ACS Combinatorial Science

RESEARCH ARTICLE

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Brominated Extracts As Source of Bioactive Compounds

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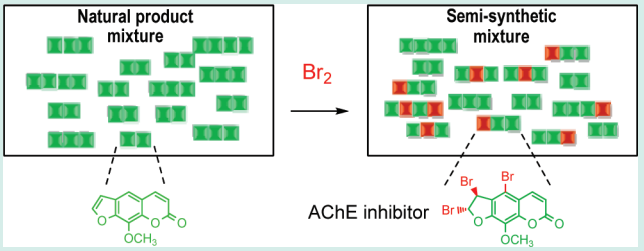
Supporting Information

ABSTRACT: The chemical composition and the biomolecular properties of a crude plant extract were altered through bromination leading to the discovery of an acetylcholinesterase inhibitor.



KEYWORDS: crude plant extract, brominated extracts, bioactive compounds, acetylcholinesterase

INTRODUCTION

Natural products are biologically validated starting points for the development of new drugs.[1] They are the outcome of a long evolution process that has resulted in a unique assortment of squeletons with high affinity for biomolecules. Consequently, natural products represent the source of inspiration for the design of natural product-like combinatorial libraries.[2] Additionally, several approaches have been proposed to increase the diversity of natural product mixtures such as the diversification of natural product mixtures by combinatorial biosynthesis[3] and related techniques.[4]

Recently, we have introduced a strategy to generate bioactive compounds through chemical diversification of inactive natural product mixtures (i.e., natural extracts).[5] Like in Houghten's libraries from libraries approach,[6] one library of compounds is transformed into another library with the expectation of altering its biomolecular properties. The main difference between both strategies is that in Houghten's approach the starting material is a fully characterized solid supported combinatorial library whereas we use natural extracts (NEs). Since NEs are mixtures of complex composition,[7] mostly uncharacterized, that usually contain a high number of molecules with different scaffolds and functionalities, the chemical transformation of a high proportion of their component molecules can be challenging. This can be carried out through the transformation of chemical functionalities which are very common in natural products such as carbonyls, −OH groups or amines; and has led to the generation of an antifungal compound[5] and of β-glucosidase inhibitors.[8] Here we report for the first time the utility of double bonds and aromatic rings as entry points toward the transformation of NEs through a bromination protocol, and the discovery of a new semisynthetic acetylcholinesterase inhibitor.

RESULTS AND DISCUSSION

To analyze the potential impact of the transformation of these groups on the chemical composition of natural products mixtures, we determined the number of molecules containing them in four sets (S1−S4) of 17 groups of compounds or "virtual extracts" selected from the Dictionary of Natural Products database[9] through the use of four different filters. In set one, each group of compounds contained every molecule within the database found in one particular plant species. Despite that the plant species selected were those with higher numbers of compounds described in the database, the number of compounds described for each plant species is still rather low. Therefore these data were complemented with the set two where each group of compounds contained every molecule within the database found in one particular plant genus. Again, the plant genus selected were those with higher number of compounds described in the database. In the third set, each of the 17 "virtual extracts" included only one type of secondary metabolite (i.e., flavonoids, alkaloids, etc.). This filter was used to analyze the distribution of the analyzed groups within the different types of metabolites. In set four each virtual extract included all the molecules in the database with molecular weights starting with arbitrarily defined values (i.e., 10, 20, etc.). This filter generates the virtual extract by grouping molecules using a characteristic that is unrelated to the functional groups that they contain.

The average frequency of molecules containing one or more double bonds (excluding the aromatic ones) in the four sets of virtual mixtures of natural products was between 0.65 and 0.74 and the standard deviation values are below 0.22 regardless of the filter used (Figure 1). This suggests that chemical transformation

Received: November 25, 2010
Published: December 17, 2010

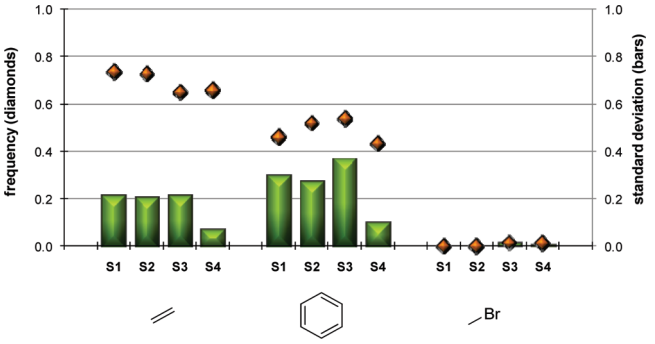


Figure 1. Standard deviations (bars) and frequencies (diamonds) of structures containing double bonds, aromatic rings, and bromine atoms in four sets of ``virtual extracts'': plant species (S1), plant genus (S2), type of metabolite (S3), and molecular weight (S4).

