J. Fac. Appl. Biol. Sci.,
Hiroshima Univ. (1979), **18** : 65 – 73

Distribution of Quaternary Ammonium Bases in Seven Species of Marine Algae

Kanji HORI, Takahiro YAMAMOTO, Keisuke MIYAZAWA and Keiji ITO

Faculty of Applied Biological Science, Hiroshima University, Fukuyama

Received April 18, 1979
(Figs. 1–2 , Tables 1–4)

INTRODUCTION

Marine algae as well as other marine organisms have been recently expected the utilization as a source for biochemicals and biomedicals other than human food. In this point of view, substances, which show some biological activities, have been investigated in certain species of marine algae. Some available components have been found.[1] Particularly, we know that kainic acid[2] from *Digenia simplex* and domoic acid[3] from *Chondria armata* possess an anthelmintic activity, and that laminine[4–7] from *Laminaria angustata* shows an hypotensive activity. Each of these compounds contains a nitrogen as an imino or a quaternary ammonium base. As for quaternary ammonium bases, choline, stachydrine, trigonelline, homarine and glycine betaine other than laminine as mentioned before have been so far detected in marine algae by several workers.[8–13] Recently, ABE and KANEDA,[14–17] during the course of their study on the hypocholesterolemic constituents in marine algae, have isolated β-homobetaine and ulvaline from a green alga *Monostroma nitidum*, and γ-butyrobetaine from a red alga *Porphyra yezoensis*. It is, therefore, interesting to examine marine algae for the presence of quaternary ammonium bases. The distribution of the bases in marine algae has not yet been examined adequately.

In this study, we examined for quaternary ammonium bases in seven species of marine algae which were found to contain relative large amounts of positive compounds to the Dragendorff reagent in our preliminary experiment.

This paper reports the results.

MATERIALS AND METHODS

Materials

Specimen of *Ahnfeltia paradoxa*, *Polysiphonia fragilis* and *Gelidium amansii* were collected at the intertidal region of Iwate Prefecture, *Plocamium leptophyllum*, *Cladophora densa* and *Enteromorpha compressa* were collected at the similar region at Hiroshima city, *Boodlea coacta* was collected at the beach near Ibusuki, Kagoshima Prefecture, between 1976 and 1977, as shown in Table 1. After being collected, they were frozen with dry-ice and brought to our laboratory.

Preparation of the basic fraction containing quaternary ammonium bases

Each fresh specimen of the algae was soaked in 3 volume of 70% ethanol for 2 weeks

Table 1. The specimens of marine algae used for materials

Division	Japanese name	Scientific name	Locality	Date of collection
Red algae	Harigane	*Ahnfeltia paradoxa*	Iwate	Jun. 1977
	Hosoyukari	*Plocamium leptophyllum*	Hiroshima	May. 1976
	Kuroitogusa	*Polysiphonia fragilis*	Iwate	Jun. 1976
	Makusa	*Gelidium amansii*	Iwate	Jun. 1977
Green algae	Aomogusa	*Boodlea coacta*	Kagoshima	May. 1977
	Asamidori shiogusa	*Cladophora densa*	Hiroshima	Dec. 1976
	Hiraaonori	*Enteromorpha compressa*	Hiroshima	Apr. 1976

at room temperature and was occasionally stirred. This mixture was filtered by a filter paper. The residue was once more extracted with 70% ethanol in the same manner. The ethanolic extracts were combined and condensed under reduced pressure in order to remove the ethanol, and then were defatted with diethyl ether. The aqueous solution obtained was applied to a Dowex 50W-X8 column (H^+ form, 20–50 mesh). After the column was washed with water, the adsorbed portion was eluted with 3*N* ammonium hydroxide. Subsequently, the eluate was passed through a Dowex 1W-X8 column (OH^- form, 50–100 mesh) after being removed the remaining ammonia using a evaporator, and the column was washed with water. After the effluent and washings were rechromatographed with Dowex 50W-X8 (H^+ form, 50–100 mesh), the basic fraction containing quaternary ammonium bases was obtained.

