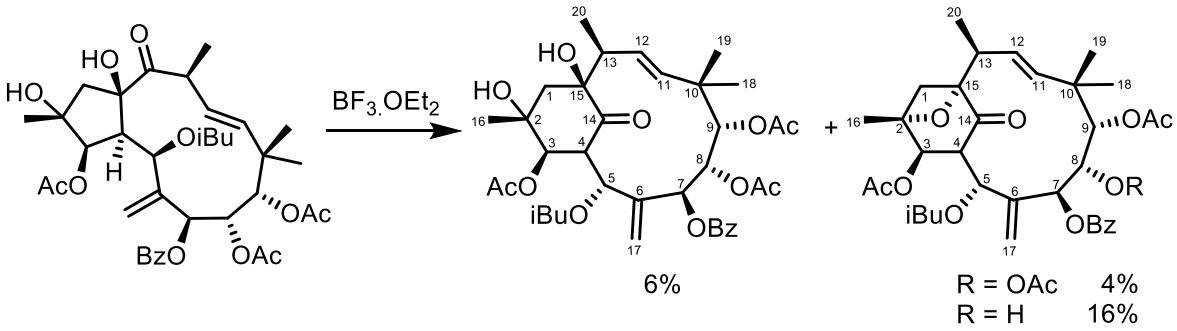
Figure 2. Diterpene esters molecular network from *E. dendroides* extract and modified extracts by Lewis acids. Node size depending on the sum of precursor intensity, and edge size are consistent with the cosine score. Reference compounds and analogs mode allowed to annotate jatrophane (green), terracinolide (blue), and phorbol (pink) groups.

To identify the ~~possible~~ products obtained from Lewis acid treatment of diterpene esters present in the extracts, we decided to apply the most promising conditions, *i.e* the use of $BF_3.OEt_2$, on pure isolated metabolites. Euphodendroidin E (**1**) and F (**2**) (Figure 3) are two jatrophane esters previously obtained in multigram amounts from *Euphorbia. dendroides* latex extract.[19] These two compounds have a different oxidation state with an additional oxygenated center at the C-2 position for euphodendroidin F (**2**). Euphodendroidins E and F were (each) treated with 10 eq of $BF_3.OEt_2$ in dichloromethane for 1 h. From euphodendroidin F (**2**), a mixture of three compounds was obtained: abeodendroidine F (**3**) in 6% yield, previously isolated from *E. dendroides*,[27] and two new compounds named oxetanabeodendroidine A (**4**) and B (**5**) in 4% and 16% yield, respectively (Scheme 1 and Table S1, Supporting Information). No modified jatrophane ester was obtained with compound **1** using the same protocol. This observation highlighted the potential role of the alcohol group at C-2 as necessary for the acid-mediated rearrangement in the jatrophane skeleton. This result confirms the hypothesis of a probable artefactual origin of abeodendroidin F **3**. A Wagner Meerwein ***α***-ketol rearrangement could explain the formation of abeodendroidin F **3** with a 1,2-alkyl shift leading to a ring expansion (Scheme 2).[27] In the presence of Lewis acid, the carbocation formed by hydroxyl elimination could be trapped by the alcohol at the C-2 position to give the oxetane compounds **4** and **5**. In these conditions, a partial deprotection of the acetate group in C-8 was observed in the case of oxetanabeodendroidin B **5**.

