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Identification of Granatane Alkaloids from *Duboisia myoporoides* (Solanaceae) using Molecular Networking and Semisynthesis

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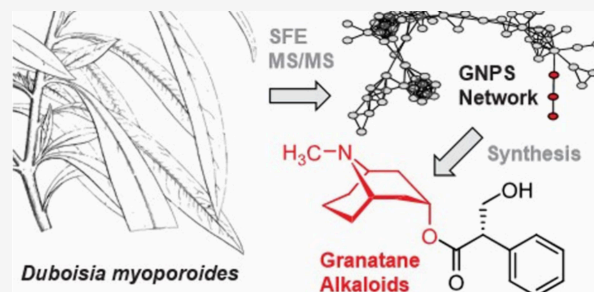
ABSTRACT: The Solanaceae plant family contains at least 98 genera and over 2700 species. The *Duboisia* genus stands out for its ability to produce pyridine and tropane alkaloids, which are relatively poorly characterized at the phytochemical level. In this study, we analyzed dried leaves of *Duboisia spp.* using supercritical CO_2 extraction and ultra-high-pressure liquid chromatography coupled to high-resolution tandem mass spectrometry, followed by feature-based molecular networking. Thirty-one known tropane alkaloids were putatively annotated, and the identity of six (atropine, scopolamine, anisodamine, aposcopolamine, apoatropine, and noratropine) were identified using reference standards. Two new granatane alkaloids connected in the molecular network were highlighted from *Duboisia myoporoides*, and their *α*-granatane tropate and *α*-granatane isovalerate structures were unambiguously established by semisynthesis.



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The Solanaceae or nightshade plant family contains more than 2700 species that occur predominantly in temperate and tropical regions of the western part of the world.[1] The Solanaceae includes important agricultural species such as potatoes, tomatoes, eggplant, and tobacco.[2,3] Furthermore, various genera within Solanaceae, *Atropa*, *Brugmansia*, *Datura*, *Duboisia*, *Hyoscyamus*, *Mandragora*, and *Scopolia*, are rich sources of tropane alkaloids. These tropane alkaloids, including (−)-hyoscyamine (**1**, synonym: (±)-atropine) and (−)-scopolamine (**4**, synonym: (−)-hyoscine) (Figure 1a),[4] have a long history of involvement in magico-religious rituals and medicine[5,6] due to their potent peripheral and central anticholinergic activities.[7,8] The *Duboisia* genus is endemic to Australia and New Caledonia[9,10] and consists of 4 species: *D. myoporoides*, *D. hopwoodii*, *D. leichhardtii*, and *D. arenitensis*.[11] *D. myoporoides* and *D. leichhardtii-myoporoides* hybrids are cultivated in southeastern Queensland for commercial production of (−)-hyoscyamine (**1**) and (−)-scopolamine (**4**).[12,13] *D. hopwoodii* leaves which, besides tropane alkaloids, are also rich in (−)-nicotine and (−)-nornicotine, are used to make the *Pituri* drug chewed by Indigenous Australians.[7,14−18]

Considering the importance of the *Duboisia* species as traditional and contemporary sources of pharmacologically active alkaloids, we aimed to reinvestigate their metabolomes using state-of-the-art methodologies. To do so, the use of untargeted profiling approaches based on (ultra)high-performance liquid chromatography coupled to high resolution tandem mass spectrometry ((U)HPLC-HRMS/MS) has become essential for natural product dereplication. However, the difficulties related to mass spectral interpretation still represent a major bottleneck to the global identification of plant metabolites in complex matrices.[19] In this context, over the past decade, molecular networking has been introduced as a promising approach for the annotation of known and unknown metabolites.[20−23] Its concept relies on MS2 mass spectral similarities where compounds with similar fragments will be linked together according to their degree of chemical similarity based on cosine scores.[23] The more similar the fragment ions patterns are, the higher the score will be. Compounds having similarity will be grouped as a distinct cluster where a common core structure or scaffold may be assigned.[24]

Nowadays, molecular networking can be performed using the online and open-access Global Natural Products Social Molecular Networking (GNPS) platform.[20,25] Among the ever growing list of tools available in GNPS, the feature-based molecular networking (FBMN) option allows to compute molecular networks from HPLC-MS/MS data after peak picking using data processing software such as MZmine, MS-DIAL, or XCMS.[26,27] Moreover, GNPS contains several MS2 libraries, some being continuously iterated, which in combination with spectral grouping can assist compound annotation. However, although molecular networks represent a

