Letter

A new fluorinated tyrosinase inhibitor from a chemically engineered essential oil

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A new fluorinated tyrosinase inhibitor from a chemically engineered essential oil

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KEYWORDS: diversification, essential oil, fluorination, tyrosinase, bioactive compound

Abstract. The generation of fluorinated essential oils as a source of bioactive compounds is described. Most of the components of the natural mixtures were altered leading to the discovery of a new fluorinated tyrosinase inhibitor.

Natural products are biologically validated starting points for the development of new drugs. They are the outcome of a long evolution process that has resulted in a unique assortment of skeletons with high affinity for biomolecules.[1]

Essential oils (EOs) are natural multicomponent systems[2] composed of low-molecular weight lipophilic compounds derived from different biosynthetic pathways.[3] In general their production by plants is diversity oriented, with the generation of complex mixtures of compounds that have the potential to regulate plant–insect and plant–mammal interactions. This bioactive volatilome is now emerging as a novel potential source of interesting lead structures for drug discovery.[3]

Several approaches have been proposed to increase the diversity of natural product mixtures such us combinatorial biosynthesis[4] and related techniques.[5] Chemically engineered extracts (CEEs) represent alternative sources of molecules for the search of new bioactive compounds based on natural skeletons.[6-9] In this strategy, natural mixtures are chemically altered through reactions directed towards the incorporation of molecular fragments or elements that are relevant for bioactivity and rarely found in secondary metabolites.[10]

Fluorine is one such element, the incorporation of which into a molecule can modulate physicochemical properties such as pKa, lipophilicity, hydrogen bonding and electrostatic interactions, as well as metabolic stability (oxidative metabolism, hydrolytic metabolism, *in vivo* racemization).[11] The strategic use of fluorine substitution in drug design has led to the production of some of the key drugs available on the market.[12, 13] The average proportion of fluorine in drugs is significantly higher than in natural products.[14] Natural organofluorines represent less than 1% of the naturally occurring organohalogens.[15]

Considering that (a) small molecule natural products have had a significant impact on drug discovery, (b) 20-25% of drugs in the pharmaceutical pipeline contain at least one fluorine atom,[11] and (c) organofluorine compounds are virtually absent as natural products,[16] it becomes interesting to evaluate the effect of fluorination on the biological properties of natural mixtures of small molecules such as EOs (Figure 1).

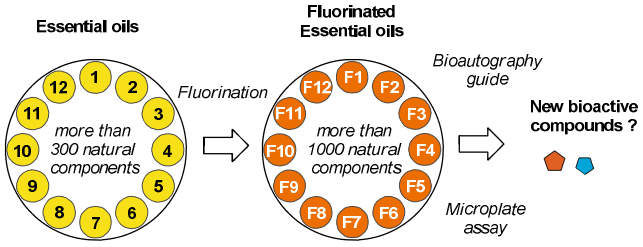


Figure 1. Diversification of essential oil mixtures by fluorination to generate libraries of biologically active compounds.

For EOs diversification we selected Selectfluor as a highly reactive fluorinating reagent that is safe, nontoxic, stable, and easy to handle.[17-20] Selectfluor can introduce fluorine atoms into molecules by reaction with double bonds, aromatic rings, and through the transformation of carbon–hydrogen bonds to carbon–fluorine bonds at saturated secondary and tertiary carbon sites.[21] According to the Dictionary of Natural Products,[22] 78% of the essential oil constituents include at least one non-aromatic carbon–carbon double bond in their structures, 22% contain at least one aromatic group, and 84% contain the types of C-H bonds that may be reactive. In addition, Selecfluor has been applied to the fluorination of other functional groups[23] that are present in essential oil components such as enols (6.8%) and alkynes (2.5%). The fluorination of essential oils has not been reported to date.

The success of the CEEs approach lies in the power of numbers: in order to increase the chances of generating a bioactive compound, it is important to produce many compounds that incorporate the desired chemical feature or element. A series of 12 essential oils was fluorinated by reaction with Selectfluor in refluxing acetonitrile, and changes in chemical composition and bioactivity were evaluated. Incorporation of fluorine into the EOs constituents was confirmed by ^{19}F NMR analysis, with new peaks appearing between -97 ppm and -197 ppm as expected for aromatic fluorine, α-fluoroketones and α-fluoroenones.

