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Isolation of a novel arachidonic acid metabolite 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE) from the yeast *Dipodascopsis uninucleata* UOFs-Y128

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When arachidonic acid (AA) is added to the yeast *Dipodascopsis uninucleata* UOFS Y128, one of the major metabolites isolated and purified with the help of thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) is 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE). The structure of this new AA metabolite was elucidated mainly by electron impact (EI) mass spectrometry (MS). Strikingly, the formation of this new metabolite was found to be inhibited by aspirin.

Dipodascopsis uninucleata; 3-HETE; TLC; HPLC; EI-MS; Aspirin-sensitive

1. INTRODUCTION

The transformation of arachidonic acid (AA) occurs enzymatically into a variety of oxygenated species primarily via cyclooxygenase and lipoxygenase enzyme systems [1]. Besides mammalian cells, also several fungi, such as *Rhizopus arrhizus, Aspergillus ochraceus* and *Curvularia lunata* are capable of synthesizing eicosanoids [2]. A previous report also showed that the yeast *Dipodascopsis uninucleata* caused a chemical change in the synthetic eicosanoid analogues which were added exogenously [3]. In our laboratory, we have investigated approx. 1000 strains representing almost 200 yeast species for fatty acid composition [4]. Many of them have been found to produce the unsaturated fatty acid precursors, which are prerequisite for the synthesis of eicosanoids [5].

In the present paper, we report the isolation and structure of a stable novel AA metabolite from the yeast *Dipodascopsis uninucleata* fed with exogenous AA. This metabolite demonstrates distinctly different chromatographic properties to those of usual cyclooxygenase products and its formation can be inhibited by aspirin. We propose its structure as 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE).

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2. MATERIALS AND METHODS

Eicosanoids were purchased from Cayman Chemicals (Ann Arbor, USA). Labeled compounds were supplied by Amersham International (UK). The trimethylsilylation kit was obtained from Pierce Chemicals (USA).

2.1. *Cultivation of yeast* Dipodascopsis uninucleata *labelled with AA*

Dipodascopsis uninucleata was grown to stationary phase and harvested as described before [4]. Labeled AA, 10 μCi (sp. act. 210 mCi/mmol) and unlabeled AA (10 mg) were added in the stationary phase of the growth. After 36 h the suspension was centrifuged.

2.2. *Extraction of AA metabolites from the yeast*

The pellet obtained above was mixed with absolute ethanol to a final concentration of 80% ethanol. The suspension was kept at 5°C for 18 h and then filtered. The filtrate was adjusted to 15% aqueous ethanol. The sample was acidified to pH 3.0 with formic acid and chromatographed on a preconditioned Sep-Pak C_{18} cartridge (Millipore, USA) as described [6]. The eicosanoids were finally eluted with 5 ml of freshly distilled ethyl acetate. The eluate was evaporated under a stream of nitrogen and the residue was taken in 500 μl chloroform for TLC-Autoradiography.

2.3. *TLC*

Fifty μl aliquots of the chloroform fraction were chromatographed on silica gel thin layer plates (Merck, Germany) as described [7]. Autoradiography was performed as described by Hurst et al. [7]. The migration distances of known eicosanoids were compared with radioactive spots on the autoradiograms. The band at R_f 0.13 was denoted as compound *X*. It was scraped off and extracted with chloroform/methanol (9:1, v/v) to yield almost 80% pure compound *X*.

For quantitative isolation of compound *X* from TLC plates and HPLC the arachidonic acid metabolites obtained from the Sep-Pak C_{18} cartridge were separated from the other hydrophobic compounds by applying their triethylamine salts onto Sep-Pak silica gel cartridges and eluting them with 15% ethanol. Following evaporation of ethanol