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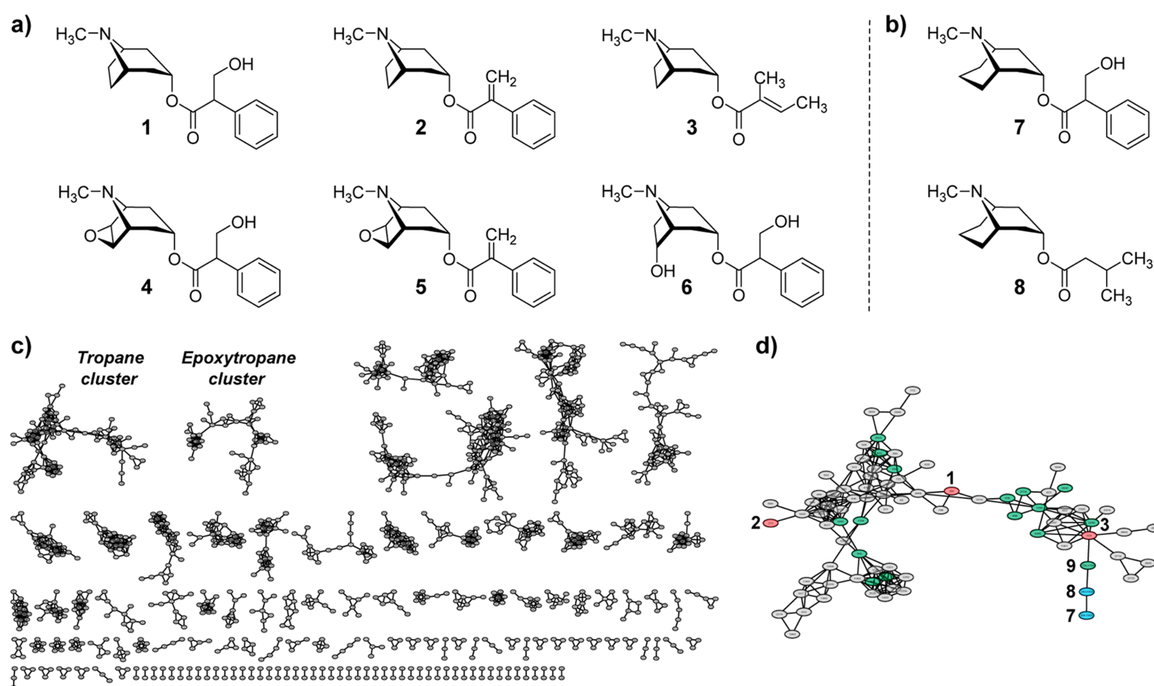

Figure 1. a) Structures of tropane alkaloids (**1**–**6**) identified in *Duboisia spp*.; b) Structures of granatane alkaloids (**7**–**8**) identified in *D. myoporoides*; c) Molecular network for natural products of *Duboisia spp*. including the clusters of tropane and epoxytropane alkaloids (singletons not shown for clarity); d) tropane cluster depicting identified compounds in red (atropine (**1**), apoatropine (**2**), tigloidine (**3**)), putatively annotated structures in green (Supporting Information Table S1), unknown structures in gray, and the two newly identified granatane alkaloids (**7**–**8**) in blue.

great leap forward for metabolite annotation, a significant portion of a plant's metabolome will typically remain unannotated and require other tools, including in-silico fragmentation software (e.g., Sirius and CSI:FingerID) and/or specific isolation or synthesis followed by conventional structure elucidation schemes.

This manuscript describes the analysis of three *Duboisia* species using extraction with supercritical carbon dioxide ($scCO_2$)[28,29] and UHPLC-HRMS/MS analysis, followed by molecular network generation using the GNPS platform.[20,25] This analysis resulted in the annotation of several tropane alkaloids and highlighted yet unidentified granatane alkaloids, which were unambiguously characterized by semisynthesis and coinjection with the natural products. Identifying granatane and other tropane alkaloids indicates that both Δ1-piperidinium and Δ1-pyrrolinium cations serve as biosynthetic intermediates during alkaloid biosynthesis in *Duboisia spp.*

RESULTS AND DISCUSSION

Dried leaves of *D. myoporoides*, a *D. hopwoodii* × *D. myoporoides* hybrid, and *D. leichhardtii* were extracted in triplicate using $scCO_2$. Different extraction conditions and modifiers (i.e., ethanol (EtOH), see Experimental Section) were tested to cover as much chemical space as possible. Samples were analyzed using UHPLC-HRMS/MS in positive mode. The resulting data were pooled to generate a molecular network for *Duboisia* metabolites, revealing 3128 nodes (2031 singletons) with 4281 edges and 132 clusters (Figure 1c).