The impact of the reaction over the chemical composition of the mixtures was evaluated by GC-MS, showing that most of the EO components were transformed by the reaction, expanding the chemical diversity of the mixtures. At least 60 % of the peaks observed in the chromatograms of EOs disappeared after the reaction, and at least 88% of the peaks present in the gas chromatograms of the resulting fluorinated essential oils (FEOs) were absent in the chromatogram of the precursor EO (Figure 2a). The average number of major compounds detected in the mixtures increased from 37 to 155 due to the fluorination reaction (Figure 2b). This suggests that, on average, four products were generated from each natural precursor.

Changes in the composition of the mixtures were also evident from GC-MS coupled to Principal Component Analysis (PCA). The score plot showed discrimination between two groups by PC1 and PC2: one corresponding to the FEOs (Figure 2, red triangles) and the other corresponding to the EOs (Figure 2c, blue circles). Similarly, PCA of ^{1}H NMR spectra of the 24 mixtures showed discrimination between two groups by PC1, PC2 and PC3 (Fig. S1 in Supporting Information).

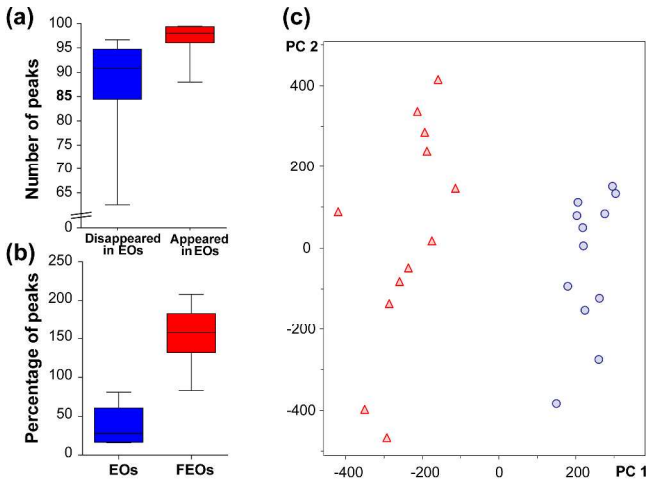


Figure 2. Box and whiskers plot for a) percentage of peaks that disappeared from the chromatograms because of the reaction (blue) and that appeared in the chromatograms after the reaction (red), b) number of peaks from EOs (blue) and FEOs (red) detected in the GC-MS chromatograms. c) Score plot of PCA of GC-MS data: EOs (blue circles) and FEOs (red triangles).

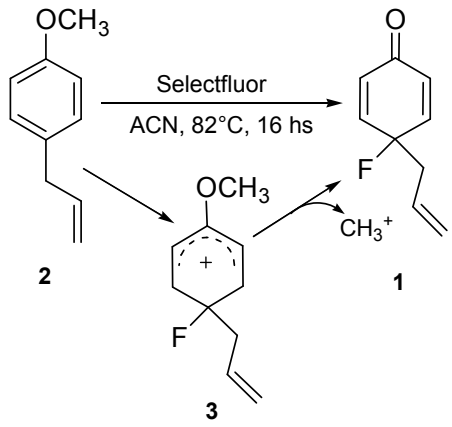
The effect of the reaction on the biomolecular properties of the mixtures was evaluated by comparing the tyrosinase inhibitory properties of the fluorinated and the non-fluorinated mixtures. The discovery of tyrosinase inhibitors is attractive due to their potential applications in cosmetic, medicinal and agricultural industries. This enzyme catalyses the production of melanin and other pigments by oxidation of L-tyrosine.[24] Various dermatological disorders such as melasma, age spots and sites of actinic damage, arise from an excessive level of epidermal pigmentation.[25] Additionally, the browning observed in vegetables and fruits after harvest is associated with tyrosinase activity, which produces a less attractive appearance and loss in nutritional quality.[26]

The tyrosinase inhibition properties of the mixtures were surveyed by TLC bioautography,[27] a technique particularly well suited for the analysis

of mixtures.[28] This methodology allows the evaluation of inhibitory properties of a sample developed onto a TLC plate that is covered with a gel that contains enzyme and substrate. When applied to tyrosinase, fluorination was observed to enhance the inhibitory properties of two mixtures: the FEOs from *Ocimum basilicum* L., Lamiaceae (FOB) and *Artemisia dracunculus* L., Asteraceae (FAD) showed intense inhibition spots that were absent in the non-fluorinated EOs. A follow-up microplate assay[29] showed that the IC_{50} values for *O. basilicum* oil decreased from 278.4 ± 1.38 μg/mL to 174.4 ± 1.45 μg/mL after fluorination. Similar results were obtained for *A. dracunculus*, in which IC_{50} decreased from 232.2 ± 1.19 μg/mL to 125.5 ± 1.56 μg/mL.

