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Fatty Acid Methyl Ester from *Neurospora Intermedia* N-1 Isolated from Indonesian Red Peanut Cake (Oncom Merah)

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Abstract: The objective of this study was to identify the Fatty Acid Methyl Ester (FAME) from *Neurospora intermedia* N-1 that isolated from Indonesian red peanut cake (oncom). FAME profiles have been used as biochemical characters to study many different groups of organisms, such as bacteria and yeasts. FAME from *N.intermedia* N-1 was obtained by some stages of extraction the orange spores and fractination using a chromatotron. The pure compound (1) was characterized by 500 mHz NMR (^{1}H and ^{13}C), FTIR and LC-MS. Summarized data's of ^{1}H and ^{13}C NMR spectra of compound 1 contained 19 Carbon, 34 Hydrogen and 2 Oxygen ($C_{19}H_{34}O_2$). The position of the double bonds at carbon number 8 and 12 were indicated in the HMBC spectrum (2D-NMR). LC-MS spectrum indicates molecular weight of the compound 1 as 294 which is visible by the presence of protonated molecular ion [M+H] at m/z 295. Methyl esters of long chain fatty acids was presented by a 3 band pattern of IR spectrum with bands near 1249, 1199 and 1172 cm^{-1}. We suggested that the structure of the pure compound 1 is methyl octadeca-8,12-dienoate. The presence methyl octadeca-8,12-dienoate in *N. intermedia* is the first report.

Key words: Orange spores, chromatotron, NMR, FTIR, LC-MS

INTRODUCTION

FAME profiles have been used as biochemical characters to study many different groups of organisms, such as bacteria and yeasts. While fatty acids of many filamentous fungi have been characterized, few studies have addressed taxonomic issues. Those addressing taxonomy approach as opposed to using more rigorous cladistic methods. The morphological data on this study suggested that during spore development, inner walls are laid down sequentially (Bentivenga and Morton, 1996). Mummey *et al*. (2002) applied FAME biomarkers to monitor the recovery of ecosystems following surface mine reclamation. In this study, it was found that the ratio of FAME bacterial to fungal biomarkers reflected changes in other indicators of soil health suggesting that this ratio is a useful indicator of reclamation progress. The use of fatty acid methyl esters as biomarkers to determine aerobic, facultatively aerobic and anaerobic communities in wastewater treatment systems. FAME analysis has also been used to evaluate changes in microorganisms due to their exposure to toxic substances. It has been proposed that specific groups of microorganisms contain characteristic fatty acid profiles that can be used as biomarkers (Quezada *et al*., 2007). Fresh soils were analyzed for their fatty acid methyl ester (FAME) profiles by an Ester-Linked (EL) method and the method of MIDI. With this method, microbial cells are saponified by heat and the addition of a strong base. Once fatty acids are cleaved from lipids, they are methylated to form FAMEs (Schutter and Dick, 2000).

In Phycomyces and Basidiomycetes, the C16 and C18 fatty acids predominate. Approximately 70 to 80% of the total fatty acids in membrane lipids of the yeast *S. cerevisiae* consist of the unsaturated fatty acids palmitoleic acid (16 : 1) and oleic acid (18 : 1). Most other fungi contain the di- and trienoic acids linoleic acid (18: 2) and α-linoleic acid (18: 3) (Moat *et al*., 2002). *Neurospora crassa* synthesizes virtually all of its fatty acid by a multifunctional cytosolic fatty acid synthase. The active enzyme consists of two subunits, each subunit of the synthase carries out a subset of the complete set of enzymatic reactions, leading to the biosynthesis of the major product of the synthase, palmitate (16:0). Other fatty acids are derived from 16:0 after its release from the fatty acid synthase, via elongation and desaturation reactions. Trace amounts of short-chain (<16:0) fatty acids are synthesized in mitochondria utilizing a membrane-localized acyl carrier protein and associated proteins (Goodrich-Tanrikulul *et al*., 1999). By gas