The GNPS platform provided putative annotation for 192 compounds, while 2936 nodes remained unknown. The generated network was uploaded into Cytoscape for visualization. Two clusters containing either tropane alkaloids (n = 82 nodes, e.g., atropine (**1**)) or epoxytropane alkaloids derivatives (n = 44 nodes, e.g., scopolamine (**4**)) were detected (Figure 1c). Members of the tropane cluster were characterized by a fragment ion at m/z 124.1121 $[C_8H_{14}N]^+$ from neutral loss of the variable ester moieties. Structure assignments of atropine (**1**), apoatropine (**2**), and tigloidine (**3**) could be confirmed by comparison with commercially available standards. In addition, 17 compounds were putatively annotated based on elemental composition, fragmentation similarities, and in silico annotation by Sirius[30] (Supporting Information Table S1). In the epoxytropane cluster, the most characteristic fragment ion corresponded to m/z 138.0913 $[C_8H_{12}NO]^+$ from neutral loss of the variable ester moieties. Annotation of scopolamine (**4**), aposcopolamine (**5**), and anisodamine (**6**) by GNPS and Sirius could be confirmed by comparison with authentic standards. In addition, 8 compounds were putatively annotated based on elemental composition, fragmentation similarities, and *in silico* annotation by Sirius (Supporting Information Table S2). Among the 31 identified or putative annotated alkaloids, three did not display the characteristic fragment ions at m/z 124.1121 $[C_8H_{14}N]^+$ or m/z 138.0913 $[C_8H_{12}NO]^+$ but instead exhibited an intense fragment ion at m/z 138.1277 $[C_9H_{16}N]^+$. These molecules were tightly connected in the tropane cluster (Figure 1d) and displayed molecular ion signals at m/z 304.1904 $[C_{18}H_{26}NO_3]^+$ (**7**), m/z 240.1956 $[C_{14}H_{26}NO_2]^+$ (**8**), and m/z 238.1797 $[C_{14}H_{24}NO_2]^+$ (**9**) (Supporting Information Table S1). Inspection of extracted ion chromatograms revealed that alkaloids **7** − **9** were most abundant in *D. myoporoides* and particularly enriched upon CO_2 extraction with 15% EtOH (v/w) but still about 300 times less abundant than the dominating atropine (**1**) and scopolamine (**4**), which made their identification challenging.

