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Antioxidant and Anticancer Activity of the Marine Sponge Clathria Basilana

Karyawati, A.T[1] Manalu, W[2] Suparto, I.H[3] Mauboy, R.S[1]
1.Department of Biology, Faculty of Science and Engineering, Nusa Cendana University, Kupang, Indonesia
2.Department of Anatomy, Physiology and Farmacology, Faculty of Veteriner, Bogor Agricultural University, Bogor, Indonesia
3.Major of Primatology, Faculty of Multidicipline, Bogor Agricultural University, Bogor, Indonesia
Corresponding Author: Department of Biology, Faculty of Science and Engineering, Nusa Cendana University, Kupang, Indonesia, Kode Pos 85361

Abstract
This study aims to determine antioxidant and anticancer activity of Clathria basilana extracts. The sponges were collected from Tablolong - Kupang , East Nusa Tenggara , Indonesia . The sponges were extracted with methanol. The crude extract was partitioned by n - hexane. The antioxidant activity of sponge extracts were tested by DPPH method . Anticancer activity of sponge extracts were tested by MTT method. Fraction of methanol - water at concentration of 500 ppm showed antioxidant activity of more than 50 % , while the fraction of n - hexane at concentration of 500 ppm showed antioxidant activity only 10 % . The test results showed that the fraction of methanol - water and n - hexane had anticancer activity of more than 50 % at concentration of 125 ppm.
Keywords: Clathria basilana, extraction, fractination, antioxidant, anticancer.

1. Introduction

Cancer is a disease what is a burden on society and government that need special attention and careful planning to anticipate in the future. Our dependence on modern cancer drugs are part of the development of health problems. The high price of cancer drug products cause health problems encountered many obstacles, especially the allocation of funds to be disbursed for this purpose.

Search of new cancer drugs continue to be done by synthesizing derivatives of known anticancer drugs or to explore new active anticancer ingredient from natural materials, including resources liver of land and sea. Search of new anticancer active ingredient of biological resources is not only intended for cancer treatment measures (curative), but also for the development of an active material for the purpose of prevention (preventive) cancer.

One of the origin of biological resources potentially oceans as a source of the active ingredient is a group of anticancer compounds from sponge (*Clathria basilana*). Results of research showed that sponge extracts of Clathria basilana at concentrations above 640 ppm can inhibit cancer cell growth of human lung epithelial A549 (Karyawati 2010). This suggests that this species has the potential to be developed as a source of anticancer compounds active ingredient.

Efforts to develop anticancer active ingredient of Clathria basilana should pay attention to two facts of scientific studies that have been done. The first fact, various studies indicate a close relationship between the antioxidant and anticancer activities, particularly in an effort to find the source of natural active ingredients to carry out preventive measures against cancer. The second fact, the development of modern medicine natural medicine requires lengthy research phase to be utilized by the public.

Observing this fact it is well placed to develop a candidate antioxidant and anticancer active ingredient of sponge Clathria basilana in traditional drug development workflow. Candidate antioxidant and anticancer active ingredient is an extract of the active developed standardized, not a pure compound. Although only in the form of extracts, active ingredients have complete data on the efficacy and safety evidence, thus can get two benefits at once. First obtained candidate antioxidant and anticancer active ingredient which can be used as part of traditional medicine (standardized extract). Second, the candidate obtained the antioxidant and anticancer active ingredient which can be further investigated for raw materials of modern medicine through clinical testing scheme.

Chemotherapy and radiation therapy are used to treat cancer, but they show serious side effects. Additionally Events economy most people are not sufficient due to the high cost of treatment that is necessary to find an alternative. This led to the development of research to find new drugs continues to grow, including the nature of the material that is currently being widely investigated for the treatment of cancer (Abdel-Hady et al. 2011; Djajanegara and Prio 2009; Fitrya and Lenny 2009; Anderson 2001; Radji 2004) part of the development of health problems. The high price of the drug product causes cancer health services encountered many obstacles.