of double bonds could be an interesting entry point to the chemical transformation of a significant number of the component molecules of NEs. A different situation was observed for aromatic rings (Figure 1). The average frequency of molecules containing at least one aromatic ring was between 0.44 and 0.54, and the standard deviation ranged between 0.10 and 0.37 depending on the filter used. Thus chemical transformation of aromatic rings may not result attractive enough to alter the composition of NEs in a significant way. However, the use of reactions that transform both chemical characters, aromatic rings, and double bonds could be interesting.

Aiming at the diversification of the components of NEs through chemical alteration of aromatic rings and double bonds, we tested the bromination reaction. Several studies demonstrate that the average proportion of halogens in drugs is significantly higher than in natural products.[10]

In the four sets of virtual extracts, the average frequency of natural molecules containing at least one bromine atom is clearly lower than the average frequency of natural molecules containing double bonds or aromatic rings (Figure 1). Most of the naturally occurring organobromine compounds are produced by marine organisms;[11] and several brominated metabolites with antibacterial,[12] antitumor,[13] antiviral,[14] and antifungal activity[15] have been isolated from seaweeds, sponges, corals, molluscs, and others. In contrast, terrestrial plants account only for a few bromine-containing compounds.[11] Since bromine is a powerful oxidation agent and a strong electrophile, several reactions can be envisaged of this reagent with different functional groups present in natural products. Considering the target molecular fragments, double bonds and aromatic rings, addition and substitution reactions could be expected. The process would result in the exchange of one hydrogen atom with one bromine atom or in the addition of a bromine atom to a sp^2 carbon atom. Both changes increase the hydrophobicity and the size of the molecule, affecting as well its shape and electronic properties.

Ten crude plant extracts were treated with bromine in dichloromethane at room temperature. All extracts were prepared from plant species regarded as weeds. Changes in composition of the mixtures introduced by the reaction were evident from ^{1}H NMR coupled to Principal Component Analysis (PCA) (Supporting Information, Figures S1 and S2).

Differences between the biological properties of brominated and NEs were studied by thin layer chromatography (TLC) bioautography, a technique particularly suited for the analysis of mixtures.[16a] This methodology allows the evaluation of inhibitory properties of a sample spotted onto a TLC plate covered with a

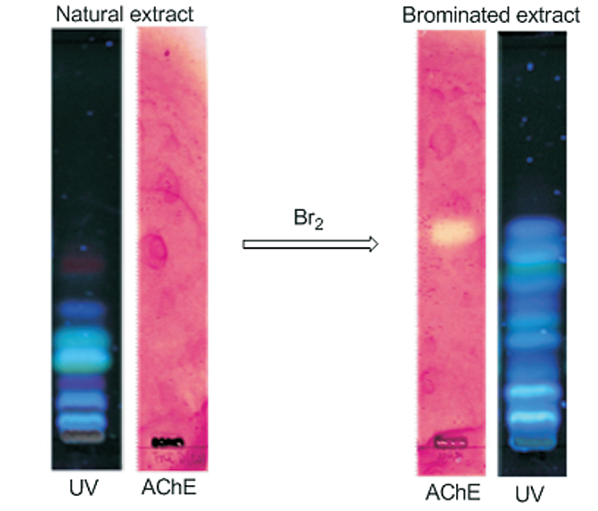


Figure 2. TLC of NE and BE of *C. maculatum* observed under UV light 365 nm and revealed by measuring acetylcholinesterase activity.

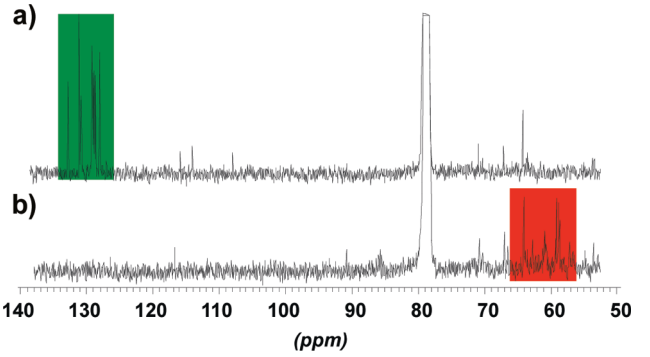


Figure 3. (a) ^{13}C NMR spectra of NE, (b) ^{13}C NMR spectra of BE.

gel that contains enzyme, substrate, and a revealing reagent for the product. Using this methodology we investigated inhibitory activities of the enzymes acetylcholinesterase,[16] xanthine oxidase,[17] and β-glucosidase,[18] all involved in different pathological processes.