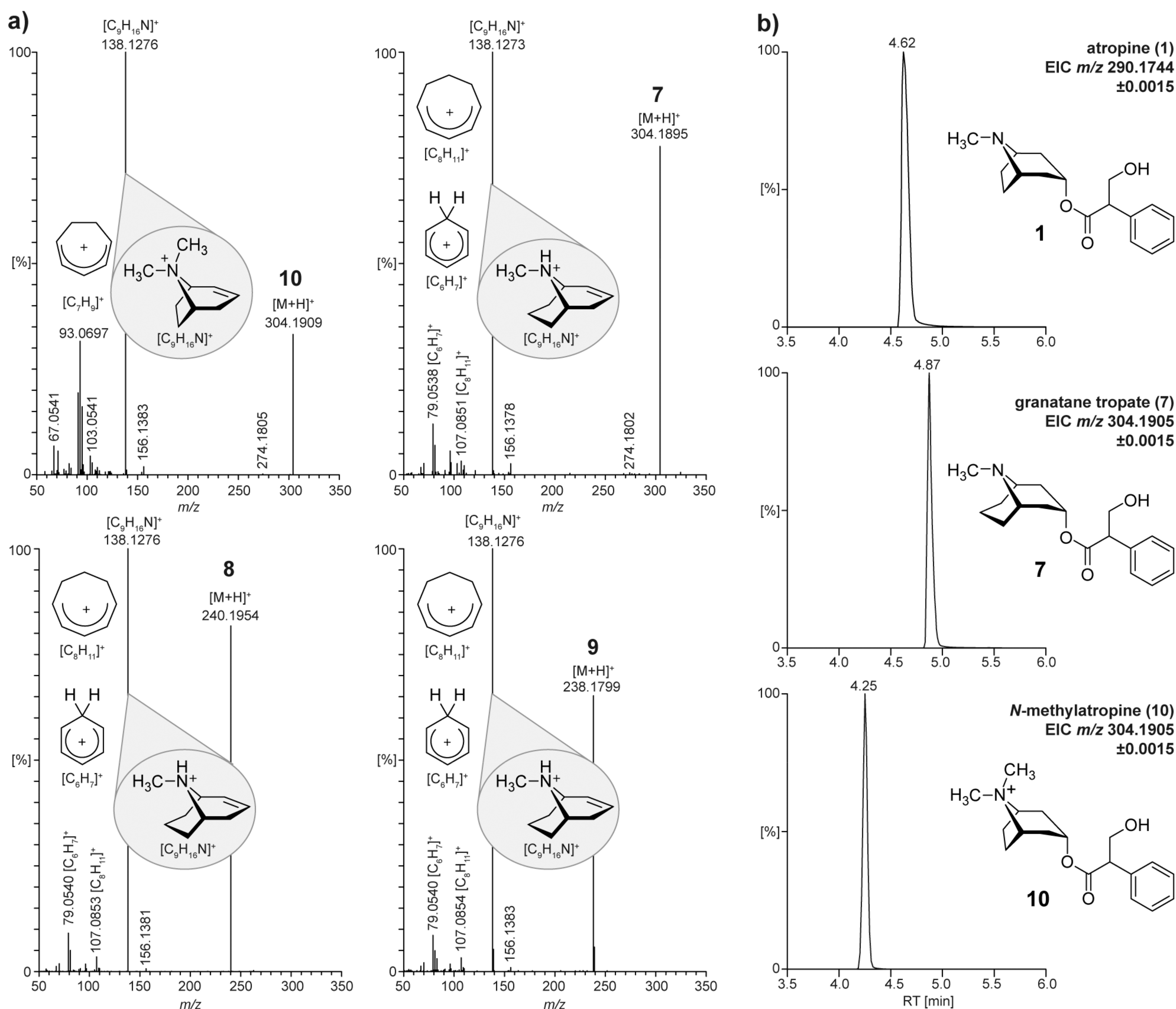

Figure 2. Structure assignment of the granatane alkaloids (**7**−**9**). a) ESI-(+) HRMS/MS spectra of granatane tropate (**7**), granatane isovalerate (**8**), a putative granatane senecioate or tiglate (**9**), and *N*-methyl atropine (**10**) showing a common fragment ion signal at *m*/*z* 138.1277 $[C_9H_{16}N]^+$ along with characteristic fragment ions at *m*/*z* 107.0854 $[C_8H_{11}]^+$ for the granatane alkaloids (**7**−**9**) versus 93.0697 $[C_7H_9]^+$ for the tropane alkaloid (**10**); b) UHPLC-HRMS chromatograms of ternary alkaloids atropine (**1**) and granatane tropate (**7**) versus quaternary *N*-methyl atropine (**10**).

Comparative analysis of the HRMS/MS spectra suggested the presence of alkaloid esters composed of a common $C_9H_{17}NO$ building block esterified with tropic acid ($C_9H_{10}O_3$) (**7**), pentanoic acid ($C_5H_{10}O_2$) (**8**), or pentenoic acid ($C_5H_8O_2$) (**9**) (Figure 2a). The presence of ternary granatan-3-ol esters was supported by comparative MS/MS analysis of the putative granatane tropate (**7**) and *N*-methylatropine (**10**), which both display a common fragment ion at *m*/*z* 138.1277 $[C_9H_{16}N]^+$ (Figure 2a).

In contrast, the chromatographic retention time of the quaternary *N*-methyl atropine (**10**) was shorter than that of the ternary atropine (**1**, tropane tropate) or of the ternary granatane tropate homologue (**7**) (Figure 2b). Furthermore, the MS/MS spectrum of quaternary *N*-methylatropine (**10**) showed a fragment ion at *m*/*z* 93.0697 $[C_7H_9]^+$ corresponding to the dihydrotropylium fragment that was not observed for **7**, **8**, and **9**. Instead, the granatan-3-ol esters **7**−**9** displayed a common fragment ion at *m*/*z* 107.0854 $[C_8H_{11}]^+$ (Figure 2a), which suggested the homologous 8 carbon ring structure. Considering the previous identification of isopelletierine (granatan-3-one) in *D. myoporoides*,[31] also reported in the root bark of pomegranate, *Punica granatum* (Lythraceae),[32] the presence of granatan-3-ol esters was proposed for alkaloids **7**−**9**, although such esters have not yet been identified as natural products in this genus. Considering the structures of the dominating tropan-3-ol esters, the acid moieties of the granatan-3-ol esters were proposed to be tropic acid for **7** and either isovaleric or 2-methylbutyric acid for **8** (Figure 3).

Although compounds **7**−**9** were enriched in the *D. myoporoides* extract, their relative concentrations were far too low for isolation by semipreparative HPLC followed by NMR-based structure assignment. We thus selected a semisynthetic approach which confirmed structure assignments, as shown in Figure 3. Esterification of a diastereomeric mixture of α- and β-

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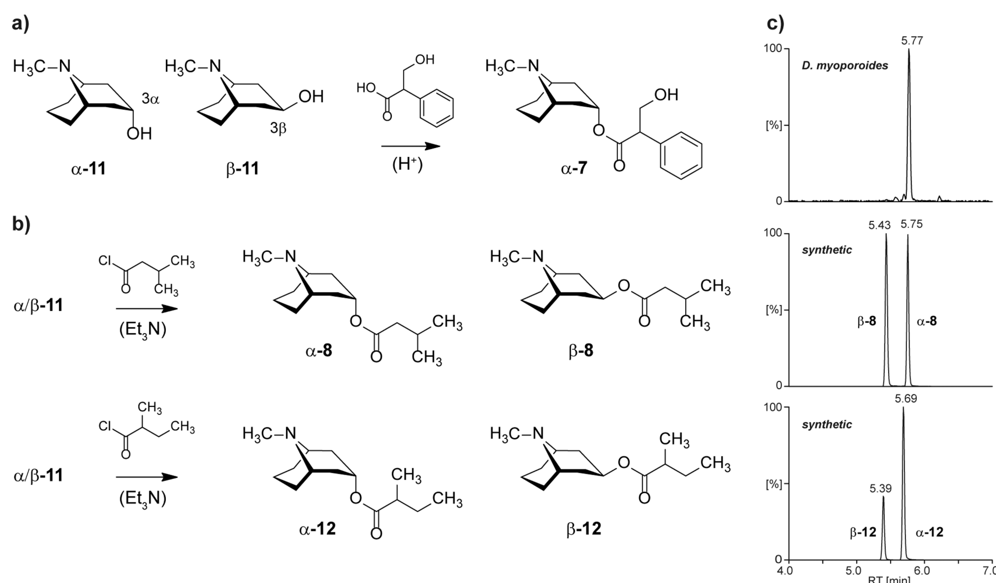