Efforts on cancer prevention measures (preventive) using natural ingredients developed the concept of thought that connects antioxidant with potential anticancer activity. Yin et al. (2009) stated that the chemical

substance with a potential catcher of ROS has potential to improve health and cancer chemotherapy. People who eat lots of fruits and vegetables are healthier than other with the risk of degenerative diseases including cancer are low. This is believed to be due to the protective nature of the content of various types of antioxidants found in fruits and vegetables. This fact is consistent with the results of research on the culture and animal experiments suggest that oxidative DNA damage is part of the carcinogenesis. Therefore, it is logical that it is recommended to consume antioxidants in the diet as part of a cancer prevention (Silalahi 2006).

As we know, the process of cancer formation through four phases, starting with DNA mutations as an initiation stage. The second stage is the stage of promotion ie multiplication of the cells that have mutations. Cancer cells with genetic changes that appear real on stage progression. Next on stage four, the cancer cells to expand to other tissues via the blood or lymph vessels, and form secondary tumors in other tissues. Role of free radicals is often used as a starting point to explain the incidence of cancer, particularly at the initiation stage.

Based on what was raised by Silalahi (2006) is then in the initial study of the candidate active ingredients necessary to test the antioxidant and anticancer activities in parallel that can be known reference concentration of the extract that has synergy effects between antioxidant activity and anticancer function. A lot of studies in vitro and in vivo have shown that oxidative stress is closely related to many acute and chronic diseases. As mediators of oxidative stress, reactive oxygen species (ROS), such as superoxide radical anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide have a close relationship with various human diseases such as amyotrophic lateral sclerosis, arthritis, cancer, and cardiovascular. Cellular targets of ROS is DNA, proteins and lipids. There is a close relationship between increased formation of ROS with cellular proliferation, mutation, and gene instability (Yin et al. 2009).

Yan Li et al. (2007) suggested that the results of his research found a positive linear relationship between antioxidant activity and anticancer effects of five herbal extracts examined. Wang and Kim (2007) suggested that the results of his research found the antioxidant activity of antioxidant enzymes in strawberries is a potential material for cancer prevention. The study results also showed that strawberries can inhibit cancer cell human lung epithelial (A549). Levels of antioxidant capacity, antioxidant enzyme activity, nonenzim components, and antiproliferative activity on A549 in strawberries have a high positive correlation. Research conducted by Fayet (2009) also showed association between antioxidant and anticancer activity of the research to extract *Citrus reticulate* and *Pelargonium graveolens*. The results Shirzad et al. (2011) showed that there is a correlation between the antioxidant capacity and anticancer activity of garlic.

Based on the above search new active ingredient for purposes of treatment (curative) and prevention (Preventive) cancer is the unity of the search for new active ingredients of anticancer and antioxidant activity. Search of new active ingredients of anticancer and antioxidant for cancer and cancer prevention is not only done on the biological resources on land but also the sea biological resources. One of the ocean biological resources as a potential source of antioxidant and anticancer active ingredient is sponge *Clathria basilana*.

MTT assay results of methanol extract of the sponge *Clathria basilana* showed the percentage inhibition of cancer cell growth in human lung epithelial (cells A-549) between 4.5 to 6.4%. However, cell morphology observation showed that the maximum safe concentration used for normal cell A-549 is 640 ppm. This suggests that the methanol extract of the sponge *Clathria basilana* at 640 ppm concentration had toxic effects on cells A-549 (Karyawati 2010).

Based on these results it is necessary to attempt to develop *Clathria basilana* sponge extracts as antioxidant and anticancer active ingredient. This effort includes the development of screening antioxidant and anticancer activity of *Clathria basilana* sponge extracts to obtain candidate antioxidant and anticancer active ingredients, physical and chemical characteristics of candidate antioxidant and anticancer active ingredient and safety testing candidate antioxidant and anticancer active ingredient.

2. Material and methods

Material for this research are sponge *Clathria basilana*, 96 % ethanol, filter paper, methanol, n-hexane, dichloromethane, ethyl acetate, 2.2-diphenylpicrylhydrazyl (DPPH), 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazolium bromide (MTT), A549 cell lines (Human Lung Carcinoma ATCC CCL 2) from Microbiology and Immunology Laboratory of Primate Research Center-Bogor Agricultural University, Dulbecco's Modified Eagle Medium (DMEM), Phosphate Buffer Saline, Bovine Serum Albumin, Penicillium, Streptomycin, deionized water, Tryphan Blue, Trypsin, distilled water, HCl, NaOH, Phytochemistry Kit.

