J. Microbiol. Biotechnol. (2001), **11**(6), 1018–1023

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Production of Oleamide, a Functional Lipid, by *Streptomyces* sp. KK90378

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Received: August 1, 2001
Accepted: November 3, 2001

Abstract Oleamide (*cis*-9-octadecenamide) is an endogenous primary amide of fatty acid that is produced in small amounts in animal brains. It is known to induce sleep and to lower temperature by destroying the lipid plasma membrane structure of cells, thereby disclosing gap junction channels. To develop a new biological production method for oleamide, a screening program was conducted to isolate a microorganism producing oleamide. Among 1,500 soil microorganisms tested, KK90378 exhibited a potent positive reaction with Dragendorff's reagent, used to detect the primary amide of oleamide. KK90378 was identified as a *Streptomyces* species based on cultural and morphological characteristics, the presence of diaminopimelic acid in the cell wall, and the sugar patterns for the whole-cell extract. *Streptomyces* sp. KK90378 produced oleamide 3 days after culture at 28°C, pH 7.2. A series of purification steps, including hexane extraction, silica gel column, and preparative thin layer chromatographies, were performed for the purification of oleamide. A spectrophotometric analysis using ^{1}H, ^{13}C-NMR, and GC-MS confirmed that the chemical structure of the purified oleamide was identical to that of authentic oleamide.

Key words: Oleamide, microbial production, *Streptomyces* sp., sleep inducing agent

Oleamide (*cis*-9-octadecenamide, Fig. 1) is an endogenous primary amide of fatty acid that accumulates in the cerebrospinal fluid under the condition of sleep deprivation, and is known to induce physiological sleep in rats [5]. The centrally placed *cis* double bond is expected to perturb the packing of saturated alkanes in the membrane, whereas the amide appears to cause the formation of stacks of oleamide, much like the β-sheet of proteins. *In vivo*, oleamide potentiates

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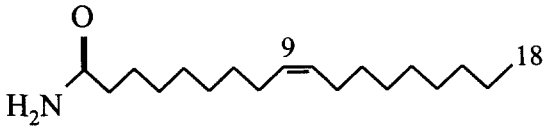

Fig. 1. Chemical structure of oleamide.

serotonin receptors [4] and closes gap junction channels [9]. Recent studies have shown that oleamide modulates serotonin-dependent neurotransmission [19]. In addition, oleamide is known to inhibit the proliferation of human breast cancer when it is treated with arachidonoylethanolamide. This may result from the competitive inhibition of arachidonolyethanolamide degradation by oleamide [1]. Besides, oleamide has been utilized in various industry fields, such as dairy [11] and plastic production as a functional feed and enhancer of surface migration in plastic production, respectively [8]. These biological activities and the industrial application of oleamide have also attracted much interest to develop oleamide as a new functional lipid [9].

In vivo, oleamide can be synthesized enzymatically from acyl-glycine precursors by peptidylglycine-amidating monooxygenase(PAM)-dependent reactions analogous to the biosynthesis of carboxyl terminally-amidated peptide hormones [14]. The rapid formation of oleamide (2.4 nmol mg protein^{-1} in 30 min) from the reaction mixture of oleic acid with neuronal $N_{18}TG_2$ cell membrane proteins [18] under alkaline conditions has also been reported [2]. For the catabolic degradation of oleamide, fatty acid amide hydrolase (FAAH), a membrane-bound enzyme, is known to hydrolyze oleamide to oleic acid [12]. FAAH is a serine hydrolase, and ser-217 and ser-241 are known to be essential for the enzyme activity [15]. Several FAAH inhibitors, such as 2-octyl γ-bromoacetoacetate [16], trifluoromethyl ketones, sulfonyl fluorides, and fluorophosphonates [6], have been also developed for the elucidation of the sleep-

inducing mechanism of oleamide. Biochemical and chemical biological studies on the synthesis and hydrolysis of oleamide suggest that the physiological concentration of oleamide is tightly regulated by the activities of two reversible enzymes when controlling the physiological sleep. As such, the amount of oleamide in the cerebrospinal fluid (CSF) of sleep-deprived cats is generally very low (0.1–5 pmol/100 μl) [13].