Separation and identification of quaternary ammonium bases

Each basic fraction dissolved in a small amount of 2*N* hydrochloric acid, was put onto a Dowex 50W-X12 column (H^+ form, 200–400 mesh) and eluted stepwisely with 2, 4 and 6*N* hydrochloric acid after the manner of CHRISTIANSON *et al.*.[18] According to the size of column, fractions of 5 to 20 m*l* were collected and monitored with the ammonium reineckate reagent after KONOSU and KASAI.[19] Each tube containing quaternary ammonium bases was further subjected to thin layer chromatography (TLC) using a cellulose microcrystalline (Merck) and 1-propanol-2.5*N* ammonium hydroxide (7:3, v/v). After development, the plate was stained with the Dragendorff reagent. Fractions which gave only a single spot with the same Rf value were combined and applied for identification of the base.

The quaternary ammonium bases isolated from the algae were compared to the authentic or synthesized specimens on their mobilities in TLC using a cellulose microcrystalline and three solvent systems; (a) 99.5% ethanol-28% ammonium hydroxide (85:15, v/v), (b) 1-butanol-acetic acid-water (4:1:2, v/v) and (c) 1-propanol-2. 5*N* ammonium

hydroxide (7:3, v/v). The Dragendorff and ninhydrin reagents were used for detection. The bases, which show the same Rf values as those of the authentic or synthesized specimens, were crystallized respectively from the aqueous ethanol as far as possible, and were further compared to the corresponding authentic or synthesized specimen on their infrared (IR) spectra and melting points. After the examination mentioned above, the bases were identified.

Preparation of the authentic and synthesized quaternary ammonium bases.

For comparison with the bases in the algae, thirteen different specimens of quaternary ammonium bases were prepared by commercial means or syntheses. Glycine betaine hydrochloride, L-carnitine hydrochloride, trigonelline, trimethylamine oxide and choline chloride were purchased from the Nakarai Chemicals Co., Ltd. or the Katayama Chemical Industry Co., Ltd.. β-Homobetaine hydrochloride, γ-butyrobetaine hydrochloride, stachydrine hydrochloride, DL-valine betaine hydrochloride, homoserine betaine hydrochloride and candicine chloride [p-(2-trimethylaminoethyl) phenol] were synthesized by methylation of the amino nitrogen after the manner of ABE and KANEDA [17] from β -alanine, γ-aminobutyric acid, L-proline, DL-valine, L-homoserine and hordenine sulfate, respectively. Laminine dioxalate was synthesized from L-lysine after TAKEMOTO *et al.*[4] and betonicine hydrochloride was synthesized by treating hydroxy-L-proline with methyl iodide in the presence of silver oxide after PATCHETT and WITKOP.[20] These synthesized specimens were comfirmed the identities by determining the melting points and IR spectra. Subsequently, those bases were subjected to TLC and Dowex 50W-X12 (H^+ form, 200–400 mesh) chromatography with 2*N* hydrochloric acid as eluent for comparison with the bases in the algae.

RESULTS

The results of TLC and Dowex 50W-X12 column chromatography of the authentic quaternary ammonium bases are shown in Table 2 and Fig. 1, respectively.

Quaternary ammonium bases in the marine algae

The bases in each alga are named compound I to V temporarily according to the order in which they were eluted from the Dowex 50W-X12 column. The yields and Rf values of the quaternary ammonium bases isolated from algae are summarized in Table 3.

1. Harigane, *Ahnfeltia paradoxa*

Five bases were detected. Compound I was the most dominant component and the Rf values in TLC with three solvent systems were coincided with those of the synthesized betonicine. However, comparison of the IR spectra of the isolated and synthesized specimens showed distinct differences which probably reflect differences in stereoisomers. From these results, it can be assumed that compound I is betonicine (N, N-dimethyl hydroxyproline), yet the steric configuration still remains to be determined