Sponge *Clathria basilana* was collected in January 2013 from Tablolong, Kupang, East Nusa Tenggara. The sponges was washed and dried in a drying oven temperature of 37°C for 4 x 24 hours. Dry sample was milled then sieved using a 100 mesh sieve. A total of 500 grams of powder was extracted by maceration using solvents methanol for 5 days, then maserat was separated from the residue by filtration using filter paper. Maserat obtained the solvent was evaporated using a rotary evaporator at low pressure and temperature of 40°C until a viscous methanol extract. The viscous extract was dried using freeze dryer to obtain dry extract.

A total of 3 grams of dried extract was dissolved in 120 mL of methanol and added 12 mL of distilled

water, and then partitioned using n-hexane solvent and solvent dichloromethane, and ethyl acetate solvent to obtain a fraction of n-hexane, dichloromethane fraction, the fraction of ethyl acetate and methanol-water fraction. Each fraction was evaporated using a rotary evaporator at low pressure and temperature of 40°C until a viscous crude fraction. Furthermore, the viscous fraction was dried using a freeze dryer to obtain dry extract of this fraction. Fractionation procedure was repeated 10 times repetitions. Drying viscous crude extracts performed in Cikaret BBIA Process Laboratory, Bogor.

The total antioxidant capacity test adapting the method proposed by Stef et al. (2009), Que et al. (2006), and Villano et al. (2006). This test uses a completely randomized design. Treatment consists of: (1) Treatment 0.5 ml extract / fraction extract *Clathria basilana* 250 ppm in methanol, (2) Treatment of a solution of 0.5 ml of extract / fraction extract *Clathria basilana* 500 ppm in methanol (3) Treatment of a solution of 0.5 ml of extract / fraction extract *Clathria basilana* 750 ppm in methanol (4) Treatment of a solution of 0.5 ml of extract / fraction extract *Clathria basilana* 1000 ppm in methanol (5) Treatment of 0.5 ml of solvent methanol as a blank. Each treatment was 3 times repetition. Each treatment was given 0.5 mL of 2.2-diphenylpicrylhydrazyl (DPPH) 0.1 mM in methanol. After 40 minutes of preparation, the absorbance was measured at 517 nm. The same procedure was carried out on a blank form of 2 mL of methanol. The parameters measured were total antioxidant capacity (TAC), which is expressed as the inhibition of free radicals (2.2-diphenylpicrilhydrazyl/DPPH) in percent. Data were analyzed by linear regression analysis.

TACDPPH (%) = (Absorban.blanko – Absorban.sample) / Absorban.blanko x 100

Anticancer test with MTT method performed by adapting the method raised by Hong Jia and Zhang Liping (2011). Cell suspension A549 (Human Lung Carcinoma / ATCC CCL 2) with concentration 2000 cells in 100 mL media Dulbecco's Modified Eagle Medium (DMEM). Extract was added after reaching confluent (24h). MTT test was performed on day 3 by adding MTT (5mg/ml) 10 mL per well, then incubated at 37°C for 4 hours. Formazan crystals were dissolved in 0.1 N HCl in isopropanol. Optical absorption was measured by using a microplate reader at a wavelength of 595 nm and the percentage of inhibition of proliferation of cancer cells A-549 was calculated using the following formula:

% inhibition of cancer cell = [1-ODsample/ODnegative control] x 100%.

3. Result

Crude extract obtained from 500 grams of sponge Clathria basilana is 45 grams. Crude extract in paste form, dark red. Crude extract is easily dissolved in methanol and water. Based on the results of the distribution of heavy crude extract obtained by weighing sponges obtained before extraction yield of 9%. This means that out of 100 gram sponge material extracted will produce 9 grams of crude extract.

Fractionation with n-hexane was repeated ten times. N-hexane fraction orange light, while the methanol fraction of the old orange. Fractionation with dichloromethane and ethyl acetate did not result in separation of the solution so that this fractionation did not followed. Thus there is only fraction of methanol-water (polar fraction) and a fraction of n-hexane (non-polar fraction.) Fractions of n-hexane and methanol-water subsequently concentrated in rotary-evaporator and then in vacuum to obtain the extract fraction. Methanol-water extract fraction was pasta shaped, dark green, this fraction soluble in methanol. Fraction n-hexane extract was pasta shaped, orange, this fraction hardly soluble in methanol so requires DMSO to dissolve it.