A positive result was obtained for the brominated extract (BE) of the weed *Conium maculatum* L. with the enzyme acetylcholinesterase, a therapeutic target to Alzheimer's disease.[19] The acetylcholinesterase TLC bioautography relies on the cleavage by the enzyme of 1-naphthyl acetate to form 1-naphthol, which in turn reacts with Fast Blue B salt to give a purple colored diazonium dye. Regions of the TLC plate which contain acetylcholinesterase inhibitors show up as white spots against the purple background.

When both NE and BE were evaluated by acetylcholinesterase TLC bioautography, a clear inhibition zone was detected only in the modified extract (Figure 2).[20]

Such change in bioactivity was associated to a clear change in composition as observed by TLC (Figure 2) and NMR analysis. Despite their expected complexity, comparison of the ^{13}C NMR spectra of the BE and the NE of *C. maculatum* revealed some interesting differences (Figure 3). The NE spectrum shows a series of signals between 125 and 135 ppm that is absent in the spectrum of the BE. These signals probably belong to carbon from double bonds, targets for the reaction. In addition, a group of signals absent in the NE, appears in the BE in the region between 55 and 65 ppm, where sp^3 carbons bound to bromine should appear.

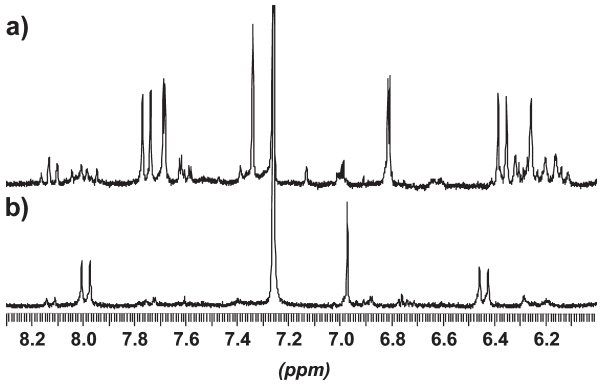
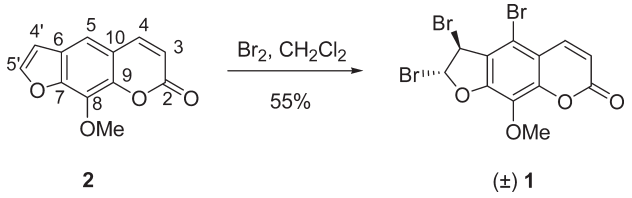


Figure 4. (a) Aromatic region of ^{1}H NMR spectra of NE. (b) Aromatic region of ^{1}H NMR spectra of BE.

Scheme 1. Synthesis of 1 from 8-Methoxypsoralen (2)



Bromination of the aromatic rings of the mixture cannot be clearly followed by ^{13}C NMR since it is expected that the signals from the targets (aromatic rings) and those of the products (partially brominated aromatic rings) will all appear in the same region. However, this process can be observed by comparing the ^{1}H NMR spectra that show how the aromatic proton signals decrease in number and intensity because of the reaction (Figure 4).

The modified *C. maculatum* extract was chromatographed on silica gel to produce only one bioautography active fraction. A second bioautography-guided chromatography column of this fraction led to the isolation of **1** that contains three bromine atoms in its structure indicating that it was produced during the diversification step (Scheme 1). Identity of compound **1** was established by ^{1}H and ^{13}C NMR, and HR-MSMS analyses and confirmed by comparison with literature[21a] and a synthetic sample (Supporting Information, Figures S3 and S4).

The HPLC-UV chromatogram of the NE recorded at 300 nm shows a peak with a retention time of 9.2 min corresponding to compound **2** (Figure 5a). Such a peak disappears after the reaction and consequently is absent in the chromatogram of the BE. Moreover, one peak at 21.4 min is present in the BE chromatogram corresponding to the brominated compound **1** (Figure 5b); this peak is absent in the original extract.

