Development of Chemical Substances Regulating Biofilm Formation

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The chemical substances which regulate biofilm formation were examined, and a bioassay system which uses marine bacteria with adhering properties was developed. This bioassay system is suitable for screening crude extracts from marine organisms. Using this system, those substances which regulate biofilm formation were isolated from marine organisms. For example, bis(deacetyl)solenolide D was obtained from the marine sponge *Psammaplysilla purpurea*. Novel nitroalkanes were also isolated from the Okinawan sponge *Callyspongia* sp. Ethyl *N*-(2-phenylethyl)carbamate isolated from the marine bacteria SCRC3P79 (*Cytophaga* sp.) inhibited biofilm formation. Furthermore, the *N,N*-dichloro, isocyanide, isothiocyanate, and dithiocarbamate derivatives of 2-(4-nitrophenyl)ethylamine, significantly inhibited the growth of marine attaching bacteria. Interestingly, most of the marine sponges examined contained anti-biofilm compounds, such as benzoic acid, aeroplysinin-I, and bromoageliferin.

Biofouling causes serious problems in the shipping business, in aquaculture and in the cooling systems of power stations. The biofouling of ship hulls increases drag, and consequently increases fuel costs. The biofouling of fishing nets in aquaculture prevents a smooth flow of sea water, followed by a serious decline in the number of fish due to insufficient oxygen supply. Metallic compounds, such as copper(I) oxide and bis(tributyltin) oxide (TBTO), have previously been used as antifouling agents. Today, however, the use of TBTO in antifouling paints is restricted in order to prevent environmental pollution. Therefore, the development of environmentally acceptable antifouling agents is essential for resolving this global problem.[1)]

The progress of biofouling on a newly immersed unprotected surface in sea water is well-documented.[2)] The surface rapidly adsorbs organic material, referred to as conditioning films, which may influence the subsequent settlement of microorganisms. Bacteria and diatoms are present soon after immersion, resulting in a biofilm covering the surface. The establishment of this microfouling biofilm layer is rapidly followed by macrofouling (Fig. 1). We have focused our attention on the formation of a microbial biofilm (microfouling), and have searched for compounds to prevent microfouling, which would consequently prevent macrofouling by barnacles, mussels and algae.

In this paper we describe: 1) a bioassay system for screening chemical substances which affect biofilm formation; 2) natural products, including bis(deacetyl)solenolide D and nitroalkanes, which regulate the biofilm formation; and 3) 2-phenylethylamine derivatives that significantly inhibit the growth of marine bacteria with adhering properties, some of which actually show antifouling activity in field tests.

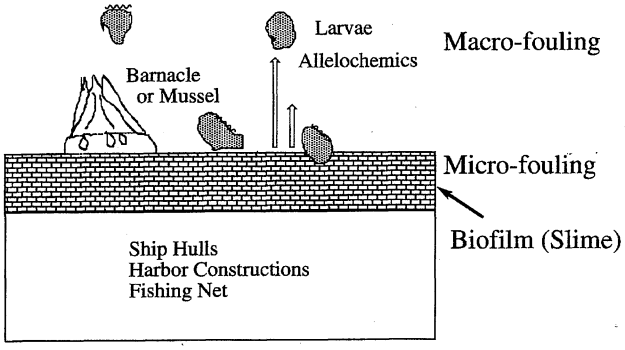
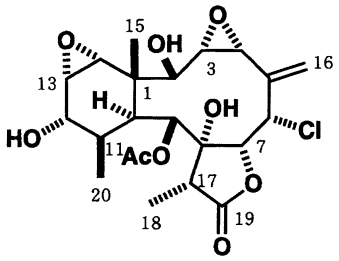


Fig. 1. Progression of biofouling.

Results and Discussion

Bioassay System 1. To identify chemical substances that regulate biofilm formation, two assay systems were developed. First, the number of marine bacteria that were attached to the surface of a glass tip was scored as an index of adhesion. The sticky acidic polysaccharides[3)] produced by marine bacteria adhering to objects are proportional to the number of attached bacteria. In an actual experiment, ground glass tips coated with test samples were immersed in the culture medium of attaching bacteria (*Rhodospirillum salexigens* SCRC 113) that had been separated from beach sand, and then the amounts of polysaccharide on the surface of the glass tips were determined by colorimetric testing with phenol–sulfuric acid.[4)] The results with coated and non-coated tips were compared. Sponges, bacteria and Actinomycetes were used as sources of active compounds. Bis(de-

acetyl)solenolide D (**1**) was isolated from the marine sponge *Psammaplysilla purpurea* as an inhibitor of biofilm formation (Chart 1). A decrease in polysaccharides was observed in this assay (Fig. 2). A 2-phenylethylamine derivative was isolated from the culture medium of the marine bacterium *Cytophaga* sp. SCRC 3P79. The active material was considered to be ethyl *N*-(2-phenylethyl)carbamate (**2**) (Chart 2), which also inhibits biofilm formation (Fig. 2). Congeners of this compound (Fig. 3) were synthesized in order to elucidate the structure-activity relationship of this series of compounds. None of the derivatives inhibited biofilm formation more than **2**. Among these derivatives, ethyl *N*-[2-(4-nitrophenyl)ethyl]carbamate (**3**) greatly inhibited the growth of the attaching bacteria. Therefore, the simple bioassay sys-



(**1**)

Chart 1.