Test results of total antioxidant capacity (TAC) with DPPH method showed antioxidant activity of the methanol-water extract fraction (Table 1). At a concentration of 500 ppm fraction of methanol-water extracts showed antioxidant activity by 52%. This showed that at concentrations of 500 ppm fraction of methanol-water extract can inhibit free radical DPPH as much as 52%. Fraction n-hexane extract did not show antioxidant activity close to 50% inhibition of DPPH radical. n-hexane extract fraction requires more than 1000 ppm concentration to inhibit 50% of DPPH free radicals.

Based on the results of the regression analysis (Figure 1),% inhibition of the methanol-water extract fraction and concentration of the methanol-water extract fraction had a very close relationship with R2 values close to 1 (R2 = 0.988). % Inhibition can be predicted by the equation y = 0.087 x + 3.954. IC50 (Inhibition Concentration 50%) or the concentration of extract that can inhibit 50% of DPPH free radicals is 530 ppm (Table 2).

Regression analysis of % inhibition fraction of n-hexane with concentration of n-hexane fraction also showed a very close relationship (R2 = 0.993). Relationship % inhibition with concentration was expressed in linear regression, the equation was y = 0.028 x + 0.157 (Figure 2). Based on the regression equation, IC50 value of the fraction of n-hexane extract was 1780 ppm (Table 3). At a concentration of 1000 ppm, % inhibition of the extract only showed 28.157%. Value of % inhibition of n-hexane extract fraction was lower than % inhibition of the methanol-water fraction.

The test result of the extract *C. basilana* was showed in Table 4. Either crude extract or fraction extract of the sponge *C. basilana* had the ability to inhibit cell growth in human lung cancer (A-549 cells). The addition of crude extract and fraction of the extract at a concentration of 125 ppm had showed % inhibition of more than

50% . At a concentration of 125 ppm methanol-water extract fraction showed % inhibition higher than the others.

Fraction of methanol-water extract had antioxidant activity with IC_{50} at a concentration 530 ppm, and this fraction also had anticancer activity at a concentration of 125 ppm with inhibition of 81.82 %. Fraction of n-hexane extract had lower antioxidant activity than methanol-water extract fraction with IC_{50} at a concentration of 1780 ppm. n-hexane fraction also had anticancer activity at a concentration of 125 ppm with % inhibition of 77.92 %. Based on these results, the candidate of antioxidant and anticancer active ingredient was recommended, that was the methanol-water fraction.

Acute toxicity test (LD50) has done by adapting the procedure proposed by Adjirni (2000), Kusmardi et al (2006), and Elya (2010) using male mice. The number of animals used in acute toxicity tests follow the opinion of Weil (1952), the 5 animal groups and each group consisted of 5 mice. Each group was given material (sponge extract of C. basilana) by mouth with a sonde. Observations of animal behavior after being administered the extract was done every 60 minutes for 6 hours. The death of animals after administration of the extract was observed for 24 hours. Acute toxicity test results expressed in the level of toxicity based on criteria Loomis (1978).

Acute toxicity test performed on strain DDY male mice aged 50-60 days with a body weight of 20-42 g. The number of mice used in the acute toxicity tests with 25 male mice. Mice were grouped into 5 cages and acclimatized for 7 days. The treatment was sponge extract of C. basilana in aquabides with a concentration of 5 mg / kg, 10 mg / kg, 15 mg / kg, and 20 mg / kg BW. Extract solution was given to mice orally or through the mouth as much as 0.1 to 1.0 ml using a sonde. Observation of the behavior of mice after the mice were treated with sponge extract were observated every 60 minutes for 6 hours.

After 24 hours of observation was not found mice that died so that the LD_{50} value could not be determined. The observation of the behavior of mice for 6 hours after administration showed normal behavior and nontoxic symptoms of mice. Based on these observations it can be concluded that the extract of C. basilana including materials that are relatively harmless in the criteria Loomis (1978).