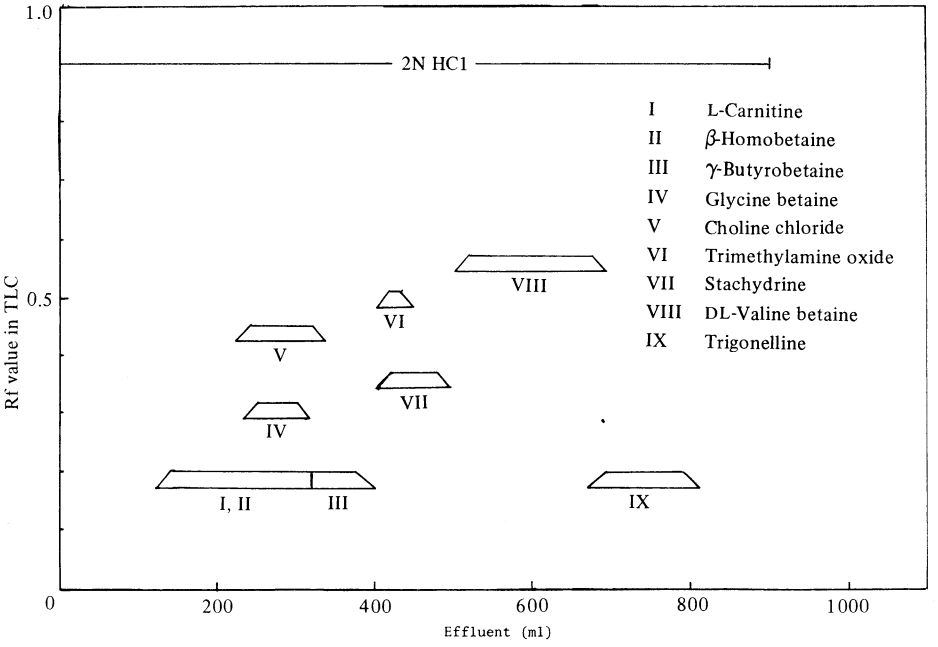

Fig. 1. The elution pattern of the authentic and synthesized quaternary ammonium bases.

Table 2. The Rf values of the authentic and sythesized quaternary ammonium bases in TLC

Bases	Solvents* (a)	(b)	(c)
Glycine betaine hydrochloride	0.31	0.24	0.34
L-Carnitine hydrochloride	0.18	0.26	0.34
Trigonelline	0.19	0.25	0.33
Trimethylamine oxide	0.50	0.41	0.51
Choline chloride	0.44	0.27	0.47
β-Homobetaine hydrochloride	0.19	0.24	0.28
γ-Butyrobetaine hydrochloride	0.19	0.28	0.28
Stachydrine hydrochloride	0.36	0.29	0.40
DL-Valine betaine hydrochloride	0.56	0.45	0.60
Homoserine betaine hydrochloride	0.31	0.24	0.36
Laminine dioxalate	0.07	0.08	0.23
Candicine chloride	0.56	0.40	0.57
Betonicine hydrochloride	0.27	0.20	0.32

* Cellulose microcrystalline plates with solvent systems; (a) 99.5% ethanol-28% ammonium hydroxide (85:15, v/v), (b) 1-butanol-acetic acid-water (4:1:2, v/v) and (C) 1-propanol-2. 5*N* ammonium hydroxide (7:3, v/v).

later. Compound II did not correspond in the Rf value to anyone of the authentic bases prepared here. Owing to insufficient sample size, the compound could not be subjected to further examination. Compound III showed the same Rf value and similar IR spectrum as those of the synthesized stachydrine. This compound was, therefore, comfirmed as stachydrine. Compound IV was positive to both the Dragendorff and ninhydrin reagents, showing the same Rf value as that of the synthesized laminine. The

Table 3. The yields and Rf values in TLC of the isolated bases in seven species of marine algae

Species	Bases	Yields (mg)	Rf values in TLC*		
			Solvent (a)	(b)	(c)
A. paradoxa	Compound I	1341	0.27	0.20	0.32
	II	2	0.19	0.35	0.29
	III	24	0.36	0.29	0.40
	IV	167	0.07	0.08	0.23
	V	32	0.56	0.40	0.57
P. leptopyllum	Compound I	12	0.31	0.24	0.34
	II	13	0.19	0.28	0.28
	III	9	0.36	0.29	0.40
P. fragilis	Compound I	7	0.31	0.24	0.34
	II	10	0.19	0.28	0.28
G. amansii	Compound I	2	0.19	0.24	0.28
	II	13	0.31	0.24	0.34
	III	5	0.19	0.28	0.28
	IV	7	0.36	0.29	0.40
B. coacta	Compound I	219	0.31	–	0.34
	II	1374	0.36	–	0.40
C. densa	Compound I	217	0.36	0.29	0.40
E. compressa	Compound I	27	0.31	0.24	0.34
	II	10	0.52	0.57	0.41

* Cellulose microcrystalline plates with solvent systems; (a) 99.5% ethanol-28% ammonium hydroxide (85:15, v/v), (b) 1-butanol-acetic acid-water (4:1:2, v/v) and (c) 1-propanol-2. 5*N* ammonium hydroxide (7:3, v/v).

dioxalate derivative of this compound was also identical with the synthesized laminine dioxalate in the IR spectrum and melting point (123–125°C). Compound IV was, therefore, identified as laminine. Compound V was identified as candicine since its chloride coincided with the synthesized candicine chloride in the Rf value, IR spectrum (Fig. 2) and melting point (256–261°C).