Since it has been difficult to prepare oleamide on a large scale from living tissue, as it only produces a very low amount of oleamide, the establishment of a new biological production method of oleamide is very attractive. To-date, several soil and marine microorganisms are known to produce functional lipids, such as DHA [10] and antifungal phospholipids [3]. However, there has been no previous report on the production of oleamide from a microorganism that can facilitate the biotechnological production of the lipid. Therefore, a screening program was conducted to isolate a microorganism producing oleamide for biological production. As a result, a novel soil microorganism, *Streptomyces* sp. KK90378, was isolated as an oleamide producer. This is the first report, to our knowledge, on the microbial production of the functional lipid, oleamide.

MATERIALS AND METHODS

Materials

Standard oleamide was purchased from Sigma chemical (St Louis, Mo, U.S.A.). Kieselgel 60 (70–230 mesh) and Kieselgel 60 F254 (Merck) were used for the silica gel column and thin layer chromatography (TLC), respectively. All solvents and reagents used were the highest grade available.

Screening and Identification of Microorganism Producing Oleamide

To isolate a microorganism producing oleamide, Dragendorff's reagent (mixture of 1.7 g basic bismuth nitrate and tartaric acid in 80 ml water, and 16 g potassium iodide in 40 ml water) staining method, which reacts with the primary amide of oleamide, and TLC analysis that shows the same R_f value with standard oleamide when using a solvent system of a chloroform/methanol (10:1) mixture, were used. One-thousand five-hundred soil microorganisms were isolated from several locations in the west provinces of South Korea during April, and used as libraries for the screening of an oleamide producer. The classification and identification of the selected strain was carried out on the basis of the ISP (International *Streptomyces* Project) method, and the strain was deposited in the Korean Collection for Type Cultures (KCTC) as KCTC18014p.

Culture of Microorganism

The oleamide producer, KK90378, was grown and maintained at 28°C on a YS medium plate (soluble starch 10 g/1 l, yeast extract 2 g/l, agar 20 g/l). For seed cultivation, an agar piece of the stock plate was cut under sterile conditions and inoculated into a 500-ml baffle flask containing 50 ml of the culture medium (G.S.S.): soluble starch (10 g/l), glucose (20 g/l), soybean meal (25 g/l), yeast extract (4 g/l), NaCl (2 g/l), K_2HPO_4 (0.25 g/l), and $CaCO_3$ (2 g/l). The pH was adjusted to 7.2 with 1 M NaOH. The flask was cultivated for 48 h at 28°C on a rotary shaking incubator (150 rpm) and then transferred to 2-l baffle flasks containing the same medium (400 ml/flask) for large-scale cultivation. All flasks were cultured for 7 days at 28°C.

Purification of Oleamide from Broth of KK90378

After cultivation, the mycelia were separated from the broth by centrifugation (5,000 rpm for 30 min). The broth was extracted with the same volume of hexane and the solvent was removed by evaporation under reduced pressure to give a white oil residue. The residue was separated by silica gel column chromatography using a chloroform/methanol (gradient from 98:2 to 50:50) mixture, and a successive preparative TLC was carried out twice, using a chloroform/methanol (98:2) and hexane/ethyl acetate (4:1) solvent system, respectively. The purity of the purified oleamide was confirmed by a reverse-phase HPLC