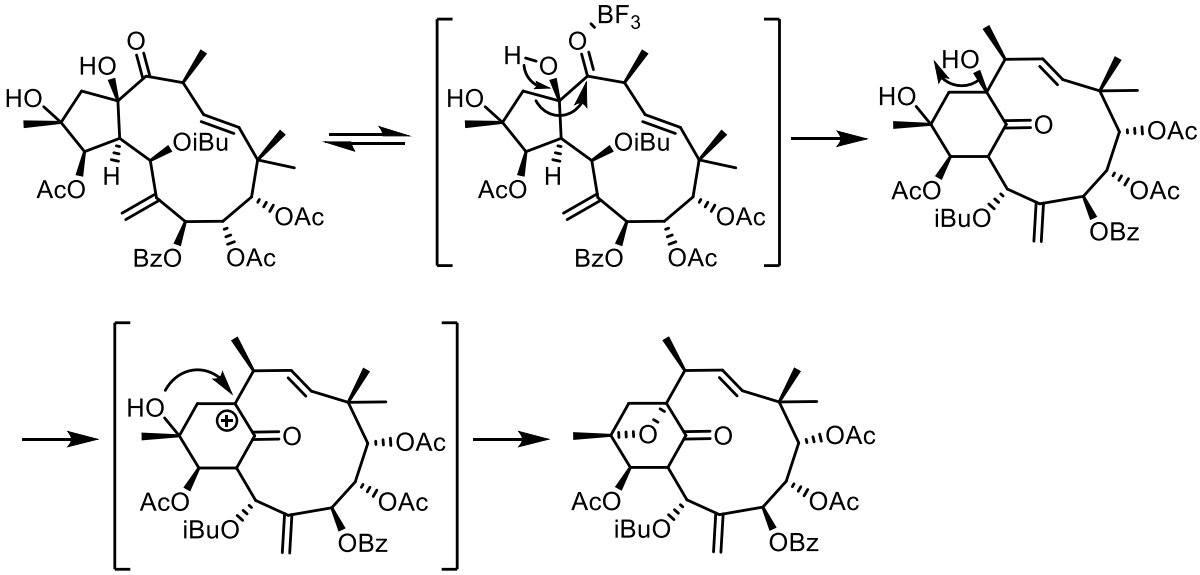


euphodendroidin E euphodendroidin F

Figure 3. Structure of jatrophane esters euphodendroidin E (**1**) and F (**2**).



Scheme 1. Reaction of euphodendroidin F (**2**) with $BF_3.OEt_2$, giving compounds **3**, **4**, and **5** in 6%, 4%, and 16% yield respectively after purification by preparative HPLC.



Scheme 2. Plausible mechanism for Wagner-Meerwein rearrangement of euphodendroidin F (**2**).

When looking at the jatrophane cluster obtained from compounds of the enriched extract (white) and the modified extract (red) with $BF_3.OEt_2$, as depicted in Figure 4, only 7% of the total number of compounds are present in the modified extract. This suggested the formation of possible new jatrophane derivatives (red nodes). The "analogs mode search" helped to define more precisely some clusters: jatrophane bearing a nicotinoyl group (nodes surrounded in green) or not, terracinolide (nodes surrounded in blue), and oxetanabeojatrophane (nodes surrounded in yellow). Most of the new derivatives generated

with $BF_3.OEt_2$ (red nodes) are surrounded in yellow, indicating the presence of the same oxetane bridge as found in oxetanabeojatrophane A (**4**) and B (**5**).