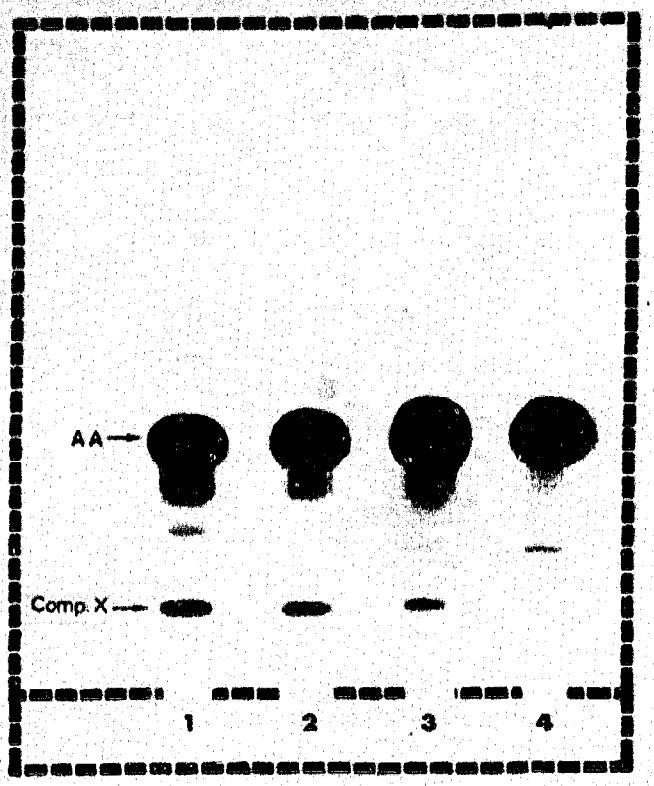


Fig. 1. TLC autoradiography of a yeast extract in which yeast cultures were fed with labeled and unlabeled AA (for details see section 2). To samples in lanes 1–4 aspirin was added to a final concentration of 0 mM, 0.01, 0.1 and 1 mM, respectively.

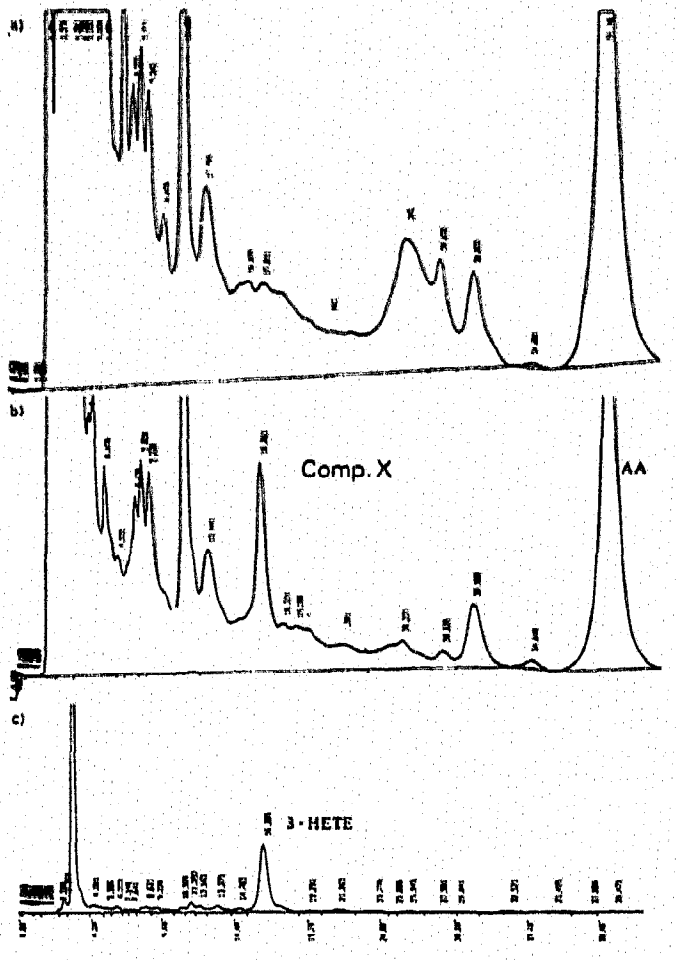


Fig. 2. HPLC chromatograms of yeast extract obtained from cultures fed with unlabeled AA in presence (a) and absence (b) of 1 mM aspirin. Chromatogram (c) is obtained from the purified 3-HETE.

under nitrogen, the sample was adjusted to pH 3.0 and extracted with ethyl acetate.

2.4. *HPLC*

HPLC of the compound *X* was carried out on a SPHERISORB C_{18} reverse-phase column (25 cm × 0.46 cm, 5 μm, Macherey-Nagel, Germany) using a mixture of 0.017 M H_3PO_4/CH_3CN (40:60, v/v) as solvent phase at a flow rate of 1 ml/min. The UV detection was performed at 193 nm.