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chromatography, myristic acid was identified as the major fatty acid after hydrolysis of isolated compounds 9 and 11. This fatty acid is efficiently synthesized in *N. crassa*. Minor fatty acids bound to the C35 carotenoid alcohols are 16:0, 18:0, 18:1 and 18:3 (Sandman *et al*., 2008). The isolation of an unknown lipid component (e.g., from a sebum sample) is indispensable for its structural elucidation. The latter is usually performed by a combination of mass spectrometry and high resolution NMR spectroscopy. Using these methods detailed information concerning the position and geometry of double bonds as well as ramification points can be obtained. Each chemical and physico-chemical information is highly valuable in order to confine the number of conceivable structures prior to a mass- or NMR spectrometric analysis (Bodoprost and Rosemeyer, 2007).

The fermented food, oncom found exclusively in West Java, Indonesia is important because it is a common and very low cost protein food derived from fermentation of legume waste residues by *Neurospora* sp., a fungus well-known in biochemical genetics. In oncom production, the producers deliberately inoculate each batch of cakes with conidia from the previous culture. Both mating types and a variety of genotypes affecting visible traits have been recovered from the same oncom cake (Perkins and Turner, 1988). On this study, FAME from *Neurospora intermedia* N-1 which isolated from Indonesian red peanut cake (oncom) was obtained by some stages of extraction the orange spores and fractination using chromatotron. The pure compound was characterized by NMR (^{1}H and ^{13}C), FTIR and LC-MS.

MATERIALS AND METHODS

This study was conducted in laboratory of Natural Products and Pharmaceutical, Research Center for Chemistry, Indonesian Institute of Sciences and School of Pharmacy, Bandung Institute of Technology on 2009.

Materials: A wild type strain of *Neurospora intermedia* N-1 was isolated from red oncom samples and is collected by Research Center for Chemistry-Indonesian Institute of Sciences, was used in the present study. It was maintained in Potato Dextrose Agar (PDA) slants. Dried solid waste of tofu processing was used for *N. intermedia* N-1 fermentation.

Solid substrates fermentation of *N. intermedia* N-1: Solid waste of tofu processing was dried at 50°C overnight and grinded by a grind blender. 100 g of this substrate was mixed with 200 mL aquadest (2:1), then autoclaved at 121°C for 15 min. One tube agar slant of *Neurospora intermedia* N-1 strain was suspended by 50 mL of sterile water and mixed with the autoclaved substrate. Fermentation was carried out in two aluminium holed trays (25×35 cm) and incubated at 30°C for 3-5 days. The spores were cultivated and the substrate was discarded.

Extraction and isolation: Extraction of spores was carried out by shaking 10 g spores with 100 mL 95% of ethanol at 150 rpm for an hour. Suspension was filtered with suction through a Buchner funnel. The residue was grinded and rinsed with cold ethanol. Extraction and filtration were repeated 3-4 times. The pigment extract was evaporated in a vacuum rotary evaporator at 35°C. The dry residue (±500 mg) was then dissolved in few drops of acetone. 100 mL of n-hexane was placed in a 500 mL separatory funnel with a teflon stop-cock and this acetone solution was added. Fifty milliliter the distillated water was added slowly by flowing along the walls of the funnel. The lower phase, aqueous-acetone phase was discarded. 10% methanolic KOH was added to the upper remaining n-hexane phase and was left in the dark at room temperature overnight. The methanolic phase was discarded and the upper n-hexane phase was washed with water to remove the alkali. n-hexane phase was dried with anhydrous sodium sulfate and evaporated to dryness. The residue was dried additionally by nitrogen purge (Amaya, 2001). Three hundred milligram of dry residue was dissolved in few drops of chloroform and filtered before being introduced to the rotating plate of chromatotron. This plate was coated with the Silica Gel-Gypsum (contains 254 nm fluorescent indicator) 1 mm the thickness of coating material (depends on the quantity of sample). Before the sample solution was introduced, the sorbent layer was completely wetted with n-hexane and at least 5 min to allow the equilibration. Sample solution was filtered by suction into the chromatotron plate. Elution of the sample was carried out by 50 mL of 0, 20, 40, 60, 80 and 100% chloroform in hexane and continued with 10, 20, 40, 60, 80 and 100% methanol in chloroform. Flow rates of the rotating plate were adjusted to 2-4 mL min^{-1}. UV lamp was used to detect the UV absorbing fractions ring. The fractions were collected and their purity examined by TLC. The pure compound (1) was characterized by one and two dimension of 500 mHz NMR ^{1}H and ^{13}C (JEOL ECA), FTIR (FT/IR-4200typeA) and LC-MS (Marimer Biospectrometry Hitachi L6200, system ESI).