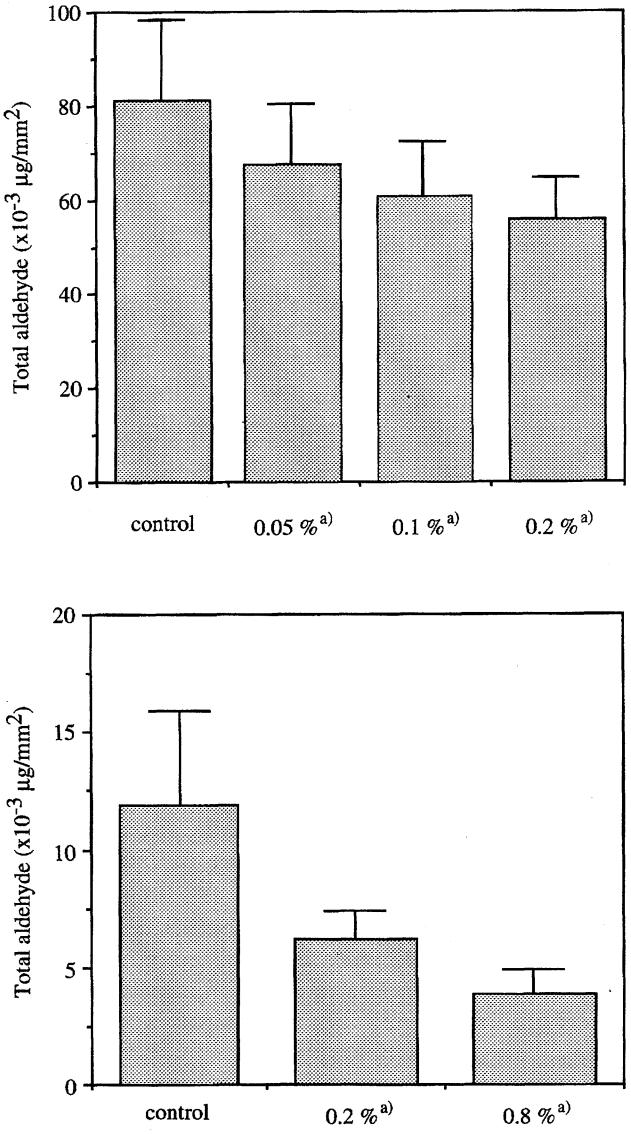
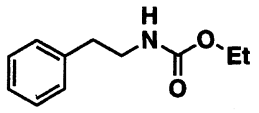
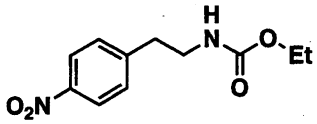


Fig. 2. Inhibitory effects of bis(deacetyl)solenolide D (**1**) (upper) and ethyl *N*-(2-phenylethyl)carbamate (**2**) (bottom) on biofilm formation. a) Concentration of the applied sample solution (e.g. 0.1% = 3 μg cm^{-2}).



(**2**)



(**3**)

Chart 2.

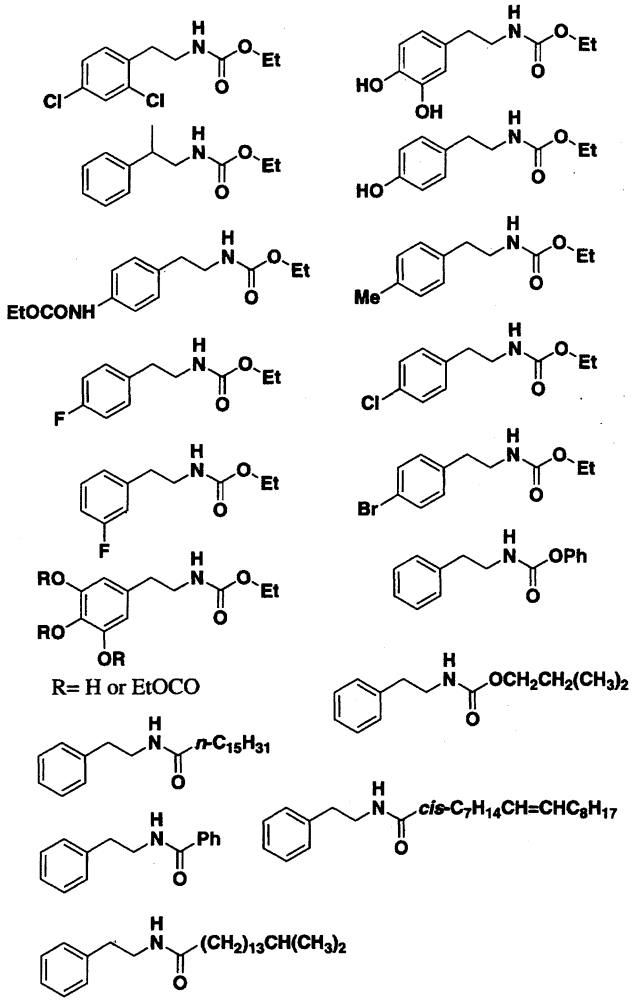


Fig. 3. Congeners of ethyl *N*-(2-phenylethyl)carbamate (**2**).

tem described above was replaced by a new method which depends on the growth inhibition of the attaching bacteria. The original bioassay system was complicated and did not provide adequate reproducibility.

Bis(deacetyl)solenolide D. Bis(deacetyl)solenolide D (**1**) was isolated from the marine sponge *Psammaplysilla purpurea*, collected on the coast of Ie island near Okinawa. The marine sponge *Psammaplysilla purpurea* (3.8 kg) was extracted with ethanol (10 L). The ethanolic filtrate was concentrated under reduced pressure to give an aqueous residue, which was partitioned between water and chloroform, and then ethyl acetate. The lipid-soluble extract was chromatographed on silica gel (eluted with a mixture of chloroform and methanol), followed by PTLC to yield bis(deacetyl)solenolide D (**1**) as a colorless glassy material [8.2 mg, 2.2×10^{-6}%, $[\alpha]_D^{20}-14.5°$ (*c* 0.51, MeOH)]. Bis(deacetyl)solenolide D (**1**) showed $(M+H)^+$ ion peaks at *m/z* 473 and 475 in FABMS, indicating a molecular formula of $C_{22}H_{29}O_9Cl$. The IR spectrum suggested the presence of two ester carbonyl groups (1760, 1740 cm^{-1}) and hydroxy groups (3420 cm^{-1}). In the ^1H NMR spectrum (Table 1) of bis(deacetyl)solenolide D (**1**), 26 protons, including one acetoxyl methyl group resonance at $\delta=2.20$ (3H, s) and six oxymethine group resonances ($\delta=3.56$; H-2, $\delta=3.35$; H-3, $\delta=3.83$; H-4, $\delta=5.21$; H-7, $\delta=5.29$; H-9, $\delta=3.53$; H-12, $\delta=3.10$; H-13, $\delta=3.16$; H-14) were observed. Since three protons disappeared in CD_3OD, the presence of three hydroxyl groups was suggested. The ^1H-^1H COSY and ^1H-^{13}C COSY spectra suggested the presence of one exomethylene group ($\delta=5.44$, 5.88; H-16, $\delta=118.2$; C-16) and one methine proton bearing a chlorine atom ($\delta=5.40$; H-6, $\delta=62.3$; C-6). A detailed analysis using the HMBC technique led us to a briarein skeleton[5] for bis(diacetyl)-solenolide D (**1**). The chemical shifts of H-13 and H-14 were at a relatively high field. Therefore, the presence of an oxirane ring between C-13 and C-14 was proposed. The methine protons, H-2 and H-12, were observed as a broad singlet in C_5D_5N. Consequently, two hydroxy groups are located at C-2 and C-12. Since H-3 and H-4 were not observed as broad signals in C_5D_5N, the epoxide is present between C-3 and C-4. Accordingly, one hydroxy group is present at C-8. The stereochemistry of bis(deacetyl)solenolide D (**1**) was determined by acetylation of **1**. Bis(deacetyl)solenolide D (**1**) was acetylated with Ac_2O/pyridine to give solenolide D.[6] The ^1H NMR spectral data and $[\alpha]_D$ were consistent with those of solenolide D.