2.5. *UV and FT-IR Spectra*

Whereas the UV spectrum of the compound isolated from TLC was recorded on-line during HPLC, the FT-IR spectrum was recorded by absorbing 100 μg of it on 5 mg of KBr in a floating cell.

2.6. *EI mass spectra*

EI mass spectra of the compound *X* were recorded on a Finnigan MAT 90 double focussing magnetic instrument at 70 eV with a probe temperature of 150°C.

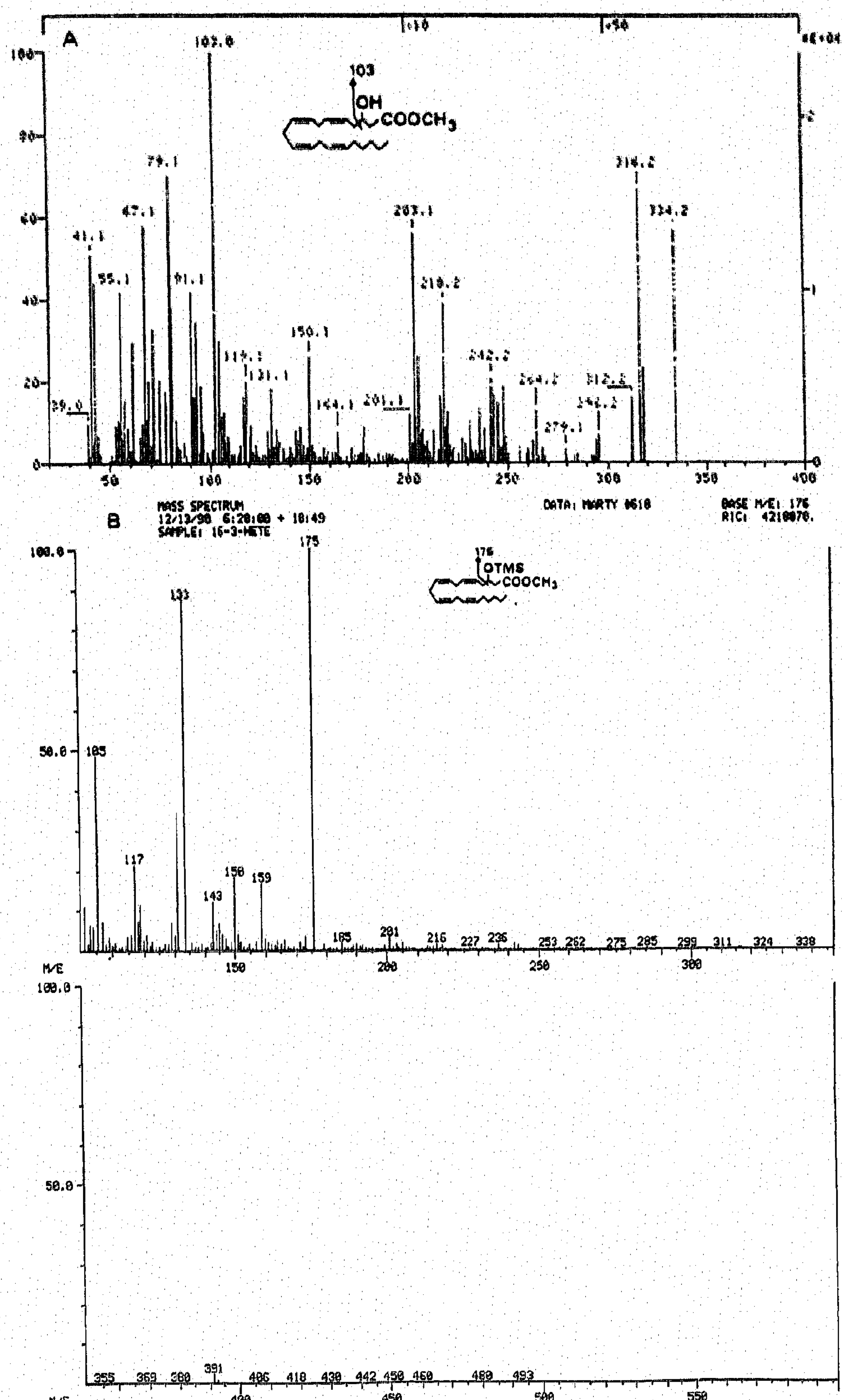
Prior to analysis, the methyl (Me)- and methyl-trimethyl-silyl (Me-TMS) derivatives of the samples were prepared as described [8], and reconstituted in 100 μl chloroform/hexane (1:4) before injection.