RESULTS

Neurospora intermedia N-1 was fermented on waste solid tofu production for at 30°C for 3-5 days. The orange spores was harvested and extracted for spectrometric

identification of fatty acid methyl ester in this fungus. Data's ^{1}H and ^{13}C NMR spectra of compound 1 contained 19 Carbon, 34 Hydrogen and 2 Oxygen, which were tabulated in Table 1. This data's was confirmed by DEPT ^{13}C NMR spectra (Fig. 1a, b), observed one of the methyl group; 12 of CH_2 atoms and 4 of CH atoms.

For a detailed assignment of both proton and carbon spectra we used ^{1}H/13C 2D HMQC and HMBC spectroscopy. HMQC and HMBC of this compound were analyzed to elaborate the direct and long-range correlations between the ^{1}H and ^{13}C NMR, respectively. Figure 2 shows the HMBC and HMQC spectra.

LC-MS spectrum indicates molecular weight of the compound 1 as 294 which is visible by the presence of protonated molecular ion [M+H] at m/z 295 and the presence of sodium adducts at m/z 317, respectively (Fig. 3). IR spectra shown that the present of =CH group indicated by absorption at wave number 3413 cm^{-1}, -CH stretching vibration at 2927 cm^{-1}, C = O absorption band at 1739 cm^{-1}. Methyl esters of long chain fatty acids was presented by a 3 band pattern with bands near 1249, 1199 and 1172 cm^{-1}, the band 1172 cm^{-1} is the strongest. IR spectra indicates present of a trans C = C which gives a strong C-H bending band near 970 cm^{-1} (Fig. 3a, b).