4. Conclusion

The conclusion of this study was recommended fraction of methanol-water extract of sponge *Clathria basilana* as candidate active ingredient of antioxidant and anticancer compound. Classes of active compound contained in the methanol-water fraction remains to be analyzed by spectroscopic analysis and tested of phytochemical compound . Result of acute toxicity had shown that sponge extract was safety for using one single dozes. Safety used of methanol-water extract fraction of the sponge *C. basilana* must be tested by sub-chronic toxicity.

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6. Tables

Table 1. Total Antioxidant Capacity (TAC) of *C. basilana* extracts

Consentration (ppm)	% TAC of ascorbic acid (positive control)	% TAC of methanol-water extract	% TAC of n-heksan extract
0	0	0	0
250	94.36	27.76	6.48
500	94.87	**52.47**	16.11
750	95.21	70.91	21.61
1000	96.24	88.21	28.29

Table 2. % inhibition based on regresion y = 0,087 + 3,954

Consentration (ppm)	% inhibition based on regresion y = 0,087 x + 3, 954
500	47.454
510	48.324
520	49.194
530	**50.064**
540	50.934
550	51.804

Table 3. % inhibition based on regresion y = 0,028 x + 0,157

Consentration (ppm)	% inhibition based on regresion y = 0,028 x + 0, 157
1700	47.757
1710	48.037
1720	48.317
1730	48.597
1740	48.877
1750	49.157
1760	49.437
1770	49.717
1780	**49.997**
1790	50.277
1800	50.557

Table 4. Result of anticancer test of *C. basilana* extracts

Consentration (ppm)	% inhibition of crude extract	% inhibition of n-hexan extract	% inhibition of methanol-water extract
3000	80.52	83.98	80.52
2500	85.28	83.55	81.39
2000	56.28	85.28	37.23
1500	83.55	83.55	80.52
1000	87.88	86.15	83.55
500	86.15	84.42	84.42
250	84.42	84.42	84.42
125	**80.52**	**77.92**	**81.82**
0	0.00	0.00	0.00