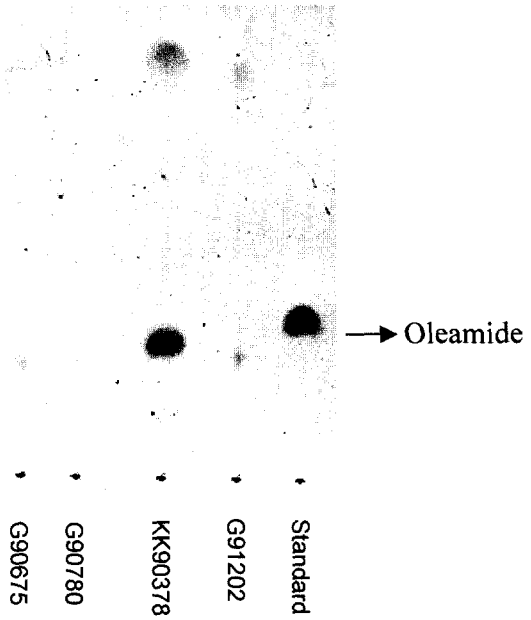

Fig. 2. TLC analysis pattern of n-hexane extract of KK90378 and other microorganisms.
Each microbial culture broth was extracted with n-hexane. The samples obtained were spotted on a Kieselgel 60 F_{254} (Merck), which was developed with $CHCl_3$-MeOH (10:1).

Table 1. Morphological characteristics of strain KK90378.

Colony surface	Smooth
Spore chain morphology	Spiral, 15–20 spores
Spore size	0.8–1.0×0.8–1.0 (μl)
Spore surface	Rod
Substrate mycelium	Gray
Soluble pigment	Colorless

(SHIMADZU) with a 50 min linear gradient of methanol from 75 to 100 in water (flow rate 1 ml/min).

Measurement of Oleamide using GC-MS

To quantify the oleamide produced by KK90378, 5 ml of cultured broth of KK90378 was extracted twice using an equal volume of *n*-hexane. The solvent was removed by evaporation and 100 μl of MeOH was added to dissolve the oleamide. One μl of the mixture was analyzed using a GC-MS (Hewlett - Packard 5890, DB-1 capillary column). The instrument settings used were as follows: initial temperature, 50°C; initial time, 5 min; rate, 20.0°C/min; final temperature, 300°C; final time, 26.71 min; injection port, 250°C; detector transfer line, 320°C; carrier gas, N_2; split ratio, 15:1. The amount of oleamide in each sample was determined by the area of the GC-MS spectra.

Identification of Chemical Structure of Oleamide from KK90378

The ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), and DEPT spectra were obtained using a JEOL JNM-LA400. Compound 1: White powder, ^{1}H-NMR (500 MHz, $CDCl_3$, δ), 5.40 (H×2, m, H-9, 10), 2.20 (2H, t, *J*=7.6 Hz, H-2), 1.98 (2H×2, each td, *J*=6.1, 6.2 Hz, H-8, 11), 1.61 (2H, q, *J*=7.1 Hz, H-3), 1.30-1.25 (methylene-H), 0.85 (3H, t, *J*=6.8 Hz, terminal methyl). ^{13}C-NMR (125MHz, $CDCl_3$, δ c), 175.60 (C-1), 129.99, 129.77 (C-9 or 10), 35.91, 31.88, 29.74, 29.68, 29.50, 29.30, 29.22, 29.19, 29.09, 27.19, 27.14, 25.50, 22.66 (methylene), 14.10 (terminal methyl).

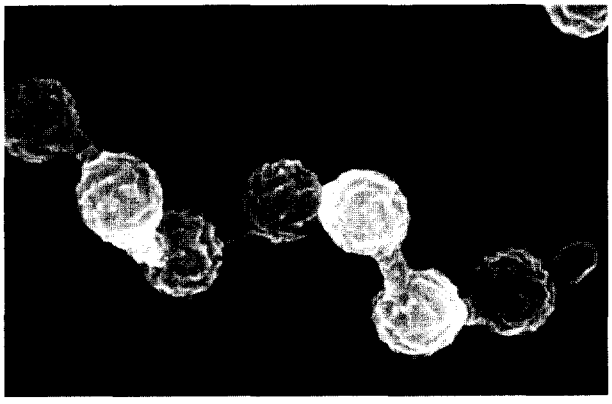

Fig. 3. Electron scanning morphology of strain KK90378 in SEM.