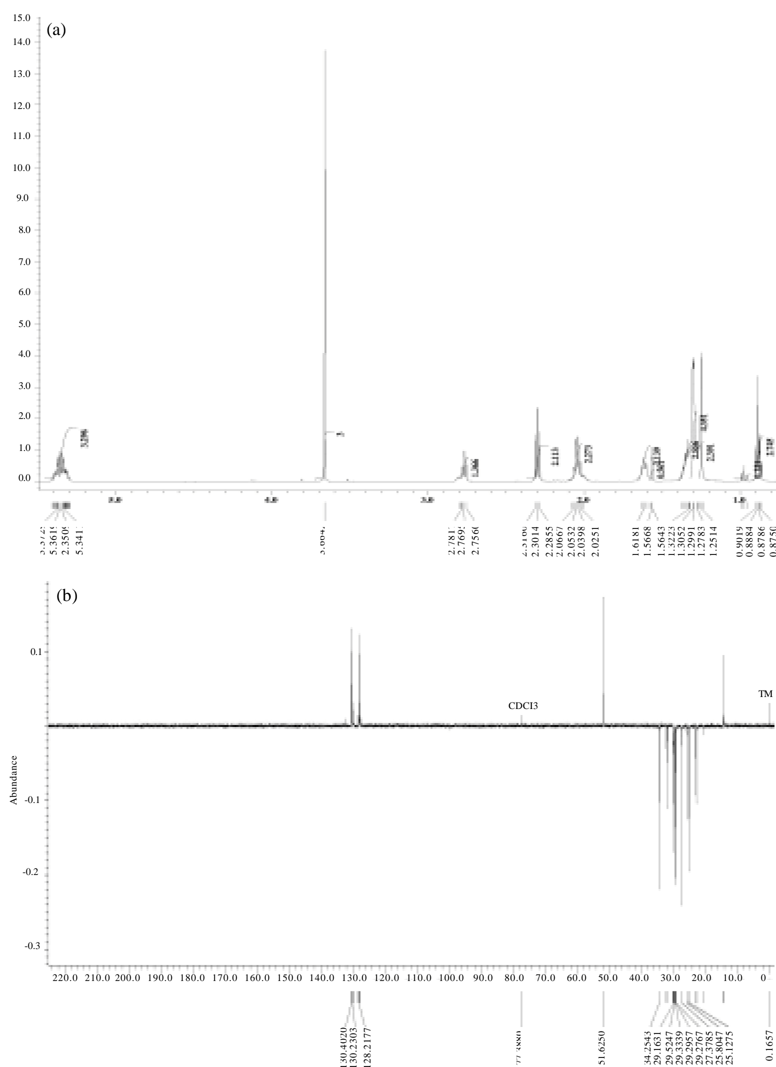

Fig. 1: (a, b) 500 MHz ^{1}H and DEPT of compound 1 in $CDCl_3$

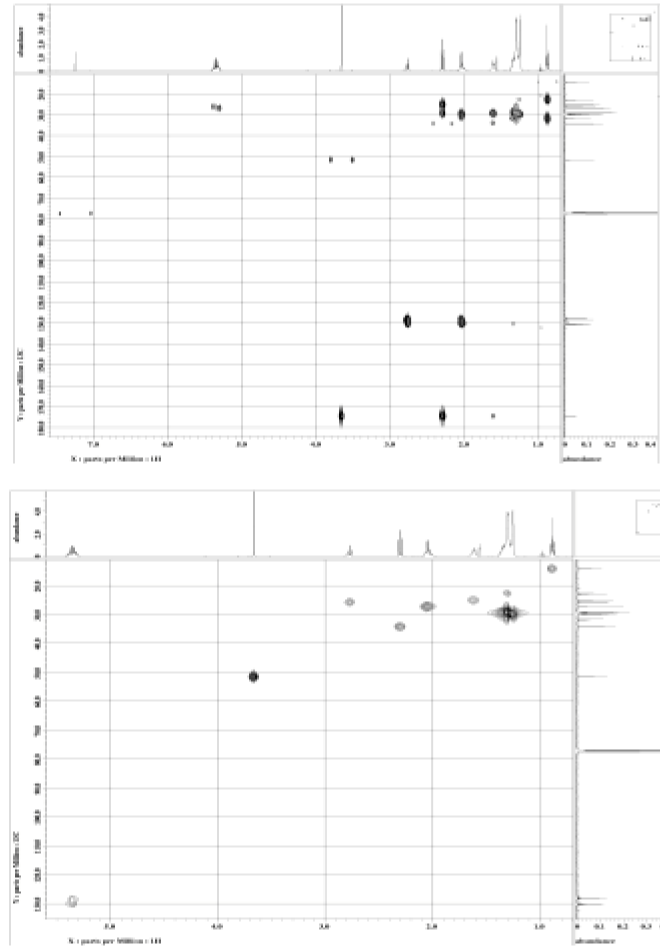

Fig. 2: HMBC and HMQC of compound 1 in $CDCl_3$

Table 1: NMR data of compound 1 in $CDCl_3$

No.	^1H	^{13}C	DEPT	HMBC ^1H
1	-	174.5	C = O	C1', C2, C3
2	2.3 (t, 2,113)	34.28	CH_2	C3, C1
3	1.62 (t, 2,139)	25.13	CH_2	C2, C4
4	1.30 (m, 2,556)	29.29	CH_2	C3, C2, C5
5	1,31(m, 2,556)	29.34	CH_2	C3, C4
6	1.30 (d, 4,351)	22.87	CH_2	-
7	-	29.54	CH_2	-
8	5.34 (m, 3,296)	130.41	CH	C9
9	5.35 (m, 3,296)	130.23	CH	C8, C11
10	1.31 (m, 2,556)	29.31	CH_2	C11, C12
11	2.77 (t, 1,366)	25.81	CH_2	C9, C13
12	5.37 (m, 3,296)	128.22	CH	C11, C14
13	5.36 (m, 3,296)	128.09	CH	C11, C14
14	2.05 (q, 2,273)	27.38	CH_2	C13, C15, C16
15	1.34 (s, 2,391)	29.77	CH_2	C14, C16
16	1.25	29.87	CH_2	C14, C15, C17, C18
17	1.30 (d, 4,351)	22.75	CH_2	C16, C18
18	0.88 (m, 2,745)	14.25	CH_3	C17, C16
1'	3.66 (s, 3)	51.63	OCH_3	C1