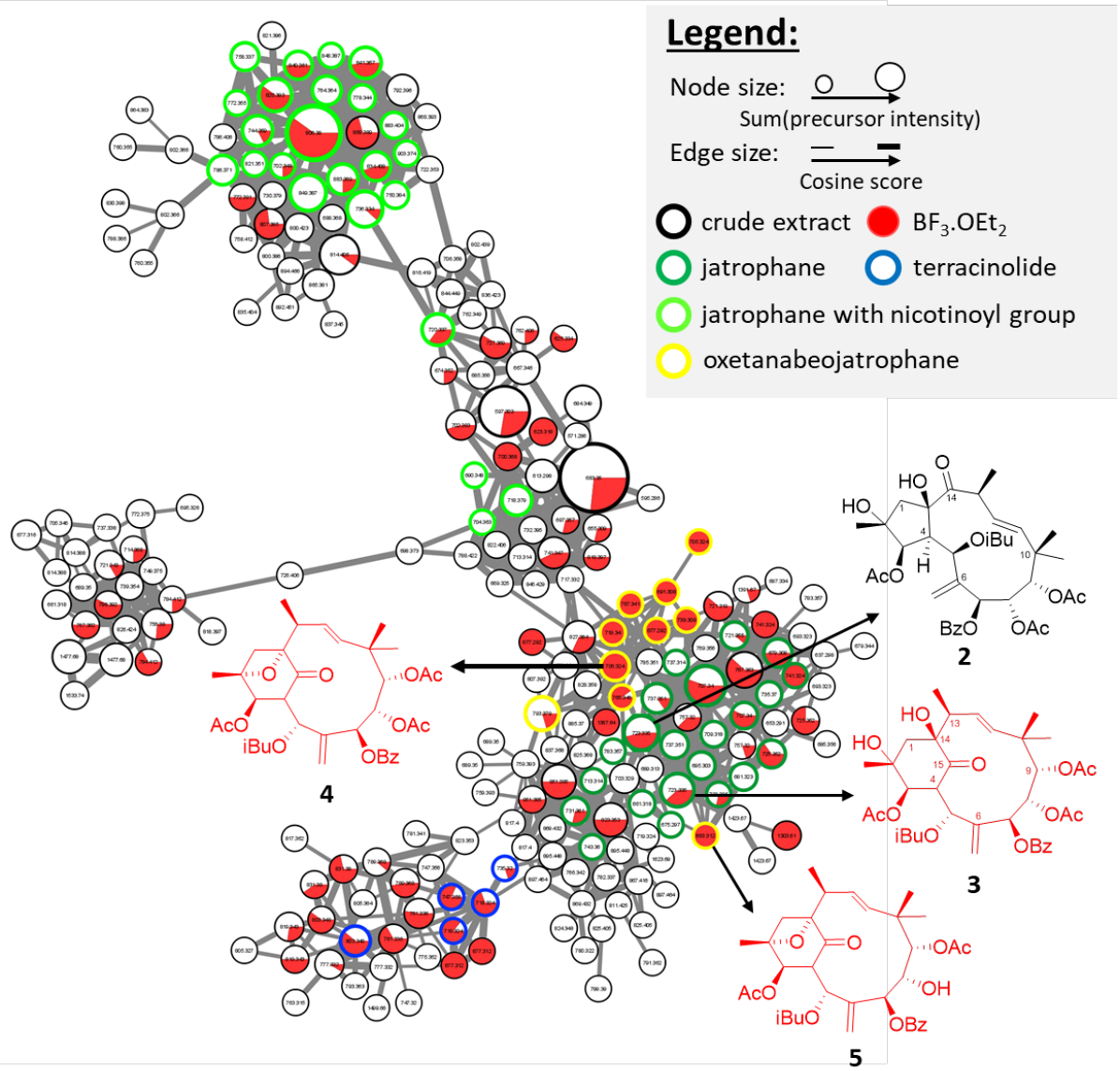


Figure 4. Jatrophane molecular network from crude extract (in white) and modified extract (in red) with $BF_3.OEt_2$. Jatrophane (surrounded in green), terracinolide (surrounded in blue), jatrophane with nicotinoyl group (surrounded in light green), and oxetanabeojatrophane (surrounded in yellow) clusters were annotated with the analog search mode.

CONCLUSIONS

In this study, we developed a new approach for generating chemical diversity by reacting different Lewis acids on a diterpene ester-enriched extract obtained from an *Euphorbia* species. Using a multi-informational molecular networking vizualisation, it is possible to quickly evaluate which are the most efficient reagents allowing a maximum of structurally new

derivatives to be obtained. Chemical modifications could be made to structurally complex molecules that could not be synthesized or isolated on a gram scale within a reasonable timeframe.

More generally, visualization by molecular network enables rapid, simple and reliable interpretation of the results at the end of an experimental procedure that is simple to implement. To avoid repetitive reactions or failures, the screening of multiple reagents on complex mixtures enables better upstream selection of the most appropriate reagent or faster targeting of the desired product.

EXPERIMENTAL SECTION

Sample preparation. In August 2012, 20 specimens of *Euphorbia dendroides* was collected for their latex, by L.-F.N. at Ficaghjola Beach (Piana), in the western region of Corsica (France). This collected latex on cotton was extracted by solid−liquid experiment to give an 24 g of EtOAc extract, and then, extracted by MeCN, as previously described.[10] The chemical reactions were performed directly on the dry MeCN extract of the fractionated latex. The diterpene esters used as reference compounds were previously isolated and identified from *E. dendroides* MeCN extract.[10–12] Euphodendroidins E (**1**) and F (**2**) were isolated from *Euphorbia dendroides* MeCN latex extract in 600 mg and 120 mg, respectively.[10]

General procedure for extract treatment with Bronsted acids. The extract was solubilized in CH_2Cl_2 (0.5 mL). Acid (10 eq) was then added to the solution among acetic acid (AcOH, 99%), trifluoroacetic acid (TFA, 99%), sulfuric acid (H_2SO_4, 96%), formic acid (HCOOH, 98%), and hydrochloric acid (HCl, 37%). The mixture was stirred for 16 h at r.t. and MeOH (0.5 mL) was then added and the resulting mixture was filtered and concentrated under reduced pressure. Water (0.5 mL) was added and the products were extracted with EtOAc (3 x 0.5 mL). The combined organic phases were then concentrated under reduced pressure.