Figure 3. a) Semisynthesis of granatane alkaloids from *D. myoporoides* using esterification of α/β-isomeric granatan-3-ols (**11**, 9-methyl-9-azabicyclo[3.3.1]nonan-3-ols) with either a) tropic acid under acidic conditions to furnish α-granatane tropate (**7**) or b) with isovaleric acid chloride and 2-methylbutyric acid chloride under alkaline conditions to furnish α/β-isomeric granatane isovalerates (**8**) and granatane 2-methylbutyrates (**12**), respectively; c) Structure assignment of α-granatane isovalerate (**8**) from *D. myoporoides* using comparative UHPLC-HRMS with isomeric mixtures of the synthetic granatane isovalerate (**8**) and 2-methylbutyrate (**12**) standards.

configurated granatan-3-ols (**11**) with tropic acid under acidic conditions furnished predominantly α-granatane tropate (**7**) (Figure 3), as previously described,[33−35] which was shown to be identical to the natural product from *D. myoporoides* (Supporting Information Figure S1).

Furthermore, esterification of α/β-isomeric granatan-3-ols (α/β-**11**) with isovaleric or 2-methylbutyric acid using the corresponding acid chlorides under base-catalyzed conditions furnished α/β-isomeric mixtures of granatane isovalerates (α/β-**8**), and granatane 2-methylbutyrates (α/β-**12**), respectively (Figure 3b). Comparative UHPLC-HRMS/MS analysis with the natural product from *D. myoporoides* confirmed its identity as the later eluting granatane isovalerate (**8**) isomer (Figure 3c). Synthetic diastereoisomers of α- and β-granatane isovalerate were isolated and their structures identified by NMR spectroscopy. The main difference between the α- and β-isomers was observed for the chemical shift values of positions 3 and 7 of the granatane ring. Transannular steric interactions are responsible for shielding carbons C-7 (−5.9 ppm) and C-3 (−4.2 ppm) in the α-isomer.[36,37] ^{1}H NMR chemical shifts exhibited similar variation for H-3 (δ_H 5.57 ppm vs 5.14 ppm) and Hα-7 (δ_H 1.77 ppm vs 1.56 ppm). NOESY correlations between H-3 (δ_H 5.57 ppm) and Hα-7 (δ_H 1.77 ppm) observed exclusively for the β-isomer confirmed the assignment of both isomers (Supporting Information Figure S2). The diastereoisomers matching the retention time of the natural compounds exhibited ^{1}H and ^{13}C chemical shift values characteristic of the α-isomers. The identity of the synthetic granatane esters and the natural products was subsequently ascertained by coinjection using alkaloid extracts of *D. myoporoides*, which confirmed the identification of α-configurated granatane tropate (**7**) and granatane isovalerate (**8**) (Supporting Information Figure S1). Compound **9** was only present in trace amounts, and its structure could not be fully identified. Nevertheless, due to its similarity with compounds **7** and **8** as well as its MS/MS spectrum (Figure 2a), we could putatively propose a granatane senecioate or tiglate structure.

The new granatane alkaloids **7** and **8** differ from the well-known tropane alkaloids in the ring extended 9-methyl-9-azabicyclo[3.3.1]nonan-3-ol skeleton. Structurally related alkaloids like granatan-3-one (pseudopelletierine) have previously been identified in the root bark of pomegranate, *P. granatum* (Lythraceae)[32,38−40] which is not related to the Solanaceae. The biosynthesis of granatanone resembles those of pelletierine and anabasine previously identified in *D. myoporoides* (Supporting Information Table S3).[31,41−48] Occurrence of granatane and tropane alkaloids like **7** and **1**, along with anabasine and nicotine in *Duboisia spp*. demonstrates that the Δ1-piperidinium and Δ1-pyrrolinium intermediates can both serve as precursors for reaction with malonyl-CoA or (nicotinic acid derived) 2,5-dihydropyridine, respectively.[49−51] Granatane alkaloids **7** and **8** could not be detected in the *D. hopwoodii* × *D. myoporoides* hybrid and *D. leichhardtii*. Granatane tropate (**7**) has been previously synthesized[35,37,52] and shown to display anticholinergic activity comparable to atropine (**1**).[53] The identification of α-configurated granatane alkaloids as minor components in *D. myoporoides* suggests that these compounds might also be present in other Solanaceae species and raises interesting questions regarding the substrate specificity and evolution of tropanone and granatanone reductases[50,54] and other enzymes involved in their biosynthesis.[32]

■ EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were acquired on a Bruker Avance Neo Ascend 600 MHz (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a BBI H-BB-D-05 Z probe. Deuterated methanol (MeOH) CD_3OD (δ_H 3.31 ppm and δ_C 49.00 ppm) served as both solvent and internal standard. All the spectra except HSQC were acquired using standard pulse programs. HSQC was recorded using Non-Uniform-Sampling with a sampling rate of 25%. NOESY spectra were recorded with a delay of 300 ms. Acquired spectra were processed using the Mnova NMR software package

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(v.14.2.0, Mestrelab Research S.L., A Coruña, Spain). LC-MS was performed using a Vanquish duo UHPLC instrument coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) using a Hypersil GOLD C18 column (150 × 2.1 mm, 1.9 μm, 0.5 mL/min) (Thermo Fisher Scientific, Waltham, MA, USA). Preparative HPLC was performed using an XSelect CSH prep C18 OBD (250 × 19 mm, 5 μm, 8.0 mL/min) (Waters Corp., Milford, MA, USA) column with an LC-20AR Shimadzu pump (Kyoto, Japan) connected to a PDA detector (Shimadzu, model SPD-20A) and a fraction collector (Shimadzu, model FRC-40).

Plant Material and Chemicals. Dried leaves of three *Duboisia* species (*D. myoporoides, D. hopwoodii x D. myoporoides* hybrids, and *D. leichhardtii*) were purchased from Alidans (San Giuliano Terme, PI, Italy). Atropine (**1**), scopolamine (**4**), and anisodamine (**6**) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Apoatropine (**2**) and aposcopolamine (**5**) were purchased from BOC Sciences (Shirley, NY, USA). Tigloidine (**3**), *N*-methylatropine chloride (**10**), and 9-methyl-9-azabicyclo[3.3.1]nonan-3-ol (**11**) were purchased from Chemspace (Riga, Latvia). Tropic acid, isovaleryl chloride, and 2-methylbutyryl chloride were purchased from Acros Organics (Geel, Belgium). Water, absolute EtOH, and LC-MS grade-acetonitrile (MeCN) were purchased from Fisher Scientific AG (Basel, Switzerland). Formic acid (FA) and triethylamine (TEA) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Deuterated MeOH CD_3OD was purchased from ReseaChem GmbH (Burgdorf, Switzerland).

Extraction. Dry leaves were ground, followed by extraction with $scCO_2$ using an SFE LAB 100 mL 1000 BAR extraction system (SFE Process, Tomblaine, France). A test portion of 3−8 g was extracted with supercritical CO_2 with and without modifier (EtOH at different percentages) to increase the polarity of extraction. Therefore, 15 extracts were generated from the three different *Duboisia* species and aliquoted in triplicates with 100× dilution before analysis, ending with a total of 45 samples analyzed to build-up the molecular network.

HRMS/MS and Molecular Networking Analysis. Separation of the phytochemicals was achieved on a Hypersil GOLD C18 column (150 × 2.1 mm, 1.9 μm) (Thermo Fisher Scientific, Waltham, MA, USA) on a Vanquish duo HPLC instrument coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). A linear gradient of water containing 0.1% FA (eluent A) and MeCN containing 0.1% FA (eluent B) from 5% B to 95% B in 13.8 min was used. The column oven temperature was set at 40 °C, and 2 μL of each sample was injected. Mass detection of metabolites was performed in positive electrospray ionization (ESI) mode by scanning a mass range of m/z 70−1050 at a mass resolution of 60,000 (full width at half-maximum (fwhm), m/z 200). The automatic gain control (AGC) target was set at 1E6 with a maximum injection time of 100 ms. Vaporizer heater temperature, capillary temperature, spray voltage, sheath gas, and auxiliary gas were set at 250 °C, 300 °C, + 3.50 kV, 50, and 15 arbitrary units, respectively. For high-energy collision dissociation (HCD), a data-dependent MS2 (Top 5, loop count: 5, dynamic exclusion: auto) of each MS1 scan, with a mass resolution of 30,000 (fwhm, m/z 200) was used with stepped normalized collision energies (NCE) of 25, 50, and 75 (AGC target of 1E5, isolation window of 2.5 Da).