Table 2. Physiological characteristics of strain KK90378.

Starch hydrolysis	+	D-Fructose	+
Skim milk hydrolysis	+	Galactose	–
Nitrate reduction	+	Inositol	+
Gelatin liquefaction	–	Mannitol	–
Melanin pigment	–	Raffinose	+
Diaminopimelic acid	LL-DAP	Rhamnose	–
Utilization of C-source		Succrose	–
D-Glucose	+	D-Xylose	+
L-Arabinose	–	Cellulose	–

Results and Discussion

Isolation of Oleamide-Producing Microorganism

To isolate an oleamide-producing microorganism, each cultural broth of 1,500 soil microorganisms was tested using a TLC analysis and the staining of the amide group using Dragendorff's reagent. One ml of a cultural broth of the microorganisms was extracted by an equal volume of n-hexane, since oleamide is a nonpolar solvent-soluble compound. The solvent fraction was then developed with standard oleamide on a TLC plate in a mixture of chloroform/methanol (10:1). As shown in Fig. 2, KK90378 exhibited a potent productivity of oleamide, showing both a positive reaction with Dragendorff's reagent and the same R_f value (R_f=0.4) with standard oleamide in the TLC analysis. Therefore, KK90378 was selected as a new microorganism producing oleamide.

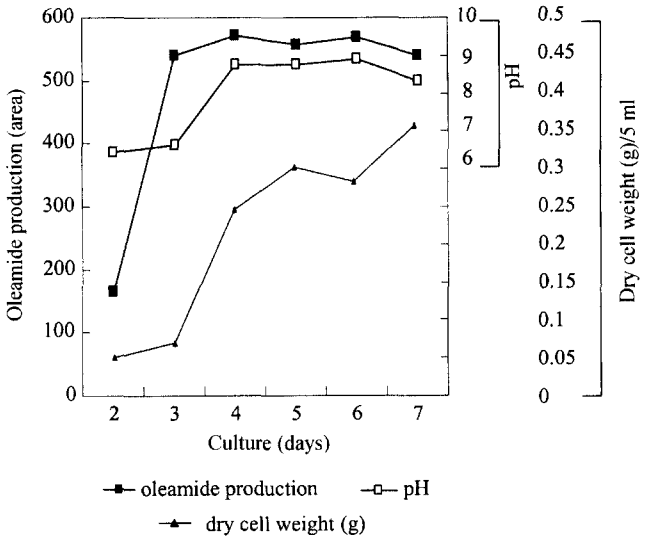

Fig. 4. Cultural properties of KK90378.
Cells were cultivated with shaking for 7 days at 28°C and pH 7.2. Five ml of culture broth was dried for 60 min to measure the cell growth (cell growth=dried weight of culture broth - media weight). The amount of oleamide was measured by gas chromatography using the area of oleamide, as described in Materials and Methods.

Table 3. Physicochemical properties of compound 1 from KK90378.

Appearance	Colorless oil
MW	281.5
Molecular formula	$C_{18}H_{35}NO$
UV λ_{max} nm (MeOH)	208
Solubility	
Soluble	AcOH, $CHCl_3$, MeOH, EtOAc
Insoluble	H_2O
R_f (silica gel $60F_{254}$) $CHCl_3$:MeOH (10:1, v/v)	0.4

MeOH, methanol; EtOAc, ethyl acetate; AcOH, acetone.

Identification of KK90378 as *Streptomyces* sp.