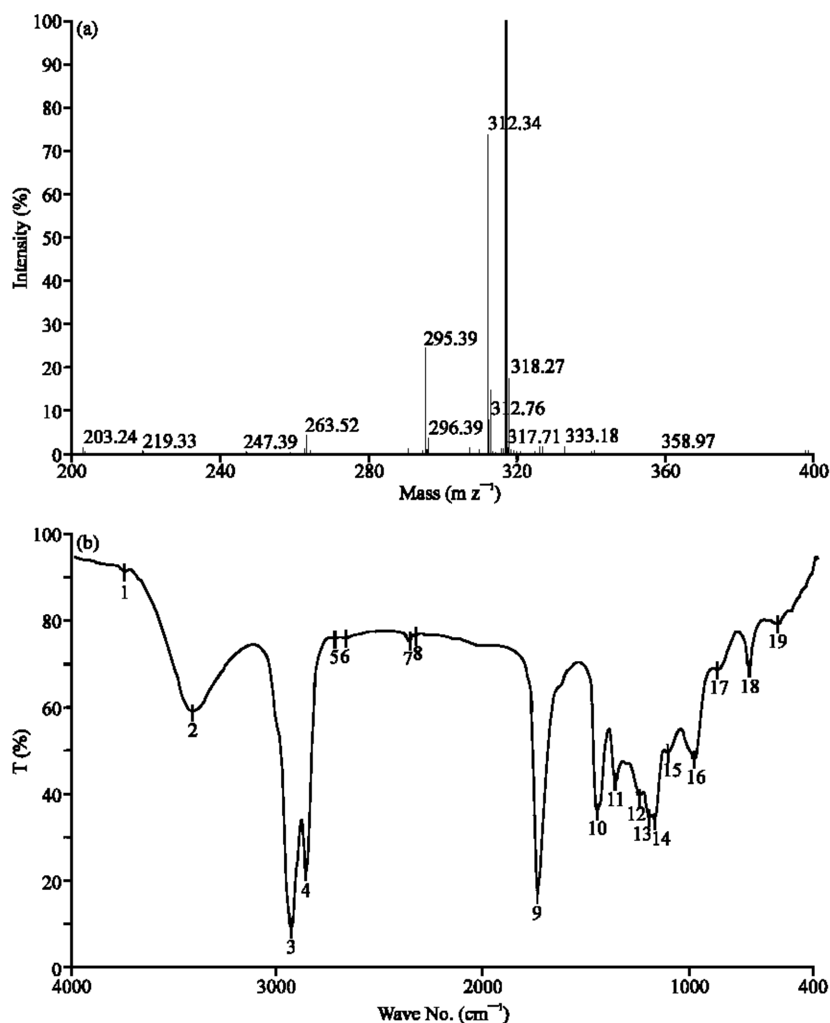

Fig. 3: (a, b) Mass spectra and IR of compound 1

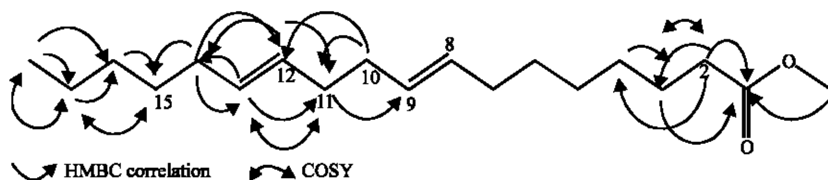

Fig. 4: The elucidated structure of compound 1

DISCUSSION

Based on NMR spectra, shielding at δ_H 3.66 (s, H3) indicates presence of one methoxy group. Mello *et al.* (2008) observed three different calibration curves based on ^{1}H-NMR spectroscopy (300 MHz) were used for quantifying the reaction yield during biodiesel synthesis by esterification of fatty acids mixtures and methanol. The integrated intensities of the hydrogens of the ester methoxy group (3.67 ppm) were correlated with the areas related to the various protons of the alkyl chain.

Carbonyl group was shown in ^{13}C NMR at 174.50. The location of the carbonyl carbon (C1) was confirmed from the HMBC correlation from C1', C2 and C3. Shielding at δ_H 5.36 (m, H 3,296) indicates presence of two un-conjugated double bonds. The position of the double bonds at carbon number 8 and 12 were indicated in the HMBC spectrum. ^{13}C-NMR was very characteristic in the

olefinic signal region, namely δ 128 to δ 131 (Shiao and Shiao, 1989). In food products from ruminants, c-9, t-11 is the most prevalent one comprising 80 to 90% of total Conjugated Linoleic Acid (CLA). Isomerization the double bond at carbon-12 position is transferred to carbon-11 position forming c-9, t-11 CLA. It is followed by the rapid hydrogenation of cis-9 bond leaving TVA. Both these steps are carried out by a group a bacteria. The enzyme responsible for the conjugation of cis-9, cis-9 double bonds was identified as linoleic acid isomerase (EC 5.3.1.5). it is a particulate enzyme bound to the bacterial cell membrane (Khanal and Dhiman, 2004). Migration of the double bond leads to shift of signals, especially when the double bond approaches one of end of the