Mass spectrometric raw data were processed with the MZmine 2.53[26] software (Supporting Information Table S4) to generate a final feature table containing m/z-RT pairs with peak height and an associated MS2 fragmentation pattern file. The two files were exported to GNPS (https://gnps.ucsd.edu)[20,25] to create a molecular network using the Feature-Based Molecular Networking (FBMN) workflow. The following parameters were used: mass tolerances of both precursor and MS/MS fragment ions 0.02 Da, minimum pairs cosine score 0.7, minimum matched fragment ions 6, network TopK 10, maximum connected component size 100, maximum shift between precursors 500 Da. The MS/MS spectra present in the network were then searched against GNPS spectral libraries. The Dereplicator was used to annotate MS/MS spectra and result files have been deposited at MassIVE Public repository (ftp://massive.ucsd.edu/v07/MSV000094640/). The molecular networks were visualized using Cytoscape software[55] (v.3.10.0).

The fragment file information was imported into Sirius 4.9.12 (https://bio.informatik.uni-jena.de/software/sirius/),[30] where a second level of annotation was performed by computing all the spectra using CSI:FingerID with Coconut, GNPS, Natural Products, and PlantCyc selected libraries.[24,56]

Synthesis of α-Granatane Tropate (7). A solution of 29 mg 9-methyl-9-azabicyclo[3.3.1]nonan-3-ol (**11**, 200 μmol) in 20 mL toluene was treated with 32 mg tropic acid (200 μmol) and 100 μL concentrated sulfuric acid. After heating under reflux for 16 h using a Dean−Stark apparatus, the reaction was quenched with 100 μL ammonium hydroxide. The products were concentrated using a stream of nitrogen. RP-C18 chromatography furnished 42 mg granatane tropate (**7**, 150 μmol, 69% yield) as a colorless oil. An analytical sample of α-granatane tropate (**7**) was isolated by preparative HPLC and shown to be identical to the natural product from *D. myoporoides*.

*α-Granatane Tropate (**7**).* ^{1}H NMR (CD_3OD, 600 MHz) δ 7.35−7.29 (m, 5H, H-4′, H-5′, H-6′, H-7′, H-8′), 5.14 (tt, J = 6.9, 1.4 Hz, 1H, H-3), 4.14 (dd, J = 10.7, 9.2 Hz, 1H, H-9′a), 3.83 (dd, J = 9.2, 5.4 Hz, 1H, H-2′), 3.75 (dd, J = 10.7, 5.4 Hz, 3H, H-9'b), 3.33 (m, 2H, H-1, H-5), 2.76 (s, 3H, N-Me), 2.54 (dt, J = 16.0, 7.0 Hz, 1H, H-2a, H-4a), 2.22 (m, 1H, H-7b), 1.89 (br d-like, J = 6.9 Hz, 2H, H-6, H-8), 1.87 (d, J = 16.1 Hz, 1H, H-2b, H-4b), 1.23 (dt, J = 14.2, 6.3 Hz, 1H, H-7a); ^{13}C NMR (CD_3OD, 151 MHz) δ 130.0−128.1 (CH, C-4′, C-5′, C-6′, C-7′, C-8′), 64.4 (CH_2, C-9'), 63.4 (CH, C-3), 55.8 (CH, C-2′), 53.7 (CH_2, C-1, C-5), 39.3 (CH_3, N-Me), 29.8 (CH_2, C-2, C-4), 26.5 (CH_2, C-6, C-8), 13.5 (CH, C-7).

MS/MS m/z 304.1901 $[M + H]^+$ (80), 156.1383 (10), 138.1276 (100), 107.0854 (5), 79.0540 (10); HR-ESI-MS: m/z 304.1904 $[M + H]^+$ (calcd. for $C_{18}H_{26}NO_3$, 304.1907).

Synthesis of α/β-Isomeric Granatane Isovalerates (8). A solution of 29 mg 9-methyl-9-azabicyclo[3.3.1]nonan-3-ol (**11**, 200 μmol) in 20 mL toluene was treated with 23 mg isovaleryl chloride (200 μmol) and 80 μL of TEA. After heating under reflux for 16 h, the products were washed twice with 20 mL water and concentrated using a stream of nitrogen. RP-C18 chromatography furnished 36 mg granatane isovalerates (150 μmol, 75% yield) as a colorless oil. Purification of the α and β-isomers was performed by preparative HPLC. α-Granatane isovalerate (**8**) was shown to be identical to the natural product from *D. myoporoides*.

*α-Granatane Isovalerate (**8**).* ^{1}H NMR (CD_3OD, 600 MHz) δ 5.14 (1H, tt, J = 7.0, 1.8 Hz, H-3), 3.41 (2H, m, H-1, H-5), 2.85 (3H, s, N-Me), 2.62 (1H, dt, J = 16.6, 7.0 Hz, H-2a, H-4a), 2.58 (1H, dt, J = 13.9, 6.1 Hz, H-7b), 2.27 (2H, d, J = 7.2 Hz, H-2'), 2.13 (1H, m, H-3′), 2.08 (1H, m, H-6a, H-8a), 1.86 (1H, d, J = 16.6 Hz, H-2b, H-4b), 1.74 (1H, m, H-6b, H-8b), 1.56 (1H, dt, J = 13.9, 6.4 Hz, H-7a), 1.00 (3H, d, J = 6.7 Hz, H-4′); ^{13}C NMR (CD_3OD, 151 MHz) δ 62.6 (CH, C-3), 53.9 (CH_2, C-1, C-5), 44.3 (CH_2, C-2'), 39.2 (CH_3, N-Me), 30.0 (CH_2, C-2, C-4), 26.7 (CH, C-3′), 26.6 (CH_2, C-6, C-8), 22.4 (CH_3, C-4′), 13.6 (CH, C-7).