The classification and identification of KK90378 was carried out according to the ISP method. The morphological feature of KK90378 was investigated using an optical and electron microscope with 14-day cultured cells (Table 1): A spore chain showed a spiral morphology and the superficies of the spore were smooth. The shape of the spore was a rod with a length of 0.8–1.0×0.8–1.0 μl (Fig. 3). The cultural character of KK90378 was investigated using several ISP media conditions with 21-day cultured cells. The strain grew well in all the media tested except for glycerol-asparagine and tyrosine containing an agar medium. KK90378 was able to use D-glucose, D-fructose, inositol, raffinose, and D-xylose as a carbon source. In addition, the strain had diaminopimelic acid in its cell wall based on an analysis using Becker's method (Table 2). Therefore, it was concluded that KK90378 belongs to a *Streptomyces* species and was named as *Streptomyces* sp. KK90378.

Cultural Conditions of KK90378

The culture conditions for oleamide production were investigated next. The dry cell weight, oleamide production, and pH of a culture broth of KK90378 were analyzed at various times after the culture. The maximum cell growth was exhibited at 7 days of culture. KK90378 started to produce oleamide after 3 days of culture and the productivity reached a maximum after 7 days. The pH and dry cell weight showed a similar pattern to the oleamide production, which increased 3 days after the culture (Fig. 4). Detailed studies on optimizing the culture conditions for KK90378 are currently under investigation and will be reported elsewhere.

Purification of Oleamide from KK90378 Culture Broth

To establish a purification method for oleamide and to identify its chemical properties, oleamide was purified from the KK90378 culture broth. Using a 7-day culture broth, the mycelia was removed from the broth using centrifugation (5,000 rpm, 30 min). The broth was then extracted with an equal volume of n-hexane and the solvent concentrated *in vacuo* to obtain a colorless oil residue. The oil residue was separated by silica gel

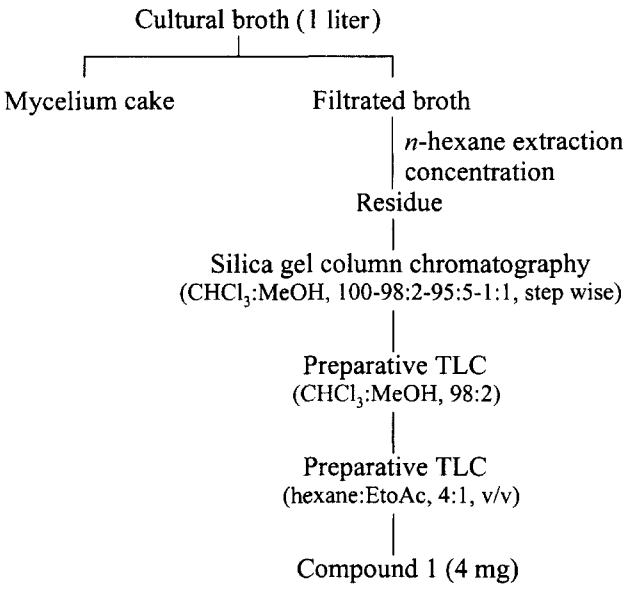

Fig. 5. Purification scheme of KK90378.
Isolation procedure of oleamide from a culture of *Streptomyces* sp. KK90378.

column chromatography using a gradient of a chloroform/methanol mixture (100:1→98:2→95:5→9:1→1:1). A TLC analysis of each fraction was performed and the chloroform/methanol mixture (95:5) eluted fraction exhibited the same R_f value as standard oleamide on the TLC plate. The fractions including oleamide were then concentrated *in vacuo* and separated by a preparative TLC using a mixture of chloroform/methanol (10:1). Based on the TLC analysis of each separated fraction, those bands showing the same R_f as standard oleamide were collected and separated again by a preparative