Table 1. ^1H and ^{13}C NMR Spectral Data[a] of Bis(deacetyl)-solenolide D

Atom	^{13}C Shift	^1H Shift	*J* values
1	40.8 s		
2	75.2 d	3.56 d	8.8
3	64.4 d	3.35 dd	8.8, 4.4
4	59.4 d	3.83 d	4.4
5	139.4 s		
6	62.3 d	5.40 d	3.7
7	80.7 d	5.21 d	3.7
8	85.8 s		
9	71.5 d	5.29 d	8.4
10	39.5 d	1.65 dd	8.4, 2.4
11	41.1 d	2.29 dd	7.4, 2.4
12	64.4 d	3.53 m	
13	61.3 d	3.10 d	3.7
14	65.1 d	3.16 d	3.7
15	17.3 q	1.08 s	
16	118.2 t	5.44, 5.88 br. s	
17	46.7 d	2.63 d	7.0
18	7.0 q	1.09 d	7.0
19	178.0 s		
20	10.5 d	0.96 d	7.4
21	173.0 s		
22	22.8 q	2.20 s	

a) In CD_3OD (**δ**/ppm; s=singlet, d=doublet, t=triplet, dd=doublet of doublet, br. s=broad singlet, and m=multiplet).

Bioassay System 2. In the second bioassay system, inhibition of the growth of marine bacteria with adhering properties was examined. Ethyl *N*-[2-(4-nitrophenyl)ethyl]carbamate (**3**) remarkably inhibits the growth of attaching bacteria. The experiment in the laboratory was performed as follows. After ground-glass tips coated with test samples were immersed in a medium, and bacteria (*Rhodospirillum salexigens* SCRC 113) were inoculated into it. The medium was cultured for 3 d and the absorbance of the culture medium was measured in order to estimate the growth of bacteria. Extracts of sponges, coelenterates, bacteria and Actinomycetes were applied as sources of active compounds. Several kinds of molecules were found to inhibit the growth of attaching bacteria. Aeroplysinin-I[7] (**4**) was isolated from the marine sponge *Psammaplysilla purpurea* (Chart 3). Bromoageliferin[8] (**5**) was isolated from the marine sponge *Agelas* sp. Oroidine[9] (**6**) was obtained from an unidentified Okinawan sponge. The known compound 13-hydroxylobolide[10] (**7**) was purified from unidentified coelenterates of Amami island in Japan. Citrinin[11] (**8**) and chaetomin[12] (**9**) were isolated from filamentous fungi. The bioactivity of these compounds is shown in Table 2. New nitroalkanes, untenines A (**10**), B (**11**), and C (**12**),[13] were successfully isolated from the marine sponge *Callyspongia* sp. (Fig. 4).

Table 2. Growth Inhibitory Effects of Compounds Isolated from Marine Organisms

Sample	IC_{50} ($\mu g\ cm^{-2}$)[a]
(**1**)	>100
(**4**)	0.66
(**5**)	1.7
(**6**)	63
(**7**)	30
(**8**)	6.6
(**9**)	0.3[b]
(**10**)	3.1[b]
(**11**)	6.1[b]
(**12**)	5.8[b]

a) IC_{50}: 50% Inhibition Coefficient. b) IC_{99}.

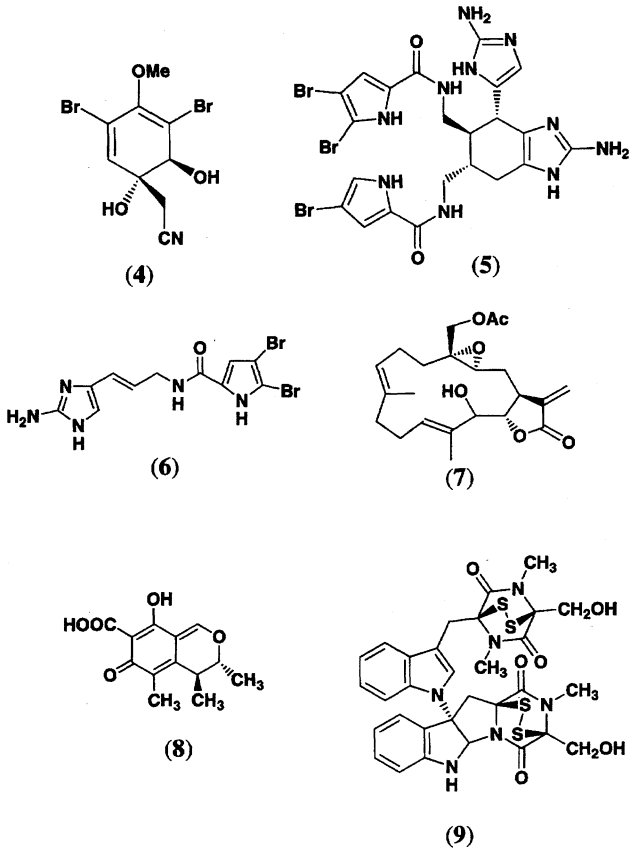


Chart 3.

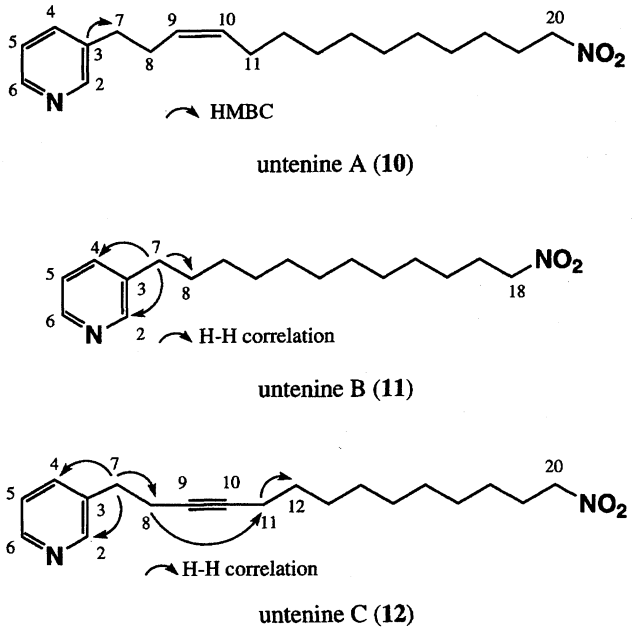
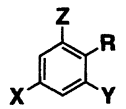


Fig. 4. Structures of untenines.