chain. This effect was discovered in early NMR studies of a full series of *cis*-octadecenoic and some acetylenic fatty acids. Prior work demonstrated this effect for double bonds near the terminal methyl group. The shifts of the olefinic protons did not differ significantly (Knothe, 2005). The sequence of compound 1 was deduced from a COSY experiment. Thus, the methyl signal at δ_H 0.88 (C18) was coupled to the methylene signals at δ_H 1.30. Another COSY experiment indicates presence of the sequence for protons with signals at δ_H 1.30 (C17), 5.36 (C13) and 1.62 (C3). COSY spectrum which showed the connectivity between proton signals and its HMBC correlation are shown in Fig. 4.

The molecular ion peak of a methyl ester of a straight-chain aliphatic acid is usually can be distinguished by a mass spectra. The ion $R\text{-}C = O^+$ gives an excellent diagnostic peak for esters. In methyl esters it occurs at M-31 (Silverstain *et al*., 1981). On this study, molecular ion peak of methyl ester is visible by the presence peak at m/z 263.5. From the NMR, LC-MS and IR data's, the elucidated structure of compound 1 was methyl octadeca-8,12-dienoat. The presence of methyl octadeca-8,12-dienoat or methyl linoleate in *N. intermedia* is the first report. In the fungus *Penicillium javanicum*, linoleic acid was preferentially synthesized in the younger cells. Linoleic acid was the major fatty acid in the spores of *P. atrovenetum.* The increase in the fatty acids during the period of spore germination and early lag phase (Van Etten and Gottlieb, 1965). According to Bentivenga and Morton (1996) FAME can be used as biochemical character of organisms. We suggested that methyl octadeca-8,12-dienoate can be used as a specific biochemical character or marker of *Neurospora intermedia*.

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