The main bioactive compound in both mixtures was identified as 4-allyl-4-fluorocyclohexa-2,5-dienone (**1**, Scheme 1). The identity of this compound was established by NMR (^{1}H, ^{19}F and ^{13}C NMR), IR, and HRMS analyses. This fluorinated derivative could have been formed from the inactive natural component methyl chavicol (**2**, Scheme 1) that is present in both *O. basilicum* and of *A. dracunculus* EOs.[30] This was confirmed by fluorination of pure **2** using the same reaction protocol previously employed for the EOs. The TLC-bioactivity profile of the reaction showed the generation of the same bioactivity spot that was previously detected in *O. basilicum* and of *A. dracunculus* FEOs.

It is interesting to note that compound **1** was a minor constituent of both bioactive fluorinated mixtures. Although comprising only 0.6% of the total peak area of the chromatogram of *O. basilicum* FEO, and 0.5% total peak area of the chromatogram of *A. dracunculus* FEO (Fig. S2 in Supporting Information), this active component was easily identified in the bioautography assay. Compound **1** could result from an addition-elimination process initiated with the generation of a *para*-fluoro cation (**3**, Scheme 1) from **2.** Release an alkyl cation from the phenolic oxygen would thus result in the formation of the observed 4-allyl-4-fluorocyclohexa-2,5-dienone (Scheme 1).[31]



Scheme 1. Proposed synthesis of 4-allyl-4-fluorocyclohexa-2,5-dienone (**1**) from methyl chavicol (**2**) with Selectfluor through *para*-fluoro cation (**3**).

The inhibitory potency of the fluorinated compound **1** (IC_{50} 59.14 ± 1.15 μM) was found to be similar to that of the known tyrosinase inhibitor kojic acid (IC_{50} 42.16 ± 1.04 μM). Under these experimental conditions the natural precursor of **1**, methyl chavicol (**2**) was inactive (IC_{50} >1000 μM).

In summary, the generation of chemically engineered extracts through fluorination is described for the first time. Chemical diversification of a series of EOs led to the transformation of most of the components of the starting mixtures, producing fluorinated mixtures of expanded diversity (four-fold increase in the number of compounds). Fluorination increased tyrosinase inhibition in two mixtures. The use of a straightforward bioautographic assay enabled the identification of a minor fluorinated compound with similar inhibitory properties to kojic acid, generated in the mixture from a natural inactive precursor.

ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures and spectroscopic data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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References

(1) Ertl, P.; Roggo, S.; Schuffenhauer, A. Natural product-likeness score and its application for prioritization of compound libraries. *J. Chem. Inf. Model.*, **2008**, *48*, 68-74.

(2) Edris, A. E. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytother. Res.*, **2007**, *21*, 308-323.

(3) Maffei, M. E.; Gertsch, J.; Appendino, G. Plant volatiles: Production, function and pharmacology. *Nat. Prod. Rep.*, **2011**, *28*, 1359-1380.

(4) Horinouchi, S. Combinatorial biosynthesis of plant medicinal polyketides by microorganisms. *Curr. Opin. Chem. Biol.*, **2009**, *13*, 197-204.

(5) Zhang, C.; Weller, R. L.; Thorson, J. S.; Rajski, S. R. Natural product diversification using a non-natural cofactor analogue of S-adenosyl-L-methionine. *J. Am. Chem. Soc.*, **2006**, *128*, 2760-2761.

(6) López, S. N.; Ramallo, I. A.; Gonzalez Sierra, M.; Zacchino, S. A.; Furlan, R. L. E. Chemically engineered extracts as an alternative source of bioactive natural product-like compounds. *Proc. Natl. Acad. Sci. U.S.A.*, **2007**, *104*, 441-444.

(7) Kawamura, T.; Matsubara, K.; Otaka, H.; Tashiro, E.; Shindo, K.; Yanagita, R. C.; Irie, K.; Imoto, M. Generation of 'Unnatural Natural Product' library and identification of a small molecule inhibitor of XIAP. *Bioorg. Med. Chem.*, **2011**, *19*, 4377-4385.