General procedure for extract treatment with Lewis acids. To a solution of the MeCN latex extract in anhydrous CH_2Cl_2 (0.5 mL) under Ar at. was added Lewis acid (10 eq) among: trimethyl aluminum ($AlMe_3$), diethyl aluminium chloride ($Et_2ClAlCl$), aluminium isopropoxide [$Al(O\mathit{iPr})_3$], boron trifluoride etherate ($BF_3.OEt_2$), iron(III) chloride ($FeCl_3$), aluminium chloride ($AlCl_3$), titanium(IV) chloride ($TiCl_4$), zinc chloride ($ZnCl_2$), lithium tetrafluoroborate ($LiBF_4$), Ytterbium(III) trifluoromethanesulfonate [$Yb(OTf)_3$], tin(IV) chloride ($SnCl_4$), and Montmorrillonite K10. The mixture was stirred for 16 h at rt and the solvent was evaporated. MeOH (0.5 mL) was then added and the resulting mixture was filtered and concentrated under reduced pressure. Water (0.5 mL) was added and the products were

extracted with EtOAc (3 x 0.5 mL). The combined organic phases were then concentrated under reduced pressure.

General procedure for the reaction of euphodendroidin F with boron trifluoride diethyl etherate. To a solution of euphodendroidin F (50 mg, 0.075 mmol) in anhydrous CH_2Cl_2 (1 mL) at r.t. under Ar atm., was added $BF_3.OEt_2$ (51.7 mg, 0.36 mmol, 5 mol. Equiv.) was added. After 1 h, water was added and the compounds were extracted with MTBE (3 times). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. A purification by semi-preparative HPLC (Kromasil C_{18}, isocratic H_2O-MeCN + 0.1% formic acid, 25:75 in 40 min), afforded three compounds: the epiabeodendroidin F (**2**, 3.1 mg) and two new compounds **3** (7.9 mg) and **4** (1.8 mg).

General compounds elucidation procedure. Optical rotations $[\alpha]_D^{20}$ were measured on a polarimeter MCP 300 Anton Paar polarimeter at 20 °C. Monochromatic light source is the sodium D-lines. MeOH was used as a solvent. UV spectra were recorded on a Varian Cary 100 scan spectrophotometer and they were measured in a 1 cm quartz cell. The 1D- (^1H and ^{13}C) and 2D- (COSY, HSQC, HMBC, and ROESY) NMR analysis were recorded in $CDCl_3$ at 300 K on a Bruker Avance 600 MHz instrument using a 1.7 mm microprobe for compound **5** and on a Bruker Avance 300 MHz instrument for compounds **4**. The data processing software was NMRnotebook (http://www.nmrtec.com/). Analytical C_{18} columns (Kromasil, 250 × 4.6 mm i.d., 5 μm, Thermo Scientific) was used for HPLC separation on a Waters autopurification system equipped with a binary pump (Waters 2525), a UV-vis diode array detector (190–600 nm, Waters 2996), and a PL-ELS 1000 ELSD Polymer Laboratory detector. This unit was controlled with Masslynx software. Semi-preparative C_{18} column (Kromasil, 250 × 10 mm; i.d. 5 μm) was used for semi-preparative HPLC separation using a Dionex autopurification system equipped with a binary pump (P580), an UV-vis array detector (200–600 nm, Dionex UVD340U), and a PL-ELS 1000 ELSD detector Polymer Laboratory. This unit was controlled with Chromeleon software. The staining reagent used for analytical TLC plates (Silica gel 60

F_{254}, Merck) was the sulfuric molybdate, which was prepared by dissolving ammonium dimolybdate (50 g) in water (450 mL) and slowly adding concentrated sulfuric acid (50 mL) in cold conditions. The plates were observed at 254 nm and 366 nm UV-wavelength. All other chemicals and solvents were purchased from SDS (Peypin, France).

Synthetic compounds characteristic.

Oxetanabeodendroidin A (**4**): amorphous powder; $[\alpha]_D^{20}$ -8 (c 1, EtOH); UV (EtOH) λ_{max} (log ε) 231 (4.06) nm; for ^1H and ^{13}C NMR spectroscopic data, see Table S1; HRESIMS *m/z* 705.2892 $[M + Na]^+$ (calcd for $C_{37}H_{46}O_{12}Na^+$, 705.2887).