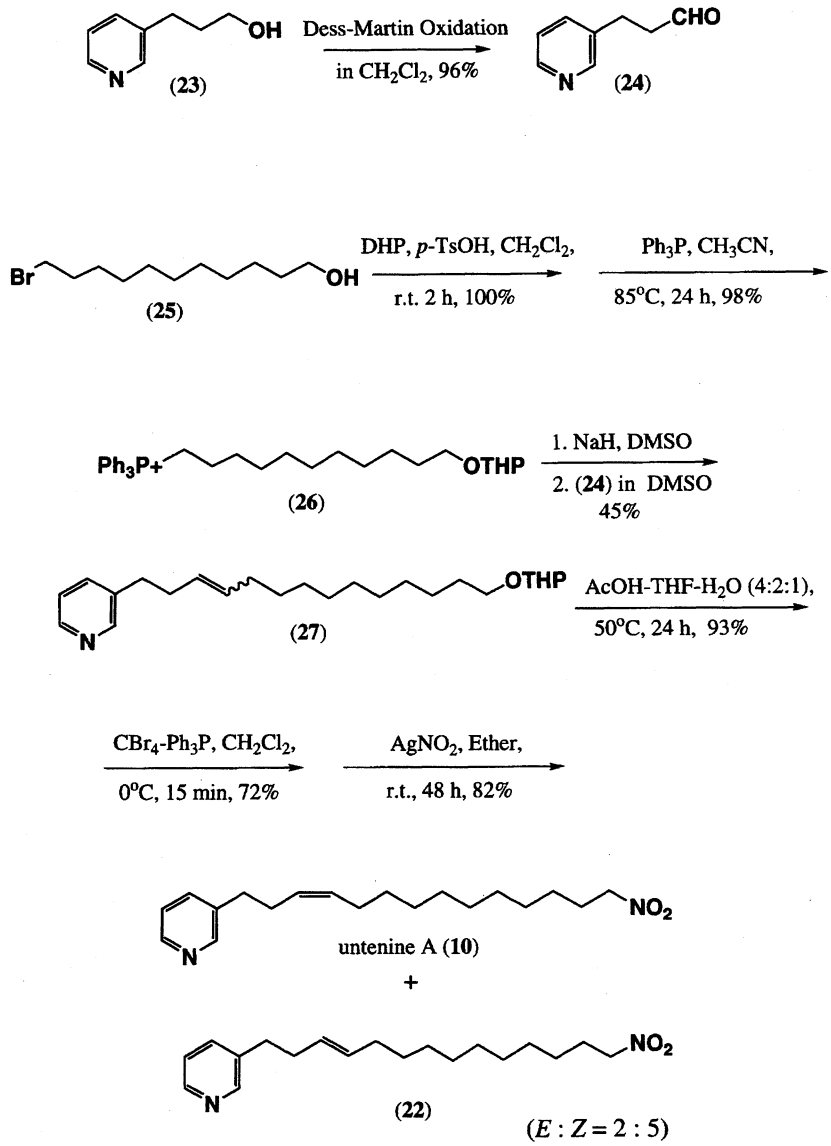
While the extract of the marine sponge *Halichondria okadai* inhibited the growth of attaching bacteria, the active substance was surprisingly benzoic acid (**13**). It should be noted that marine organisms use such simple molecules as defense substances. Various derivatives of 2-phenylethylamine and benzoic acid were tested by this assay system. The synthetic compounds, *N,N*-dichloro-2-(4-nitrophenyl)ethylamine (**14**), *N,N*-dichloro-2-(4-chlorophenyl)ethylamine (**15**), 2-(4-nitrophenyl)ethyl isocyanide (**16**), 2-(2,4-dichlorophenyl)ethyl isocyanide (**17**), 2-(4-nitrophenyl)ethyl isothiocyanate (**18**), 2-(2,4-dichlorophenyl)ethyl isothiocyanate (**19**), triethylammonium *N*-[2-(4-nitrophenyl)ethyl]dithiocarbamate (**20**), and salicylic acid (**21**) significantly inhibited the growth of attaching bacteria. The growth inhibitory activity of 2-phenylethylamine derivatives is shown in Table 3. The magnitude of this activity depends on the functional groups on the alkyl chain. Compounds containing an electron-withdrawing group, for example NO_2 or Cl, on a benzene ring, also exhibited significant activity.

Untenines. Untenines were isolated from the marine sponge *Callyspongia* sp., collected in Unten, Okinawa. Concentrates of methanolic extracts of the marine sponge *Callyspongia* sp. (350 g) were in turn extracted with ethyl acetate. The organic extract was chromatographed on SiO_2 (eluted with a mixture of hexane and ethyl acetate). Further

Table 3. Biological Activity of 2-Phenylethylamine Derivatives and Benzoic Acid Analogs



Sample No.	X	Y	Z	R	Bioassay System 2 IC_{50} ($\mu g\,cm^{-2}$)	Field experiment IC_{99} ($\mu mol\,cm^{-2}$)
(**2**)	H	H	H	$CH_2CH_2NHCOOEt$	>300	—
(**3**)	NO_2	H	H	$CH_2CH_2NHCOOEt$	300	—
(**13**)	H	H	H	COOH	93	—
(**14**)	NO_2	H	H	$CH_2CH_2NCl_2$	1.0	50
(**15**)	Cl	H	H	$CH_2CH_2NCl_2$	8.6	300
(**16**)	NO_2	H	H	CH_2CH_2NC	0.49	50
(**17**)	Cl	Cl	H	CH_2CH_2NC	1.5	—
(**18**)	NO_2	H	H	CH_2CH_2NCS	0.13	50
(**19**)	Cl	Cl	H	CH_2CH_2NCS	1.5	50
(**20**)	NO_2	H	H	$CH_2CH_2NHCS_2H$	0.5	50
(**21**)	OH	H	H	COOH	0.5	>300



Scheme 1. Synthesis of untenine A (**10**) and (**22**).[10]

purification was carried out using ODS HPLC (eluted with 70% MeCN–H_2O) to yield untenines A (**10**) (1.0 mg), B (**11**) (0.6 mg), and C (**12**) (0.6 mg). The spectral data of these untenines are given in the experimental section.