In the process of the condensation of the ethanolic extract and the successive treatment with diethyl ether for decoloration, a considerable amount of plate crystals appeared. This compound was easily recrystallized from hot water. Since it gave a distinct color with the Dragendorff reagent on paper, it is assumed to be a tertiary or quaternary ammonium derivative. Elucidation of the chemical structure of the compound is now under progress.

2. Hosoyukari, *Plocamium leptophyllum*

Three bases were detected. Compound I showed the same Rf value and similar IR spectrum as those of the synthesized glycine betaine, and is presumed to be glycine betaine. Compound II and III were identical with the synthesized γ-butyrobetaine and stachydrine, respectively, in their Rf values. However, they were not comfirmed by

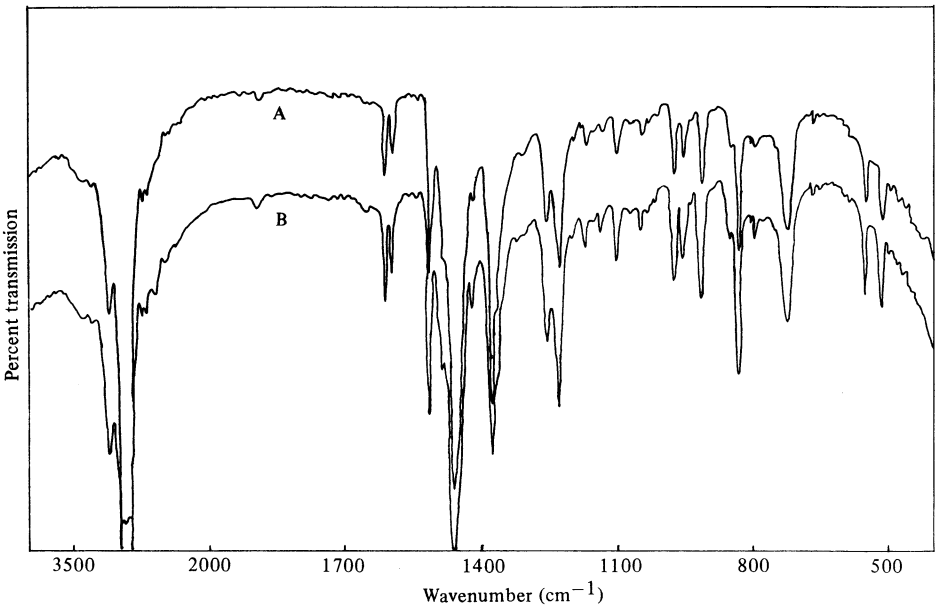

Fig. 2. The IR spectra of the isolated (A) and synthesized candicine chloride (B) in Nujol.

their IR spectra due to their low yields.

3. Kuroitogusa, *Polysiphonia fragillis*

Two bases were detected. Compound I was identical with the authentic glycine betaine in the Rf value and IR spectrum, and was identified as glycine betaine. Compound II showed the same Rf value as that of the synthesized γ-butyrobetaine. However, it was not comfirmed by the IR spectrum.

4. Makusa, *Gelidium amansii*

Four bases were detected. Compound II was identical with the authentic glycine betaine in the Rf value and IR spectrum, and was identified as glycine betaine. Compound I, III and IV were identical with the synthesized β-homobetaine, γ-butyrobetaine and stachydrine, respectively, in their Rf values. However, they were not comfirmed by their IR spectra due to their low yields.

5. Aomogusa, *Boodlea coacta*

Two bases were detected. Compound I was identical with the authentic glycine betaine in the Rf value and IR spectrum, and was identified as glycine betaine. Compound II showed the same Rf value and similar IR spectrum as those of the synthesized stachydrine, and is presumed to be stachydrine.

6. Asamidorishiogusa, *Cladophora densa*

Only one base was detected. Compound I showed the same Rf value as that of the synthesized stachydrine. However, it was not comfirmed by the IR spectrum.

7. Hiraaonori, *Enteromorpha compressa*

Two bases were detected. Compound I was identical with the authentic glycine betaine in the Rf value and IR spectrum, and was identified as glycine betaine. Compound II did not correspond in the Rf value to anyone of the authentic or

synthesized bases prepared here.

These results are summarized in Table 4.