Oxetanabeodendroidin B (**5**): amorphous powder; $[\alpha]_D^{20}$ +8 (c 1, EtOH); UV (EtOH) λ_{max} (log ε) 231 (3.67) nm; for ^1H and ^{13}C NMR spectroscopic data, see Table S1; HRESIMS *m/z* 663.2769 $[M + Na]^+$ (calcd for $C_{35}H_{44}O_{11}Na^+$, 663.2781).

LC-MS/MS analysis of plant extract and modified extracts samples. On the *E. dendroides* MeCN latex extract, diluted 10 times in MeOH, before and after chemical reactions, a quadrupole time-of-flight mass spectrometer (Agilent 6540 Q-TOF, Agilent Technologies) coupled with an HPLC system (U3000, Dionex), was employed for the LC-ESIMS/HRMS analysis. For the chromatographic method, a column with solid-core particles Accucore (C18, 150 mm × 2.1 mm × 2.7 µm, ThermoFisher Scientific) was used. The elution solvents, water, and acetonitrile with 0.1% formic acid in both solvent A and B, respectively, were chosen to follow this optimal gradient: 0−25 min linear gradient from 20% to 100% solvent B, 25−28 min isocratic plateau at 100% solvent B, 29−34 min re-equilibration at 20% solvent B, at a constant flow rate of 300 μL/min. The mass spectrometer is equipped with an ESI source operating in positive mode as follows: gas temperature 325 °C, drying gas 11 L/min, nebulizer 30 psig, capillary voltage 3500 V, and fragmented voltage 110 V. The collision-induced dissociation energy was set at 35 eV, and a data-dependent acquisition (DDA) mode, consisting of a full MS scan from *m*/*z* 100 to *m*/*z* 1700 (scan time: 100 ms), followed by DDA of MS/MS spectra of the five most intense ions (top five, 200 ms each) from *m*/*z* 200 to *m*/*z* 900, with a

minimum intensity of 2500 counts, was used. Finally, acquisitions were achieved by an "extended dynamic range" mode allowing a mass resolution of 20 000 at *m*/*z* 922 and a dynamic range over five decades.

Molecular networking parameters. First, previously LC-MS/MS data acquired were converted to .mzML format using the MSConvert[18] part of the ProteoWizard suite. Then, by employing the Global Natural Products Social Molecular Networking platform (GNPS http://gnps.ucsd.edu),[4] classical molecular networking using MS-Cluster was generated with parameters depending on the instrument used. The generated .graphml files, associated with their tables, were open and visualized using Cytoscape 3.8.2 software.[13] In this visualization tool, node size is modulated by the number of scans, edge size depends on the cosine score, and a pie chart diagram is used to visualize samples.

Molecular networking parameters for plant extract and modified extract samples. For the samples obtained from the *E. dendroides* MeCN latex extract before and after chemical modifications, the precursor and the fragment ion mass tolerance are set at 0.03 Da both. The network options were set as follows: min pairs cos. at 0.7, the network topK at 10, the minimum matched fragment ions at 20, the maximum connected component size at 500 while the minimum cluster size is at 2. For the library search options, 20 minimum matched peaks with a score threshold at 0.7 were used, in analogs search mode (mass difference set at 130 Da). No filtering was applied. The GNPS molecular networking job of chemically modified plant extract with basis, Lewis acids, and acids, can be respectively found at:

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=2f3fa801e7eb480399a6964d9c74a36c

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=69b68f18ccd8450b83909461b83bdc38

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=b133f064edee4b1aa516288783ab58c1

The GNPS molecular networking job from the crude extract and the modified extract with $BF_3.OEt_2$ was set with most of the previously described parameters, but 0.65 was used for min pairs cos., 15 for the network topK, and 13 was used for the minimum matched fragment ions.

For the library search options, 13 minimum matched peaks with a score threshold at 0.7 were used, in analogs search mode with a mass difference set at 120 Da. A peaks filter in the 50 Da window was applied. The link to the job is:

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ab89a9336baf4a6e8ce19a025218dc4f

ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C RMN data table for compounds **4** and **5,** ^{1}H NMR spectra for the compounds **3**; ^{1}H, ^{13}C, and HRESIMS data for the compounds **4** and **5**.

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Notes

All other authors declare no competing financial interest.

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GRAPHICAL ABSTRACT