The molecular formula of untenine A (**10**) was determined to be $C_{19}H_{30}N_2O_2$ by HREIMS ($m/z = 318.2286$, $\Delta - 1.9$ mmu). The ^{13}C NMR spectrum and DEPT spectrum showed that there were seven unsaturated carbons, consisting of six tertiary carbons and one quaternary carbon, twelve saturated secondary carbons and no primary or other tertiary carbons. The ^1H NMR and ^{13}C NMR data of untenine A indicated the presence of 3-substituted pyridine and one disubstituted olefin ($\delta = 8.35$; H-2, $\delta = 7.68$; H-4, $\delta = 7.33$; H-5, $\delta = 8.33$; H-6, $\delta = 5.33$; H-9, H-10, $\delta = 151.0$; C-2, $\delta = 140.2$; C-3, $\delta = 139.2$; C-4, $\delta = 125.8$; C-5, $\delta = 148.3$; C-6, $\delta = 129.6$; C-9, $\delta = 133.2$; C-10).[11] The ^1H-^1H COSY spectrum suggested a linear partial structure from C-7 to C-12 (Fig. 4). The C-9 double bond was determined to exhibit a *Z*-geometry based on the coupling constant of H-9/H-10 (11 Hz) in the *J*-resolution spectrum and the stronger shielding of the carbons ($\delta = 30.5$; C-8, $\delta = 28.9$; C-11) than in the *E*-isomer (**22**) ($\delta = 35.7$; C-8, $\delta = 34.3$; C-11). The *E*- and *Z*-isomers were synthesized as shown in Scheme 1.[13] Dess–Martin oxidation[14] of 3-pyridinepropanol[15] (**23**) gave 3-pyridinepropanal (**24**). The phosphonium salt (**26**) was obtained from 11-bromo-1-undecanol[15] (**25**) by protecting the hydroxy group of **25** with tetrahydropyranyl and reacting this mixture with triphenylphosphine in acetonitrile at 85 °C for 24 h. A Wittig reaction of aldehyde (**24**) with the ylide formed by the reaction of phosphonium salt (**26**) and NaH in anhydrous DMSO gave pyridylalkene (**27**) ($E:Z = 2:5$). Untenine A (**10**) and the *E*-isomer (**22**) were obtained from pyridylalkene (**27**) by 1) removal of the tetrahydropyranyl group in AcOH–THF–H_2O; 2) bromination with carbon tetrabromide and triphenylphosphine, followed by a treatment with cold 10% sodium hydrogencarbonate solution for 5 min; and 3) a reaction with silver nitrate by stirring in ether in the dark. The NMR resonances of H-20 ($\delta = 4.22$)

and C-20 ($\delta = 77.4$) were particularly interesting because of their gradual disappearance when untenine A was allowed to stand in CD_3OD for more than one week. The acidity of H-20 led us to assign the partial structure $-CH_2NO_2$. The connection of C-7 to C-3 in the pyridine ring was suggested by the correlation of H-7/C-3 in the HMBC spectrum (Fig. 3). Thus, the structure of untenine A was clarified.

The molecular formula of untenine B (**11**) was determined to be $C_{17}H_{28}N_2O_2$ by HREIMS ($m/z = 292.2152$, $\Delta + 0.3$ mmu). The five degrees of unsaturation were accounted for by the 3-alkyl-substituted pyridine ring and one nitro group. The saturated linear alkyl chain terminated by $-CH_2NO_2$ ($\delta = 4.42$; H-17, $\delta = 77.4$; C-17) was assigned according to NMR data and the EIMS fragments [$m/z = 246 - 14 \times n$; $(M-NO_2-CH_2)^+$ and $92+14 \times n$; $(C_5H_4NCH_2+nCH_2)^+$]. The ^1H-^1H COSY spectra showed correlation peaks of H-7/H-8 and long-range correlation of H-7/H-2 and H-4. Therefore, the structure of untenine B was clarified.

The molecular formula of untenine C (**12**) was determined to be $C_{19}H_{28}N_2O_2$ by HREIMS ($m/z = 316.2153$, $\Delta + 0.4$ mmu). In addition to the presence of the 3-alkyl-substituted pyridine ring and one nitro group, the NMR spectra also showed the existence of one acetylenic bond ($\delta = 80.4$; C-9, $\delta = 83.5$; C-10) and no olefinic bonds. The ^1H-^1H COSY spectra showed correlation peaks of H-7/H-8 and H-11/H-12, and a long-range correlation of H-7/H-2 and H-4 and H-8/H-11. Therefore, the structure of untenine C was determined.

Field Experiments. Field experiments[16] were carried out on substances that inhibited the growth of attaching bacteria in bioassay system 2 at Shimoda Marine Research Center, University of Tsukuba, Shimoda, in Shizuoka Prefecture. Compounds **14**, **15**, **16**, **18**, and **20** exhibited significant antifouling activity in this field experiment. Salicylic acid (**21**), which is the most active among the benzoic acid analogs, did not exhibit antifouling activity, since it is highly soluble in water. The relationship between the results of the field experiment and bioassay system 2 are given in Table 3.

Experimental

Bioassay System 1. Standard Curve. To a 0, 10, 30, 50 or 80 $\mu g\,ml^{-1}$ glucose solution (1 ml each) were added 1 ml of a 5% phenol–H_2O solution and 5 ml of concentrated H_2SO_4. After the resulting solution was allowed to stand for 10 min, the solution was stirred at 25 °C for 30 min. The absorbance at 490 nm was measured. The absorbance data, which reflected the total aldehydes, were plotted against the concentration of glucose. The resulting standard curve was used throughout this study.

Estimation of Biofilm Formation. A glycerol stock (100 µl) of the strain *Rhodospirillum salexigens* SCRC 113 was inoculated into a 1-L beaker containing 500 ml of a medium with the following composition: yeast extract 0.1%, peptone 0.5%, and artificial sea water diluted twice. Before sterilization, the pH was adjusted to 7.2. The culture was slowly stirred for 2 d at 25 °C. A methanolic solution (10 µl) of the test sample was applied to each side of a ground-glass tip (13 mm×26 mm), which was dried. These glass tips were mounted on a holder, and immersed into the resulting culture. The culture was slowly stirred for 5 d at 25 °C. The holder with glass tips was removed from the culture and submerged in 500 ml of a 1.5% NaCl solution to wash off the surface and to remove the reversible adhesion of bacteria. An aqueous 5% phenolic solution (1 ml) and concentrated H_2SO_4 (5 ml) were added to the test tube with the glass tip. Ten minutes later, the reaction mixtures were stirred at 25 °C for 30 min, and their absorbances at 490 nm were measured. The amount of sugar attached to the glass tip was determined from a standard curve and the inhibitory effect on biofilm formation was estimated.