(8) Kikuchi, H.; Sakurai K.; Oshima, Y. Development of diversity-enhanced extracts of *Curcuma zedoaria* and their new sesquiterpene-like compounds. *Org. Lett.*, **2014**, *16*, 1916-1919.

(9) Lin, Z.; Ma, X.; Wei, H.; Li, D.; Gu, Q.; Zhu, T. Spicarins A–D from acetylated extract of fungus Spicaria elegans KLA03. *RSC Adv.*, **2015**, 5, 35262-35266.

(10) Ramallo, I. A.; Salazar, M. O.; Mendez L.; Furlan, R. L. E. Chemically engineered extracts: source of bioactive compounds. *Acc. Chem. Res.*, **2011**, *44*, 241-250.

(11) Purser, S.; Moore, P. R.; Swallow, S.; Gouverneur, V. Fluorine in medicinal chemistry *Chem. Soc. Rev.*, **2008**, *37*, 320-330.

(12) Swallow, S. Fluorine in medicinal chemistry. *Prog. Med. Chem.*, **2015**, *54*, 65-133.

(13) Wang, J.; Sánchez-Roselló, M.; Aceña, J. L.; del Pozo, C.; Sorochinsky, A. E.; Fustero, S.; Soloshonok, V.; Liu, H. Fluorine in Pharmaceutical Industry: Fluorine-containing drugs introduced to the market in the

last decade (2001–2011). *Chem. Rev.,* **2014**, *114*, 2432–2506.

(14) Harsanyi A.; Sandford, G. Organofluorine chemistry: applications, sources and sustainability. *Green Chem.*, **2015**, *17*, 2081-2086.

(15) Gribbles, G. Organofluorines The Handbook of Environmental Chemistry. Naturally Occurring Organofluorines, Springer-Verlag Berlin Heidelberg **2002**; Chapter 5.

(16) Deng, H.; O'Hagan, D.; Schaffrath, C. Fluorometabolite biosynthesis and the fluorinase from *Streptomyces cattleya*. *Nat. Prod. Rep.*, **2004**, *21*, 773-784.

(17) Nyffeler, P. T.; Durón, S. G.; Burkart, M. D.; Vincent, S. P.; Wong, C.-H. Selectfluor: mechanistic insight and applications. *Angew. Chem. Int. Edit.*, **2004**, *44*, 192-212.

(18) Wang, W.; Jasinski, J.; Hammond, G. B.; Xu, B. Fluorine-Enabled Cationic Gold Catalysis: Functionalized Hydration of Alkynes. *Angew. Chem. Int. Edit.*, **2010**, *49*, 7247-7252.

(19) Jin, Z.; Hammond, G. B.; Xu, B. Transition-Metal-Mediated Fluorination, Difluoromethylation, and Trifluoromethylation. *J. Org. Chem.*, **2012**, *77*, 7725–7729.

(20) Jin, Z.; Hidinger, R. S.; Xu, B.; Hammond, G. B. Stereoselective synthesis of monofluoroalkyl α,β-unsaturated ketones from allenyl carbinol esters mediated by gold and selectfluor. *J. Org. Aldrichimica Acta*, **2012**, *45*, 67–83.

(21) Chambers, R. D.; Parsons, M.; Sandford, G.; Bowden, R. Electrophilic fluorination at saturated sites. *Chem. Commun.*, **2000**, 959-960.

(22) Buckingham, J. *Dictionary of Natural Products* on CD-ROM (Chapman & Hall), London, **2001**.

(23) Singh, R. P.; Shreeve, J. M. Recent highlights in electrophilic fluorination with 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate). *Acc. Chem. Res.*, **2004**, *37*, 31-44.

(24) Sánchez-Ferrer, A.; Rodriguez-Lopez, J. N.; García-Cánovas, F; García-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta*, **1995**, *1247*, 1-11.

(25) Kim Y. J.; Uyama, H. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell. Mol. Life Sci.*, **2005**, *62*, 1707-1723.

(26) Chang, T.-S. An updated review of tyrosinase inhibitors. *Int. J. Mol. Sci.*, **2009**, *10*, 2440-2475.

(27) García P.; Furlan, R. L. E. Multiresponse optimisation applied to the development of a TLC autography for the detection of tyrosinase inhibitors. *Phytochem. Anal.*, **2015**, *26*, 287-292.