This brominated derivative could have been formed from an inactive natural component of the extract such as 8-methoxypsoralen (**2**, scheme 1) that is present in the NE.[22] To confirm the origin of **1**, the suspected precursor **2** was isolated from the natural *C. maculatum* extract and treated with bromine in the same conditions previously used for the bromination of the NE (Scheme 1). As expected, the desired compound **1** was the main product.[21] The identity of compounds **1** and **2** in the HPLC chromatograms of the BE and NE were confirmed by comparing their retention times with the retention times observed for the synthetic compound **1** and commercial

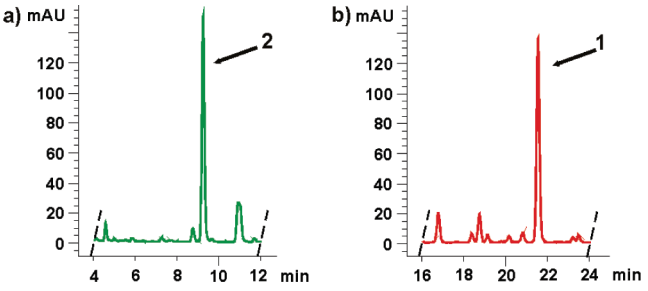


Figure 5. (a) HPLC-UV-300 nm of the NE. (b) HPLC-UV-300 nm of the BE.

compound **2** (Supporting Information, Figure S5). It is worth mentioning that both compounds are minor constituents of the NE and BE respectively (Supporting Information, Figure S6).

Interestingly, the structure of **2** includes the two target functionalities (aromatic rings and double bounds), and both reacted with bromine to produce **1**. According to NMR data compound **1** was obtained as a racemate with an *anti* relative stereochemistry between bromines attached to carbons 4′ and 5′ of the furan ring (^{1}H NMR, neighboring protons *H*-4′ and *H*-5′ are uncoupled).

The inhibitory potency of **1** and **2** was compared to that of known acetylcholinesterase inhibitors using the TLC bioautography and the microplate assay based on Ellman's reagent.[23] Compound **2** was inactive in the range of concentration tested with a pMIQ[24] value below 9.4. Quite the opposite, compound **1** showed a pMIQ of 10.65 which is within the activities observed for some of the best known inhibitors for the enzyme such as physostigmine (pMIQ = 11.81) and galanthamine (pMIQ = 10.57).[25] The pIC_{50} observed for compound **1** in the microplate assay was 6.18, again between the values reported for galanthamine (pIC_{50} = 6.46) and physostigmine (pIC_{50} = 5.93).[25]

In summary, we have described the first example of generation of biological activity in a NE by means of chemical diversification of their components through bromination. The process focuses in the transformation of chemical groups highly common in natural products, such as double bonds and aromatic rings, into chemical groups that are rarely produced by the secondary metabolism of plants.

EXPERIMENTAL PROCEDURES

Plant Material. Plants were collected in Pergamino, Buenos Aires province, Argentina, during November 2005. Voucher specimens were deposited at the Universidad Nacional de Rosario Herbarium. Collected plants were: (1) *C. maculatum* L. (Apiaceae), ID MO007; (2) *Urtica urens* L. (Urticaceae), ID MO010; (3) *Solanum diflorum* Vell. (Solanaceae), ID MO005; (4) *Morrenia brachystephana Griseb.* (Asclepiadaceae), ID MO004; (5) *Matricaria recutita* L. (Asteraceae), ID MO008; (6) *Sonchus oleraceus* L. (Asteraceae), ID MO013; (7) *Rapistrum rugosum* (Brassicaceae), ID MO014; (8) *Solanum sisymbriifolium* Lam. var. *sisymbriifolium* (Solanaceae), ID MO011; (9) *Oenothera affinis* Cambess. (Onagraceae) and ID OM012; (10) *Lamium amplexicaule* L. (Laminaceae), ID MO001.

Crude Extracts. A sample (200 g) of dried and powdered material (entire plant) was refluxed with 3 × 4 L of methanol for 45 min. The extracts were combined after filtration, and the solvent was eliminated under reduced pressure by rotary evaporation.