Bis(deacetyl)solenolide D (1). The marine sponge *Psammaplysilla purpurea* (3.8 kg) was collected in July 1991 on the coast of Ie island, Okinawa Prefecture, and frozen. The marine sponge was minced with a Waring blender and extracted with ethanol. The extracted solution was filtered and the residue was rinsed with ethanol. The ethanol extract was concentrated and the resulting concentrate was partitioned between water and ethyl acetate. The aqueous layer was further extracted with chloroform. These organic extracts were evaporated to give an oily material, which was chromatographed on silica gel using chloroform an methanol. Bis(deacetyl)solenolide D (**1**) was eluted from the column with 5% MeOH–$CHCl_3$. This eluate was concentrated and purified by PTLC (ethyl acetate, 0.5 mm), to give bis(deacetyl)solenolide D (**1**) as a colorless glassy material (8.2 mg): $[\alpha]_D^{20} - 14.5°$ (*c* 0.51, MeOH); IR (KBr) 3420, 2920, 1760, 1740, 1570, 1400, 1210, 1100, 1020, 650 cm^{-1}; FABMS $m/z = 473$ and 475 for $C_{22}H_{29}O_9Cl$.

Bis(deacetyl)solenolide D (**1**) (1.0 mg, 2.1×10^{-6} mmol) was treated with pyridine (0.4 ml) and acetic anhydride (0.2 ml). The reaction mixture was stirred for 9 h at room temperature. The solution was then evaporated to give solenolide D (0.4 mg, 37%, 7.8×10^{-7} mmol), which showed spectral data consistent with those reported previously, including the optical rotation.[6]

Bioassay System 2. A glycerol stock (50 µl) of the strain *Rhodospirillum salexigens* SCRC 113 was inoculated into a test tube containing 8 ml of a medium consisting of yeast extract 0.1%, peptone 0.5%, and artificial sea water diluted twice. Before sterilization, the pH was adjusted to 7.2. The culture was shaken for 1 d at 25 °C. A methanolic solution (10 µl) of the test sample was applied to each side of a ground glass tip (13 mm×26 mm), which was dried. The seed culture (100 µl) was inoculated into a test tube containing 8 ml of medium and this glass tip. The culture was shaken for 3 d at 25 °C. The absorbance of the resulting culture medium at 610 nm was measured. The inhibition of biofilm formation was estimated by a comparison with standard absorbance; i.e., a noncoated glass tip.

Aeroplysinin-I[7] **(4).** The marine sponge *Psammaplysilla purpurea* (3.8 kg) was collected in July 1991 on the coast of Ie Island near Okinawa, and frozen. It was then minced with a Waring blender and extracted with ethanol. The extracted solution was filtered and the residue was rinsed with ethanol. The ethanolic extract was concentrated and the resulting concentrate was partitioned between water and ethyl acetate. The aqueous layer was further extracted with chloroform. These organic layers were evaporated to give an oily material, which was chromatographed on SiO_2 using chloroform and methanol. Aeroplysinin-I[7] (**4**) was eluted from the column with 2% MeOH–$CHCl_3$. This eluate was concentrated and further purified by PTLC (7% MeOH–$CHCl_3$, 0.5 mm), to give Aeroplysinin-I (**4**) as a colorless glassy material (3.9 mg): ^1H NMR (CD_3CN, ppm) $\delta = 2.78$ (2H, s), 3.72 (3H, s), 4.10 (2H, br.), 6.36 (1H, s); ^{13}C NMR ($CDCl_3$, ppm) $\delta = 30.0$, 60.5, 74.6, 77.6, 112.6, 118.9, 121.4, 131.9, 148.3; EIMS: $m/z = 337$ (M^+), 339, 341.

Bromoageliferin[8] **(5).** The marine sponge *Agelas* sp. (1.7 kg) was collected in Unten, Okinawa in 1994 and frozen. The marine sponge was minced with a Waring blender and extracted with

methanol. After filtration, the methanolic extract was concentrated and the resulting concentrate was partitioned between water and ethyl acetate. The ethyl acetate extract was concentrated. The organic extract was partitioned between an aqueous 70% methanolic solution and hexane. The methanolic layer was evaporated to give an oily material, which was chromatographed on SiO_2 using chloroform and methanol. Bromoageliferin[8] (**5**) was eluted from the column with 50% MeOH–$CHCl_3$. This eluate was concentrated and chromatographed on ODS using an aqueous methanolic solution, to give bromoageliferin (**5**) as a colorless glassy material (5.1 mg): ^{1}H NMR (CD_3OD, ppm) δ = 2.18 (1H, m), 2.28 (1H, m), 2.49 (1H, ddd, J = 16, 8.0 and 1.5 Hz), 2.77 (1H, ddd, J = 16, 5.0 and 1.5 Hz), 3.51 (1H, dd, J = 15 and 5.0 Hz), 3.63 (1H, dd, J = 15 and 4.5 Hz), 3.73 (1H, dd, J = 15 and 4.5 Hz), 3.84 (1H, br. d, J = 7.5 Hz), 6.79 (1H, br. s), 6.86 (1H, d, J = 1.5 Hz), 6.94 (1H, d, J = 1.5 Hz), 7.03 (1H, s); ^{13}C NMR (CD_3OD, ppm) δ = 21.7, 31.3, 35.2, 38.2, 40.8, 42.0, 95.7, 98.3, 104.6, 111.1, 111.9, 113.3, 117.2, 121.0, 121.2, 125.4, 125.7, 128.5, 147.2, 147.2, 160.5, 161.0; FABMS: m/z=697 $(M+H)^+$, 699, 701, 703.

Oroidine[9] **(6).** The unidentified marine sponge ISG9601 (2.5 kg) was obtained on Ishigaki island, Okinawa in 1996 and frozen. The marine sponge was minced with a Waring blender and extracted with methanol. The extracted solution was filtrated and its residue was rinsed with ethanol. The methanolic extract was concentrated and the resulting concentrate was partitioned between water and ethyl acetate. After concentration of the ethyl acetate layer, the concentrate was partitioned between an aqueous 70% ethanolic solution and hexane. The aqueous extract was evaporated to give an oily material, which was chromatographed on SiO_2 using chloroform and methanol. Oroidine[9] (**6**) was eluted from the column with 25% MeOH–$CHCl_3$. This eluate was concentrated and chromatographed on ODS by using aqueous methanolic solution. The 50 and 75% MeOH–H_2O eluates were further purified by HPLC (TSKgel NH2-60, eluted with 15% MeOH–$CHCl_3$) to yield oroidine (**6**) as a colorless glassy material (2.5 mg): ^{1}H NMR (CD_3OD, ppm) δ = 4.00 (2H, dd, J = 6.2 and 1.3 Hz), 5.87 (1H, dt, J = 15.8 and 6.2 Hz), 6.29 (1H, dd, J = 15.8 and 1.3 Hz), 6.46 (1H, s), 6.83 (1H, s); ^{13}C NMR (CD_3OD, ppm) δ = 42.4, 99.9, 106.1, 114.3, 121.4, 121.4, 122.8, 128.9, 152.0, 161.5; EIMS: m/z=392 (M^+), 390, 388.