(28) Cieśla, Ł. M.; Waksmundzka-Hajnos, M.; Wojtunik, K. A. Hajnos, M. Thin-layer chromatography coupled with biological detection to screen natural mixtures for potential drug leads. *Phytochem. Lett.*, **2015**, *11*, 445-454.

(29) Atta-ur-Rahman; Choudhary, M. I.; Thomsen, W. J. *Bioassay Techniques for Drug Development*, Taylor & Francis, Amsterdam, **2001**.

(30) Teixeira, B.; Marques, A.; Ramos, C.; Neng, N. R.; Nogueira, J. M. F.; Saraiva, J. A.; Nunes, M. L. Chemical composition and antibacterial and antioxidant properties of commercial essential oils. *Ind. Crop. Prod.*, **2013**, *43*, 587-595.

(31) Pravst I.; Stavber, S. Fluorination of 4-alkyl-substituted phenols and aromatic ethers with fluoroxy and N-F reagents: Cesium fluoroxysulfate and N-fluoro-1,4-diazonia-bicyclo[2.2.2]octane dication salts case *J. Fluor. Chem.*, **2013**, *156*, 276-282.

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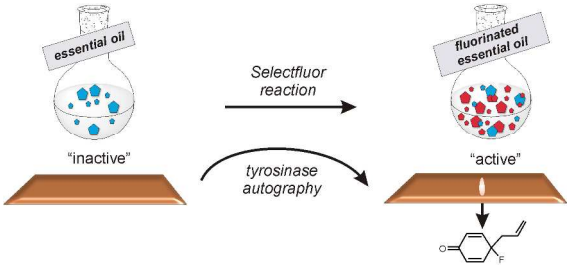

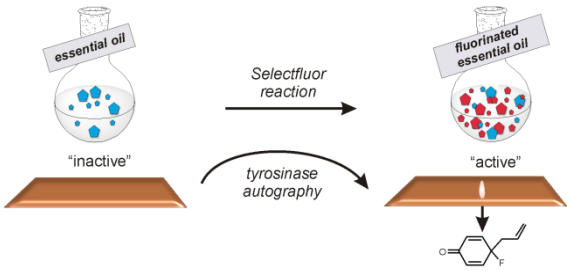
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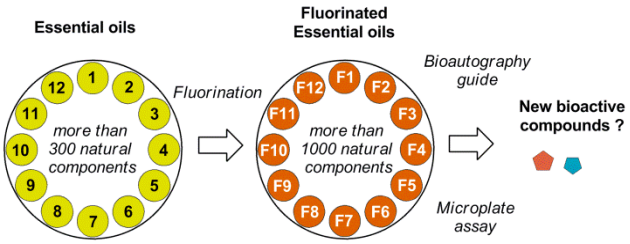
Essential oils
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Fluorination
Bioautography guide
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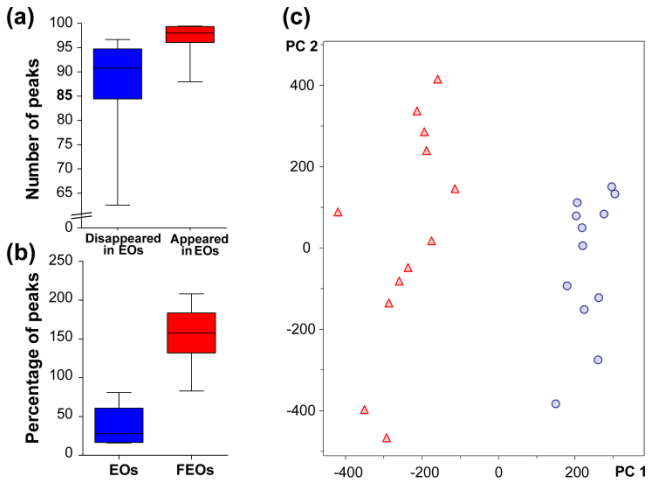
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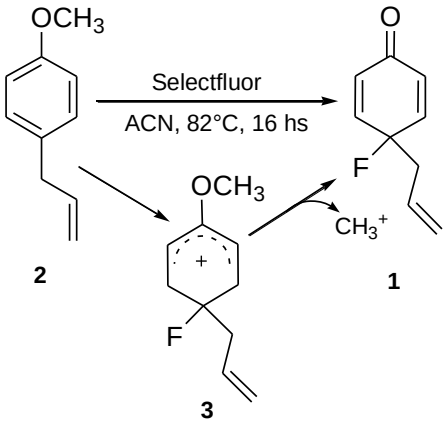
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