Table 4. The quaternary ammonium bases detected in seven species of marine algae

Division	Species	Isolated bases*	Assumed content (mg/100g fresh frond)
Red algae	*A. paradoxa*	(Betonicine)	134.1
		Unidentified	0.2
		Stachydrine	2.4
		Laminine	16.7
		Candicine	3.2
	P. leptophyllum	Glycine betaine	4.3
		(γ-Butyrobetaine)	4.6
		(Stachydrine)	3.2
	P. fragilis	Glycine betaine	2.3
		(γ-Butyrobetaine)	3.3
	G. amansii	(β-Homobetaine)	0.8
		Glycine betaine	5.2
		(γ-Butyrobetaine)	2.0
		(Stachydrine)	2.8
Green algae	*B. coacta*	Glycine betaine	10.1
		Stachydrine	63.2
	C. densa	(Stachydrine)	16.7
	E. compressa	Glycine betaine	2.2
		Unidentified	0.8

* *The bases identified only by TLC, not but by the IR spectra are given in parentheses.*

DISCUSSION

Specimen of seven kinds of quaternary ammonium bases; laminine, candicine, stachydrine, glycine betaine, betonicine, β-homobetaine and γ-butyrobetaine, two unidentified bases and one unknown tertiary or quaternary ammonium derivative were detected in seven species of marine red and green algae. Except for β-homobetaine and γ-butyrobetaine, the other five bases were identified by determination of their Rf values, IR spectra and melting points in comparison with the corresponding authentic or synthesized quaternary ammonium bases. β-Homobetaine and γ-butyrobetaine were identified only by TLC, but not by the IR spectra and melting points.

Glycine betaine and stachydrine seem to be widely distributed in the marine algae, the former compound was detected in 6 species and the latter in 5 species among the 7 species of the algae examined here. Particularly, a large amount of stachydrine was found in the green alga *Boodlea coacta* (63.2 mg/100 g of fresh frond). γ-Butyrobetaine, whose occurrence in *Porphyra yezoensis* was reported earlier by ABE and KANEDA[14], was detected in 3 species of red algae but not found in the green algae examined here. This suggests that the presence of the base is common in red algae. β-Homobetaine was detected with low contents in only one species of red alga *Gelidium amansii*, though ABE and KANEDA [14]

isolated it from a green alga *Monostoroma nitidum.*

Candicine and laminine were detected in only one red alga *Ahnfeltia paradoxa.* It is the first time that candicine was isolated from marine algae. Candicine has been known as a toxic quaternary ammonium base, and found in the family Cactaceae of the terrestrial plant[21,22] and in the skin of a few neotropical species of frog, genus Leptodactlus[23]. The toxicity of this compound has been reported by CRAIG?[24] Recently, YASUMOTO and ENDO[25]recognized the presence of the compound in the guts of a turbon-shell *Turbo argyrostoma* during the survey for ciguatoxin in the area of the southwest pacific ocean, and supposed that this toxin may be derived from benthic algae or micro-organisms as the diet of the snail.

KAWAUCHI[26] has recognized the presence of hordenine [p-(2-dimethylaminoethyl) phenol] in the same species, *A. paradoxa* collected in February. We detected candicine [p-(2-trimethylaminoethyl) phenol], but not the hordenine in our specimen collected in June. This fact may indicate that hordenine is converted to candicine through N-methylation in the alga from February to June.

Laminine has been mainly found in brown algae, particularly the genus Laminaria, but also with very low contents in a few species of other marine algae, and has been popular as a hypotensive agent derived from brown algae. However, we also found the compound with high contents in the red alga *A. paradoxa* as well as KAWAUCHI and SAKAI[27] did in the same species, suggesting that the compound is widely distributed in marine algae.

SUMMARY

Seven species of marine red and green algae were examined for quaternary ammonium bases. Nine bases were purified and isolated by ion exchange column chromatography from the ethanolic extracts of the algae. The isolated bases were identified by measuring their Rf values in thin layer chromatography, infrared spectra and melting points in comparison with specimen of thirteen kinds of authentic quaternary ammonium bases which were prepared by commercial means or syntheses. As the results, seven bases; laminine, candicine, stachydrine, glycine betaine, betonicine, β-homobetaine and γ-butyrobetaine, and two unidentified bases were detected. It was first demonstrated that candicine is present in marine algae. There is no essential difference in the distribution of the bases among the species of marine algae examined here.