13-Hydroxylobolide[10] **(7).** The unidentified marine coelecterates (0.1 kg) collected in Amami island in 1995 were minced with a Waring blender and extracted with methanol. The extracted solution was filtered and the residue was rinsed with ethanol. The aqueous extract which was obtained by evapolation of methanol was partitioned between water and ethyl acetate. The ethyl acetate layers were evaporated to give an oily material, which was chromatographed on SiO_2 using chloroform and methanol. 13-Hydroxylobolide[10] (**7**) was eluted from the column with 5% MeOH–$CHCl_3$. This eluate was concentrated and purified by PTLC (7% MeOH–$CHCl_3$, 0.5 mm) and further purified by HPLC (ODS, eluted with 70% MeOH–H_2O) to yield 13-hydroxylobolide (**7**) as a colorless glassy material (3.3 mg): ^{13}C NMR ($CDCl_3$, ppm) δ = 12.4, 15.8, 20.8, 23.7, 24.7, 31.4, 32.6, 38.5, 42.3, 60.4, 62.5, 63.9, 81.0, 82.0, 124.2, 124.4, 131.8, 132.2, 135.1, 138.5, 186.9, 170.7.

Citrinin[11] **(8).** The fungus *Eurotium* sp. (FK-1036) was inoculated into a 500-ml Erlenmeyer flack containing 250 ml of a medium with the following composition: glucose 3.0%, soy peptide ('Hi Nute SMP', Fuji Oil Co., Ltd.) 1.0%, meat extract 0.3%, Polypeptone (Nihon Pharmaceutical Co., Ltd.) 0.3%, yeast extract 0.3%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%. Before sterilization, the pH was adjusted to 5.8. The culture was shaked for 7 d at 28 °C, and filtered. The filtered medium was extracted with ethyl acetate. The ethyl acetate extract was evaporated to give an oily material, which was chromatographed on SiO_2 using chloroform and methanol to yield citrinin[11] (**8**) as a colorless glassy material (28.5 mg): ^{1}H NMR ($CDCl_3$, ppm) δ = 1.22 (3H, d, J = 7.3 Hz), 1.34 (3H, d, J = 6.7 Hz), 2.98 (1H, br. q, J = 7.3 Hz), 4.77 (1H, dq, J = 6.7 and 0.9 Hz), 15.11 (1H, s), 15.88 (1H, s); ^{13}C NMR ($CDCl_3$, ppm) δ = 9.4, 18.2, 18.5, 34.5, 81.6, 100.3, 107.3, 123.1, 139.0, 162.8, 174.5, 177.2, 183.8; EIMS: m/z = 250 (M^+).

Chaetomin[12] **(9).** The fungus *Chaetomium* sp. (FK-0994) was inoculated into five 2-L Erlenmeyer flasks each containing 1.25 L of medium with the same composition (vide supra). Before sterilization, the pH was adjusted to 5.8. The culture was shaked for 7 d at 28 °C, and filtrated. The mycelium cake was extracted with chloroform and methanol. This organic extract was evaporated to give an oily material, which was chromatographed on SiO_2 using chloroform and methanol. Chaetomin[12] (**9**) was eluted from the column with 5% MeOH–$CHCl_3$. This eluate was concentrated and purified by PTLC (5% MeOH–$CHCl_3$, 0.5 mm) yield chaetomin (**9**) as a colorless glassy material (12.5 mg): ^{1}H NMR ($CDCl_3$, ppm): δ = 2.96 (3H, s), 3.09 (1H, d, J = 15.4 Hz), 3.16 (3H, s), 3.19 (3H, s), 3.70 (1H, d, J = 15.5 Hz), 3.87 (1H, dd, J = 15.5 and 0.9 Hz), 4.31 (4H, m), 4.41 (1H, d, J = 15.4 Hz), 6.21 (1H, s), 6.79 (1H, d, J = 7.9 Hz), 6.94 (1H, dt, J = 7.5 and 0.8 Hz), 7.19—7.34 (5H, m), 7.66 (1H, m); ^{13}C NMR ($CDCl_3$, ppm) δ = 27.1, 27.4, 27.5, 28.2, 42.6, 60.6, 61.2, 73.5, 73.8, 74.8, 76.1, 76.5, 80.1, 107.7, 111.2, 111.4, 119.2, 120.4, 120.6, 122.8, 125.0, 126.5, 127.2, 130.4, 131.4, 134.0, 148.3, 163.2, 165.5, 165.5, 166.8.

Untenines. The marine sponge *Callyspongia* sp. was collected in Unten, Okinawa in 1994 and frozen. The marine sponge *Callyspongia* sp. (350 g) was crushed and extracted with methanol. The methanolic concentrate was partitioned between ethyl acetate and water. The organic layer was concentrated and chromatographed on SiO_2 (hexane and ethyl acetate), with monitoring of the growth inhibition of *R. salexigens* by bioassay system 2. The bioactive 40 and 60% AcOEt/hexane eluates were further purified by ODS HPLC (YMC-ODS-AQ AQ303, eluted with 70% MeCN–H_2O) to yield untenines A (**10**) (1.0 mg), B (**11**) (0.6 mg), and C (**12**) (0.6 mg).

Spectral Data of Untenine A (10). ^{1}H NMR (CD_3OD, ppm) δ = 1.15—1.38 (14H, m; H-12-H-18), 1.9 (2H, m; H-11), 1.96 (2H, m; H-19), 2.38 (2H, m; H-8), 2.71 (2H, t, J = 7.3 Hz; H-7), 4.42 (2H, t, J = 7.1 Hz; H-20), 5.37 (2H, m; H-9 and H-10), 7.33 (1H, dd, J = 8.0 and 4.8 Hz; H-5), 7.68 (1H, d, J = 8.0 Hz; H-4), 8.33 (1H, d, J = 4.8 Hz; H-6), 8.35 (1H, br. s; H-2); ^{13}C NMR (CD_3OD, ppm) δ = 28.1 (*), 28.9 (C-11), 29.2 (C-19), 30.5 (C-8), 30.7 (*), 30.8 (*), 31.2 (*), 31.3 (*), 31.3 (*), 31.4 (C-12), 34.6 (C-7), 77.4 (C-20), 125.8 (C-5), 129.6 (C-9), 133.2 (C-10), 139.2 (C-4),140.2 (C-3), 148.3 (C-6), 151.0 (C-2), (*; for each C-13–C-18). EIMS: m/z=318 (M^+), HREIMS: m/z = 318.2286, for $C_{19}H_{30}N_2O_2$, Δ − 1.9 mmu.