3. RESULTS AND DISCUSSION

When tritium-labeled AA along with the cold AA were fed to the yeast *Dipodascopsis uninucleata* UOFS Y128 autoradiography of ethanolic extracts on Silica Gel TLC revealed the formation of several AA metabolites (Fig. 1). The compound *X* with R_f 0.13 aroused our interest because its formation was inhibited by aspirin, thus suggesting the presence of a cyclooxygenase product. The concentration of the compound *X* could be increased by increasing the amount of the exogenous AA (data not shown). The UV spectrum of the compound *X* showed a maximum at 193 nm, which has also been found for prostaglandins [7] and excludes the possibility of any conjugated double bonds or enone-type systems. Also, the reverse-phase HPLC of purified extracts from the yeast showed the compound *X* as a major component (Figs. 2a–c), when the detection was monitored at 193 nm.

For further elucidation of the structure of this compound *X* we performed FT-IR spectroscopy (spectrum not shown). The FT-IR spectroscopy showed a C=O

Fig. 3. EI mass spectra of the methylated (A) and methyl-trimethylsilylated (B) compound *X*.


A
103
OH
COOCH3
103.0
79.1
67.1
41.1
55.1
91.1
203.1
218.2
150.1
119.1
131.1
164.1
201.1
242.2
264.2
279.1
312.2
296.2
316.2
334.2
39.0
B
MASS SPECTRUM
SAMPLE: 16-3-HETE
DATA: MARTY 0618
BASE M/E: 175
175
OTMS
COOCH3
133
105
117
150
159
143
M/E
100.0
50.0
355 369 380 391 406 418 430 442 450 460 480 493

absorption at 1710 cm^{-1} and a strong and broad absorption between 2800 and 3760 cm^{-1}, suggesting the presence of a free acid and a hydroxyl group in the compound *X*.

To determine the chemical structure, EI mass spectra of the Me- and Me-TMSi-derivative of the compound *X* were obtained (Figs. 3A, B). The spectrum of the methylated compound (3A) showed an M^+ of 334 mass units while the methylated, hydrogenated derivative gave an M^+ of 342 mass units (spectrum not shown), thus confirming the presence of 4 double bonds. The peak at mass 103 is due to the fragment [$CH_3O(CO)CH_2CHOH$] and is characteristic of 3-hydroxy fatty acids [9]. The spectrum of the Me-TMSi derivative of the compound *X* showed a base peak of *m/z* 175 [$CH_3O(CO)\cdot CH_2\cdot CHO\cdot TMSi$] and other major fragments of 391 [M^+-15 (CH_3)] and 316 [M^+-90 (TMSiOH)]. The mass unit 175 is characteristic for the hydroxylation of the fatty acid at position C-3.

The EI-MS results were also confirmed by performing ^{1}H-NMR spectroscopy (300 MHz, $CDCl_3$) of the compound *X* and AA (spectra not shown). In the spectrum of AA the protons on C-2 and C-3 showed a triplet at δ2.45 and a quintet at δ1.70. These signals were absent in the spectrum of compound *X*. Furthermore, comparison of both NMR-spectra demonstrated that the position of olefinic protons in both compounds was identical, suggesting similar configuration for double bonds. Additional support for our assumption came from the IR-spectrum (not shown) of compound *X*, in which characteristic absorption for *trans*-double bonds between 960 cm^{-1} and 970 cm^{-1} was absent, but for *cis*-double bonds between 675 cm^{-1} and 730 cm^{-1} was present. Thus, the compound *X* could be described as 3-hydroxy-5(*cis*),8(*cis*),11(*cis*),14(*cis*)-eicosatetraenoic acid (3-HETE).

On the basis of these findings, it is clear that 3-HETE cannot be classified as a normal prostaglandin type due to the definite presence of a hydroxyl group at the β position (C-3), although its formation can be inhibited by aspirin. In view of the product found, it is unlikely that the cyclooxygenase enzyme is involved in the transformation of AA into 3-HETE. In addition, the EI-MS experiments give clear-cut evidence that a cyclopentanone ring, which is characteristic for prostaglandins, is not present in the compound *X*. The biological importance of 3-HETE in plants and humans is at present under investigation in our laboratory.

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