Representative Experimental Procedure for Preparation of the BEs. To a solution of the dry extract (300 mg) in CH_2Cl_2 (15 mL) at −78 °C, bromine (56.4 μL, 1.1 mmol, 1.1 equiv) was added dropwise, and the reaction was stirred for 2 h at −5 °C. A solution of 5% $Na_2S_2O_3$ (10 mL) was added, and the reaction mixture stirred for 5 min. The organic material was then extracted with CH_2Cl_2 (3 × 10 mL), and the organic phases dried over Na_2SO_4 and evaporated under reduced pressure.

Isolation of Compound 1. The modified *C. maculatum* extract (330 mg) was chromatographed on silica gel (hexane-EtOAc gradient) to give 6 fractions. Fraction 1 (hexane/EtOAc, 92:8) resulted in the only acetylcholinesterase bioautography active fraction. Fraction 1 was repurified by column chromatography (hexane-EtOAc gradient) to obtain 6.8 mg of pure compound **1**. Compound **1** was recrystallized from methanol to give white crystals, mp. 165−166 °C.

Isolation of Compound 2. 47.3 g of crude *C. maculatum* extract were dissolved in a mixture MeOH/H_2O (9:1) and washed with hexane (2 × 200 mL). The MeOH/H_2O phase was evaporated under reduced pressure, and the remaining aqueous phase was extracted with CH_2Cl_2 (3 × 200 mL). Evaporation of the solvent followed by drying with Na_2SO_4 afforded 2.57 g of a dichloromethane fraction of *C. maculatum* extract. A 1.5 g portion of this dichloromethane fraction was chromatographed on silica gel (hexane-EtOAc gradient) to give 75 mg of impure compound **2** that was then repurified by column chromatography (hexane/EtOAc, 80:20) obtaining 22.9 mg of pure 8-methoxypsoralen (**2**). The NMR spectra was coincident with previously reported.[26]

Preparation of 1 from 2. To a solution of isolated 8-methoxypsoralen (**2**) (16.9 mg, 0.078 mmol) in CH_2Cl_2 (2 mL) at −78 °C, bromine (4.4 μL, 0.086 mmol, 1.1 equiv) was added dropwise, and the reaction was stirred for 2 h at −5 °C. A 5% solution of $Na_2S_2O_3$ (2 mL) was added, and the reaction mixture stirred for 5 min. The organic material was then extracted with CH_2Cl_2 (3 × 5 mL), and the organic phases dried over Na_2SO_4. Evaporation of the solvent under reduced pressure afforded the crude product that was then purified by column chromatography (hexane/EtOAc, 92:8) to give 19.3 mg of brominated compound **1** (55% yield). Compound **1** was recrystallized from methanol to give white crystals, mp. 165−166 °C.

(±)4′,5′-Dihydro-5,4′,5′-tribromo-8-methoxy-psoralen (1). IR film (cm^{-1}): 2920, 1744, 1586, 1461, 1085. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) = 7.97 (d, *J* = 9.8 Hz, 1H, *H*-4), 6.97 (s, 1H, *H*-5′), 6.43 (d, *J* = 9.8 Hz, 1H, *H*-3), 5.67 (s, 1H, *H*-4′), 4.11 (s, 3H, OCH_3). ^{13}CNMR (75 MHz, $CDCl_3$) δ (ppm) = 158.91 (*C*O), 149.53 (C, *C*-9), 149.07 (C, *C*-7), 141.76 (CH, *C*-4), 133.46 (C, *C*-8), 125.61 (C, *C*-6), 116.06 (C, *C*-10), 115.75 (CH, *C*-3), 111.91 (C, *C*-5), 89.35 (CH, *C*-5′), 61.53 (OCH_3), 52.75 (CH, *C*-4′). HRMS *m*/*z* 474.77904 [(M+Na); calcd: 474.77867, error 0.8 ppm].

■ ASSOCIATED CONTENT

Supporting Information. Figures S1−S6, materials, analysis of virtual extracts, ^{1}H NMR and PCA analysis of NEs and BEs, NMR and MS of compound **1**, chromatograms of compounds **1** and **2**, and methods for bioactivity determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources

This work was supported by CONICET and ANPCyT from Argentina. L.M., M.O.S., and I.A.R. thank CONICET for their fellowships.

■ ACKNOWLEDGMENT

We thank Oscar Micheloni from the Universidad Nacional del Noroeste de la Provincia de Buenos Aires for his help with Botanical identification of herbal material. We thank Dr. Andrea Escalante for her valuable help with MS analysis. R.L.E.F. is a CONICET Researcher.

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(24) pMIQ is the negative logarithm of the minimal inhibitory quantity in a mole that produced the spot with the least observable whiteness.

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