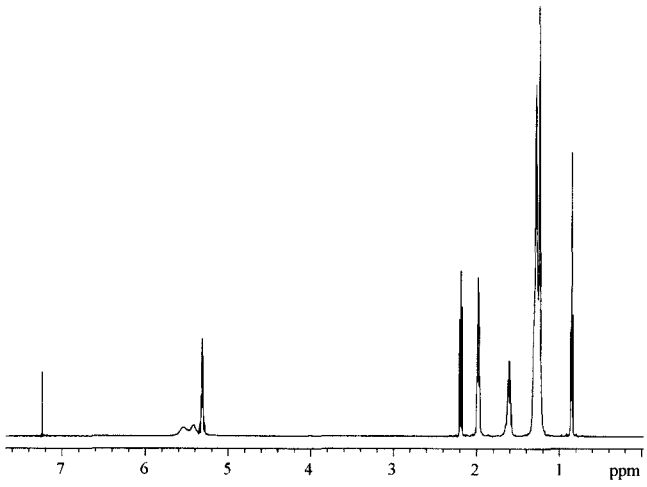

Fig. 6. ^{1}H-NMR (500 MHz) spectra of oleamide isolated from KK90378.
Signals were obtained in $CDCl_3$ 1.30–1.25 for methylene-proton and 0.85 for terminal methyl.