11. Figures

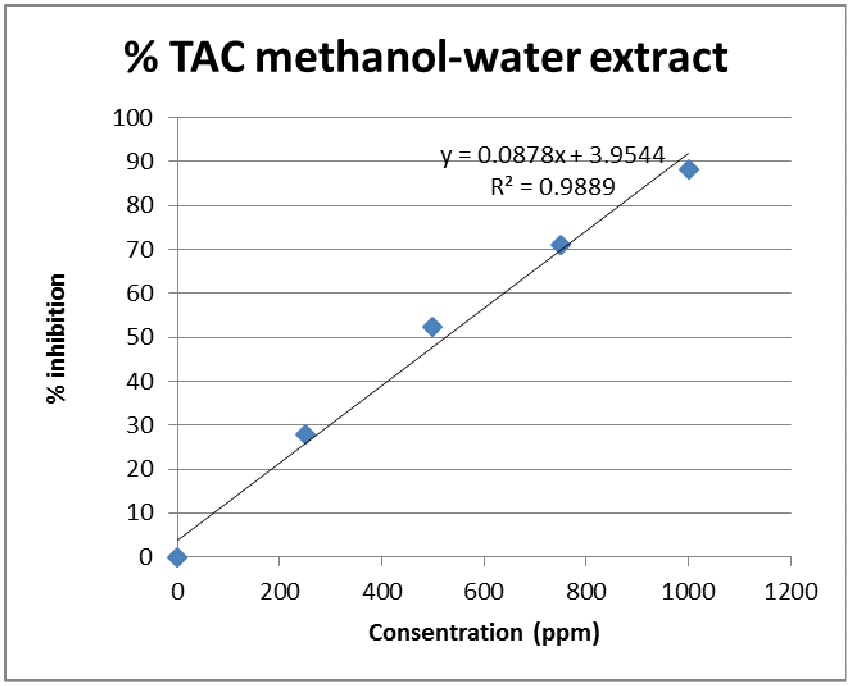


Fig.2 % TAC methanol-water extract

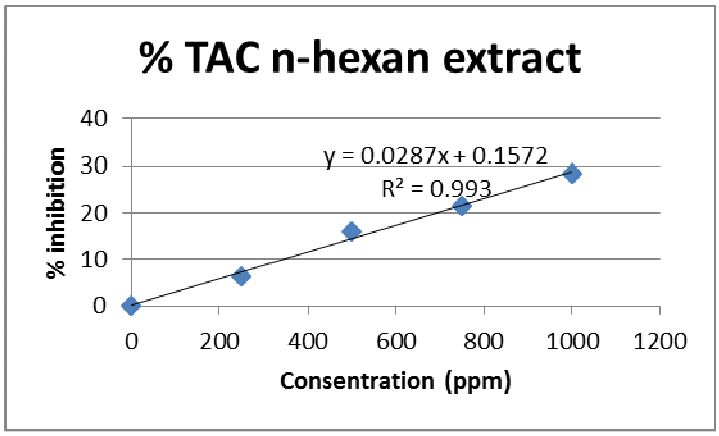
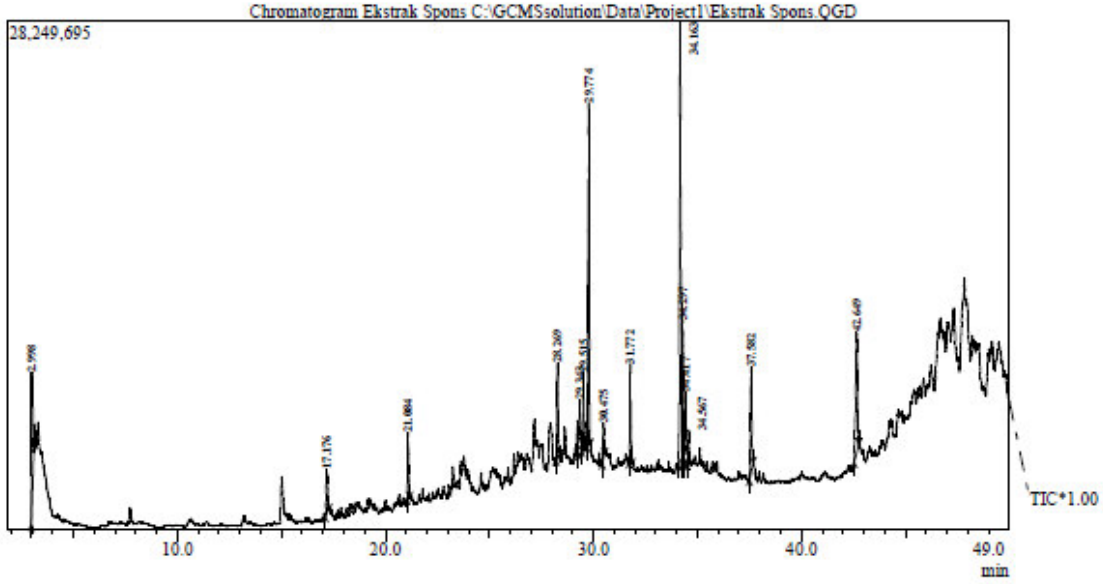


Fig.3 % TAC n-hexan extract



Peak Report TIC

Peak#	R.Time	Area	Conc%	Name
1	2.998	32879600	7.57	Carbamic acid, monoammonium salt (CAS) Ammonium carbamate
2	17.176	12634558	2.91	2-Pyrrolidinone (CAS) Pyrrolidone
3	21.084	12068109	2.78	1H-Indole (CAS) Indole
4	28.269	19389383	4.47	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-
5	29.343	13269140	3.06	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane
6	29.515	12417223	2.86	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane
7	29.774	69466598	16.00	5,10-DIETHOXY-2,3,7,8-TETRAHYDRO-1H,6H-DIPYRROLO[1,2-A;1',2'-D
8	30.475	6308855	1.45	1-Octadecanol (CAS) Stenol
9	31.772	14426105	3.32	Hexadecanamide (CAS) Amide 16
10	34.163	122757302	28.28	CITRONELLOLEPOXID (R oder S)
11	34.297	32440451	7.47	9-Octadecenamide, (Z)- (CAS) OLEOAMIDE
12	34.417	15903334	3.66	Hexadecanamide (CAS) Amide 16
13	34.567	8000036	1.84	TETRAPENTACONTAN, 1,54-DIBROMO-
14	37.582	22808418	5.25	Hexadecane, 1-(ethenyloxy)-
15	42.649	39304609	9.05	9-Octadecenamide, (Z)- (CAS) OLEOAMIDE
		434073721	100.00	