MS/MS m/z 240.1956 $[M + H]^+$ (90), 156.1382 (2), 138.1277 (100), 107.0854 (5), 79.0540 (10); HR-ESI-MS: m/z 240.1956 $[M + H]^+$ (calcd. for $C_{14}H_{26}NO_2$, 240.1958).

β-Granatane Isovalerate. ^{1}H NMR (CD_3OD, 600 MHz) δ 5.58 (tt, J = 10.6, 7.7 Hz, 1H, H-3), 3.37 (m, 2H, H-1, H-5), 2.79 (s, 3H, N-Me), 2.20 (d, J = 7.2 Hz, 2H, H-2'), 2.19 (m, 1H, H-3′), 2.15 (m, 1H, H-7b), 2.14 (m, 2H, H-6a, H-8a), 2.10 (m, 1H, H-6, H-8), 2.06 (m, 1H, H-6b, H-8b), 1.77 (m, 1H, H-7a), 0.97 (d, J = 6.8 Hz, 3H, H-4'); ^{13}C NMR (CD_3OD, 151 MHz) δ 66.8 (CH, C-3), 56.0 (CH_2, C-1, C-5), 44.0 (CH_2, C-2'), 39.5 (CH_3, N-Me), 32.1 (CH_2, C-2, C-4), 26.6 (CH, C-3′), 26.6 (CH_2, C-6, C-8), 22.3 (CH_3, C-4′), 19.5 (CH, C-7).

MS/MS m/z 240.1956 $[M + H]^+$ (85), 156.1381 (2), 138.1276 (100), 107.0853 (2), 79.0540 (8); HR-ESI-MS: m/z 240.1956 $[M + H]^+$ (calcd. for $C_{14}H_{26}NO_2$, 240.1958).

Synthesis of α/β-Isomeric Granatane 2-Methylbutyrates (12). A solution of 29 mg 9-methyl-9-azabicyclo[3.3.1]nonan-3-ol (**11**, 200 μmol) in 20 mL toluene was treated with 23 mg 2-

methylbutyryl chloride (200 μmol) and 80 μL TEA. After heating under reflux for 16 h, the products were washed twice with 20 mL water and concentrated using a stream of nitrogen. Comparative HPLC-MS analysis demonstrated that resulting α/β-isomeric granatane 2-methylbutyrates (**12**) were not identical to the natural products from *D. myoporoides*.

Preparative Liquid Chromatography. The α-granatane alkaloids **7** and **8** were purified by preparative HPLC using an LC-20AR Shimadzu pump (Kyoto, Japan) connected to a PDA detector (Shimadzu, model SPD-20A) a fraction collector (Shimadzu, model FRC-40) set for continuous collection. The column was an XSelect CSH prep C18 OBD (250 × 19 mm, 5 μm, Waters Corp., Milford, MA, USA), and the mobile phases were solvent A, water +0.05% FA; solvent B, MeOH + 0.05% FA. The flow rate was set to 8.0 mL/min. Elution was monitored by UV detection at 216 and 260 nm. Chromatographic fractions were analyzed by LC-MS and the purities were evaluated by ^{1}H NMR analysis. α-Granatane tropate (**7**) was purified using isocratic conditions with 8.0% B from 0−3 min, followed by a linear ramping gradient of 8 to 18% B from 3−65 min, of 18 to 100% B from 65−70 min, and a hold at 100% B from 70−80 min. Fractions containing the pure compound were combined to provide α-granatane tropate (**7**) (2.4 mg).

α-Granatane isovalerate (**8**) was purified using isocratic conditions with 8.0% B from 0−3 min, followed by a linear ramping gradient of 8 to 30% B from 3−65 min; of 30 to 100% B from 65−70 min, and a hold at 100% B from 70−80 min. Fractions containing the pure α-granatane isovalerate (**8**) were combined to provide 1.2 mg of the α-isomer (**8**). Fractions containing pure α-granatane isovalerate were combined to provide 0.9 mg of the β-isomer.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.4c00304.

> Supplementary Figure 1: Coinjection of natural extract with authentic standard. Figure 2: Structure of α and β granatane and NOESY interaction. Figures 3−14: NMR spectra of semisynthetic compounds. Tables S1−S2: Annotated compounds within tropane and epoxytropane clusters. Table S3: Alkaloids previously described from *Duboisia spp.* Table S4: MZmine parameters used for data processing (PDF)

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