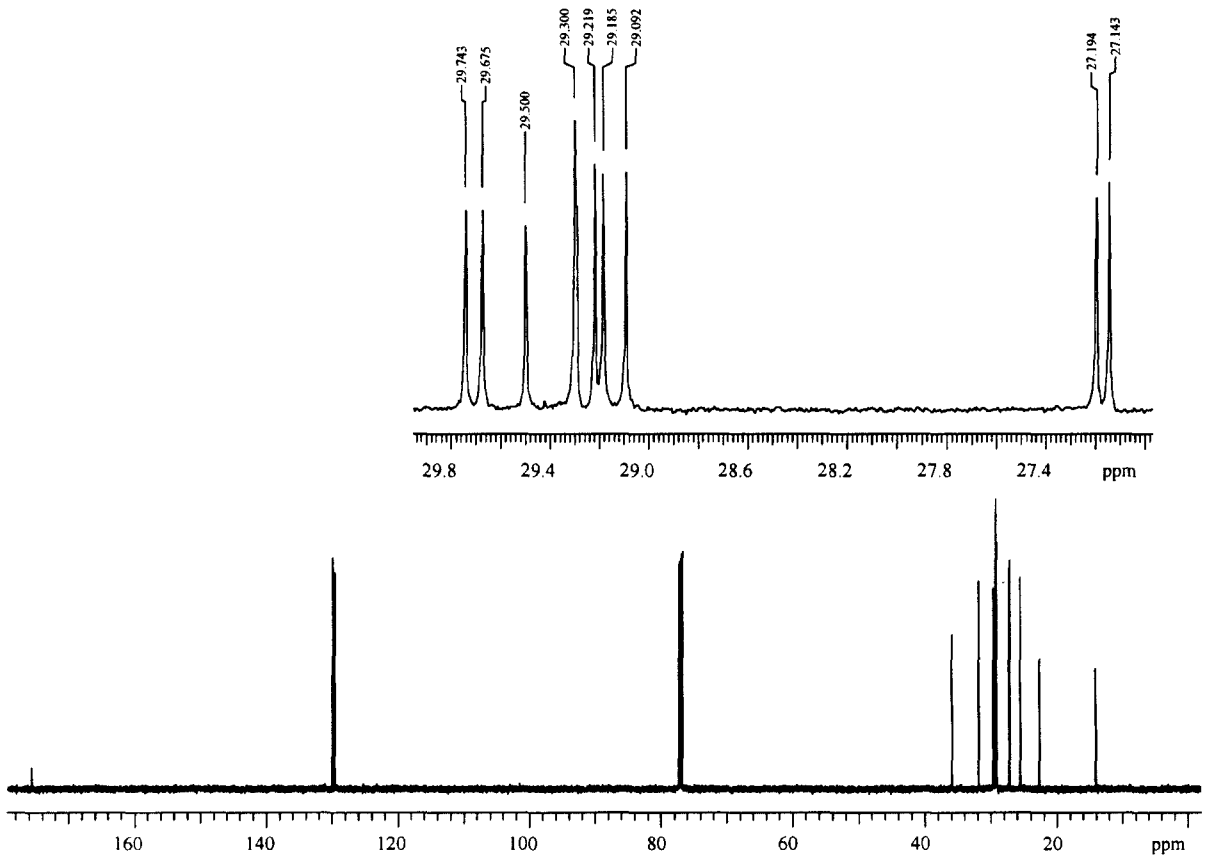

Fig. 7. ^{13}C-NMR(125 MHz) spectra of oleamide isolated from KK90378.

TLC using a mixture of hexane/ethyl acetate (4:1). Finally, 4 mg of oleamide (compound 1) was obtained from 1 l of KK90378 culture broth.

Chemical Structure Identification of Oleamide from KK90378

To confirm the chemical structure of the purified oleamide (compound 1), several spectrophotometric analyses were carried out using NMR, GC-MS, and EI-MS. Compound 1, obtained as a white powder in $CHCl_3$-MeOH, exhibited a UV absorbance band at 210 nm. The ^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of compound 1 exhibited two olefinic methines at δ 5.40, many methylenes at δ 1.61 and δ 1.30–1.25, three allyl methylenes at δ 2.20 (2H, t) and 1.98 (2H×2, each td), plus terminal methyl signal [δ 0.85 (3H, t, J=6.8 Hz)] proton signals. These data indicate that compound 1 would appear to be a fatty acid with one double bond (Fig. 6). In addition, in the ^{13}C-NMR spectrum (125 MHz, $CDCl_3$) of compound 1, one carbonyl (δ c 175.6), two olefinic methines (δ c 129.99, 129.77), fifteen methylenes (δ c 39.91–22.66), and one methyl (δ c 14.10) carbon signals were obtained. These results show that compound 1 was composed of 18C fatty acids (Fig. 7). Furthermore, compound 1 was confirmed as an amide in which nitrogen was substituted for the acid part of the carboxyl group, resulting in a molecular weight of 281 and orange color when sprayed with Dragendorff's reagent. Finally, compound 1 was identified as oleamide (*cis*-9-octadecenamide) through a comparison of its EI-MS data with corresponding constituents reported in previous literature and its chemical characteristics with those of standard oleamide (Sigma).

Acknowledgments

This research was supported by a grant to HJK from the Korean Small and Medium Business Administration (2000) and to JS from the Ministry of Science and Technology of Korea (PN 99384).

References

1. Bisogno, T., K. Katayama, D. Melck, N. Ueda, L. De Petrocellis, S. Yamamoto, and V. Di Matzo. 1998. Biosynthesis and degradation of bioactive fatty acid amides in human breast cancer and rat pheochromocytoma cells; implications for cell proliferation and differentiation. *Eur. J. Biochem.* **254:** 634–642.