REFERENCES

1) DER MARDERSIAN, A. H. : *Lloydia,* **32**, 438–465 (1969).
2) MURAKAMI, N., TAKEMOTO, T. and SHIMIZU, N. : J. Pharm. Soc. Japan, **73**, 1026–1029 (1953).
3) DAIGO, K. : *ibid.,* **79**, 353–355, 356–360 (1959).
4) TAKEMOTO, T., DAIGO, K. and TAKAGI, N. : *ibid.*, **84**, 1176–1179, 1180–1182 (1964).
5) TAKEMOTO, T., DAIGO, K. and TAKAGI, N. : *ibid.,* **85**, 37–40, 843–845 (1965).
6) TODA, S. : *J. Biochem. Japan,* **2**, 433–436 (1923).
7) OZAWA, H., GOMI, Y. and OTSUKI, I. : *J. Pharm. Soc. Japan,* **87**, 935–939 (1969).

8) ZELLER, A. : *Biochem. Z.*, **268**, 187–188 (1934).
9) OGINO, C. : *Bull. Japan. Soc. Sci. Fish.*,**10**, 156–158 (1941).
10) OGINO, C. : *ibid.*, **12**, 48–51 (1943).
11) YABE, K., TSUJINO, I. and SAITO, T. : *Sci. Paper Hokkaido Fish. Sci. Inst.*, **16**, 273–277 (1966).
12) TAKEMOTO, T. and SAI, T. : *J. Pharm. Soc. Japan*, **84**, 1224–1227 (1964).
13) TAKAGI, N., HSU, H. Y. and TAKEMOTO, T. : *ibid.*, **90**, 899–902 (1970).
14) ABE, S. and KANEDA, T. : *Bull. Japan. Soc. Sci. Fish.*, **39**, 239, 383–389 (1973).
15) ABE, S. and KANEDA, T. : *ibid.*, **40**, 1199 (1974).
16) ABE, S. and KANEDA, T. : *ibid.*, **41**, 567–571 (1975).
17) ABE, S. and KANEDA, T. : *ibid.*, **39**, 391–393 (1973).
18) CHRISTIANSON, D. D., WALL, J. S., DIMMLER, R. J. and SENTI, F. R.: *Anal. Chem.*, **32**, 874–878 (1960).
19) KONOSU, S. and KASAI, E. : *Bull, Japan. Soc. Sci. Fish.*, **27**, 194–198 (1961).
20) PATCHETT, A. A.and WITKOP, B. : *J. Am. Chem. Soc.*, **79**, 185–192 (1957).
21) RABTZCH, G. : *Planta Med.*, **6**, 103–104 (1958).
22) RETI, L. : *C. R. Soc. Biol.*, **114**, 811–814 (1933).
23) CEI, J. M., ERSPAMER, V. and ROSGHINI, M. : *Systematic Zoology*, **16**, 328–342 (1967).
24) CRAIG, L. E. : *Chem. Rev.*, **42**, 285–410 (1948).
25) YASUMOTO, T. and ENDO, M. : *Bull. Japan. Soc. Sci. Fish.*, **40**, 841–845 (1974).
26) KAWAUCHI, H. and SASAKI, T. : *ibid.*, **44**, 135–137 (1978).
27) KAWAUCHI, H. and SASAKI, T. : At annual meeting of Japan. Soc. Sci. Fish. in April (1977).

7種海藻中における第4級アンモニウム塩基の分布

堀貫治・山本孝弘・宮沢啓輔・伊藤啓二

最近，海洋生化学資源の開発が重要視され，海産物由来のいくつかの有効物質が単離・同定されて，その生化学的および薬理学的役割が検討されてきている。その中で，海藻中にも2，3の有効物質が明らかにされてはいるものの，その数は少ない。本研究では，海藻中の有効物質が含窒素化合物に多いことから，海藻中の第4級アンモニウム塩基を7種の紅藻および緑藻を用いて検索した。

各海藻の70％ エタノール抽出液中の第4級アンモニウム塩基をイオン交換カラムクロマトグラフィーを用いて単離・精製し，そのTLC上のRf値，IRスペクトルおよび融点を測定して，購入および合成した既知の第4級アンモニウム塩基のそれらと比較することにより同定した。その結果，laminine，candicine，stachydrine，glycine betaine，betonicine，β-homobetaineおよびγ-boutyrobetaineと2種の未同定塩基を検出した。candicineが海藻中から単離・同定されたのは，今回が最初と思われる。これら塩基の海藻種間における分布に関しては，特に特異性は認められなかった。