2. Bisogno, T., N. Sepe, L. De Petrocellis, R. Mechoulam, and V. Di Marzo. 1997. Biosynthesis of 2-arachidonoyl-glycerol, a novel cannabimimetic eicosanoid, in mouse neuroblastoma cells. *Adv. Exp. Med. Biol.* **433:** 201–204.
3. Cho, K. W., Y. Seo, T. Yoon, and J. Shin. 1999. Purification and structure determination of antifungal phospholipids from a marine *Streptomyces*. *J. Microbiol. Biotechnol.* **9:** 709–715.
4. Cravatt, B. F., D. K. Giang, S. P. Mayfield, D. L. Boger, R. A. Lerner, and N. B. Gilula. 1996. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **6604:** 83–87.
5. Cravatt, B. F., O. Prospero-Garcia, G. Siuzdak, N. B. Gilula, S. J. Henriksen, D. L. Boger, and R. A. Lerner. 1995. Chemical characterization of a family of brain lipids that induce sleep. *Science* **5216:** 1506–1509.
6. Deutsch, D. G., R. Omeir, G. Arreaza, D. Salehani, G. D. Prestwich, Z. Huang, and A. Howlett. 1997. Methyl arachidonyl fluorophosphonate: A potent irreversible inhibitor of anandamide amidase. *Biochem. Pharmacol.* **53:** 255–260.
7. Guan, X., B. F. Cravatt, G. R. Ehring, J. E. Hall, D. L. Boger, R. A. Lerner, and N. B. Gilula. 1997. The sleep-inducing lipid oleamide deconvolutes gap junction communication and calcium wave transmission in glial cells. *J. Cell Biol.* **29:** 1785–1792.
8. Hanus, L. O., H. M. Fales, T. F. Spamde, and A. S. Basile. 1999. A gas chromatographic-mass spectral assay for the quantitative determination of oleamide in biological fluids. *Anal. Biochem.* **270:** 159–166.
9. Huidobro-Toro, J. P. and R. A. Harris. 1996. Brain lipids that induced sleep and novel modulators of 5-hydroxytrypamin receptors. *Proc. Natl. Acad. Sci. USA* **15:** 8078–8082.
10. Hur, B.-K., H.-B. Hu, and H.-S. Yun. 2000. The distribution and position of fatty acid in glycerides hydrolyzed from fish oil by lipase. *J. Microbiol. Biotechnol.* **10:** 502–506.
11. Jenkins, T. C. 2000. Feeding oleamide to lactating Jersey cows. 1. Effects on lactation performance and milk fatty acid composition. *J. Dairy Sci.* **83:** 332–337.
12. Lerner, R. A., G. Siuzdak, O. Prospero-Garcia, S. J. Henrikse, D. L. Boger, and B. F. Cravatt. 1994. Cerebrodiene: A brain lipid isolated from sleep-deprived cats. *Proc. Natl. Acad. Sci. USA* **20:** 9505–9508.
13. Lerner, R. A. 1997. A hypothesis about the endogenous analogue of general anesthesia. *Proc. Natl. Acad. Sci. USA* **94:** 13375–13377.
14. Merkler, D. J., K. A. Merkler, W. Stern, and F. F. Fleming. 1996. Fatty acid amide biosynthesis: A possible new role for peptidylglycine α-amidating enzyme and acyl-coenzyme A: Glycine N-acetyltransferase. *Arch. Biochem. Biophys.* **330:** 430–434.
15. Omeir, R. L., G. Arreaza, and D. G. Deutsch. 1999. Identification of two serine residues involved in catalysis by fatty acid amide hydrolyase. *Biochem. Biophys. Res. Commun.* **264:** 316–320.
16. Patricelli, M. P., J. E. Patterson, D. L. Boger, and B. F. Cravatt. 1998. An endogenous sleep-inducing compound is a novel competitive inhibitor of fatty acid amide hydrolase. *Bioorg. Med. Chem. Lett.* **8:** 613–618.
17. Shiring, E. B. and D. Gottlieb. 1966. Method for the charactrization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* **16:** 313–340.
18. Sugiura, T., S. Kondo, T. Kodaka, T. Tonegawa, S. Nakane, A. Yamashita, Y. Ishima, and K. Waku. 1996. Enzymatic synthesis of oleamide (cis-9, 10-octadecenoamide), an endogenous sleep-inducing lipid, by rat brain microsomes. *Biochem. Mol. Biol. Int.* **40:** 931–938.
19. Thomas, E. A., M. J. Carson, M. J. Neal, and J. G. Sutcliffe. 1997. Unique allosteric regulation of 5-hydroxytrypamin receptor-mediated signal transduction by oleamide. *Proc. Natl. Acad. Sci. USA* **25:** 14115–14119.