Spectral Data of Untenine B (11). ^{1}H NMR (CD_3OD, ppm) δ = 1.20—1.40 (16H, m; H-9–H-16), 1.63 (2H, m; H-8), 1.96 (2H, m; H-17), 2.65 (2H, t, J = 7.2 Hz; H-7), 4.42 (2H, t, J = 7.1 Hz; H-18), 7.33 (1H, dd, J = 8.0 and 4.8 Hz; H-5), 7.68 (1H, d, J = 8.0 Hz; H-4), 8.32 (1H, d, J = 4.8 Hz; H-6), 8.35 (1H, br. s; H-2); ^{13}C NMR (CD_3OD, ppm) δ = 28.1 (*), 29.2 (C-17), 30.0—31.5 (*), 34.1 (C-7), 77.4, (C-18), 125.6 (C-5), 139.1 (C-4), 140.5 (C-3), 148.4 (C-6), 151.1 (C-2), (*; for each C-8–C-16). EIMS: m/z = 292 (M^+), HREIMS: m/z = 292.2152, for $C_{17}H_{28}N_2O_2$, Δ +0.3 mmu.

Spectral Data of Untenine C (12). ^{1}H NMR (CD_3OD, ppm) δ = 1.20-1.45 (14H, m; H-12—H-18), 1.96 (2H, m; H-19), 2.07 (2H, m; H-11), 2.46 (2H, m; H-8), 2.80 (2H, t, J = 7.3 Hz; H-7),

4.44 (2H, t, $J=7.1$ Hz; H-20), 7.34 (1H, dd, $J=8.0$ and 4.8 Hz; H-5), 7.74 (1H, d, $J=8.0$ Hz; H-4), 8.37 (1H, d, $J=4.8$ Hz; H-6), 8.42 (1H, br. s; H-2); ^{13}C NMR (CD_3OD, ppm) $\delta=20.0$ (C-11), 22.0 (C-8), 28.1 (*), 29.1 (C-19), 30.5—31.5 (*), 34.2 (C-7), 77.4 (C-20), 80.4 (C-9), 83.5 (C-10), 125.6 (C-5), 139.1 (C-4), 140.6 (C-3), 148.6 (C-6), 151.2 (C-2), (*; for each C-13–C-18). CIMS: $m/z=317$ $(M+H)^+$, HREIMS: $m/z=316.2153$, for $C_{19}H_{28}N_2O_2$, Δ +0.4 mmu.

Field Experiments. Field experiments were carried out at Shimoda Marine Research Center, University of Tsukuba. In this field experiment,[16] circles 5.0 cm in diameter were drawn on a 25 cm×25 cm poly(vinyl chloride) resin plate, and the test samples and 150 mg of poly(vinyl butyral) resin were applied to each circle. These plates were immersed in a moderate stream of sea water and observed every four weeks.

Synthesis of Ethyl *N*-[2-(4-Nitrophenyl)ethyl]carbamate (3). To a solution of 2-(4-nitrophenyl)ethylamine hydrochloride (2.0 g), 4-(dimethylamino)pyridine (0.1 g) and pyridine (4.5 g) in CH_2Cl_2 (50 ml), ethyl chloroformate (2.6 g) was added at 0 °C under Ar. The mixture was stirred at room temperature for 50 min and concentrated. H_2O (100 ml) was added to this mixture and the aqueous solution was extracted with three portions of ethyl acetate (100 ml). The ethyl acetate layers were combined and dried with sodium sulfate and concentrated to give 2.4 g of ethyl *N*-[2-(4-nitrophenyl]-carbamate as yellow crystals.[17] ^{1}H NMR ($CDCl_3$, ppm) $\delta=1.18$ (3H, t, $J=7.1$ Hz), 2.90 (2H, br. t, $J=7.0$ Hz), 3.43 (2H, q, $J=6.7$ Hz), 4.06 (2H, q, $J=7.1$ Hz), 4.89 (1H, br. s), 7.33 (2H, d, $J=8.7$ Hz), 8.11 (2H, d, $J=8.7$ Hz). EIMS: $m/z=238$ (M^+); mp = 92.5—93.0 °C.

Synthesis of *N,N*-Dichloro-2-(4-nitrophenyl)ethylamine (14). To a suspension of 2-(4-nitrophenyl)ethylamine hydrochloride (500 mg) in $CHCl_3$ (50 ml), 20% NaOH was added. The $CHCl_3$ extract was dried over Na_2SO_4 and concentrated. To a solution of this product in dried benzene (5 ml), *t*-butyl hypochlorite (0.7 ml)[18] in dried benzene (10 ml) was added at 10 °C. The mixture was stirred at room temperature for 2 h and concentrated.[19] The crude mixture was purified by SiO_2 column chromatography (benzene/hexane = 1/1) to give 535 mg of *N,N*-dichloro-2-(4-nitrophenyl)ethylamine as colorless crystals. ^{1}H NMR ($CDCl_3$, ppm) $\delta=3.17$ (2H, t, $J=7.0$ Hz), 3.89 (2H, t, $J=7.0$ Hz), 7.39 (2H, d, $J=8.7$ Hz), 8.18 (2H, d, $J=8.7$ Hz). EIMS: $m/z=234$ (M^+), 236, 238; mp = 63.8—64.1 °C.

Synthesis of *N,N*-Dichloro-2-(4-chlorophenyl)ethylamine (15). To a solution of 2-(4-chlorophenyl)ethylamine (5 g) in dried benzene (10 ml), *t*-butyl hypochlorite (8.5 ml)[18] in dried benzene (20 ml) was added at 10 °C. The mixture was stirred at room temperature for 2 h and concentrated.[19] The crude mixture was purified by SiO_2 column chromatography (benzene/hexane = 1/1) to give 6.8 g of *N,N*-dichloro-2-(4-chlorophenyl)ethylamine as a brown oil. ^{1}H NMR ($CDCl_3$, ppm) $\delta=3.04$ (2H, t, $J=7.3$ Hz), 3.84 (2H, t, $J=7.3$ Hz), 7.15 (2H, d, $J=8.4$ Hz), 7.29 (2H, d, $J=8.4$ Hz). EIMS: $m/z=223$ (M^+), 225, 227.

Synthesis of 2-(4-Nitrophenyl)ethyl Isocyanide (16). To a solution of 2-(4-nitrophenyl)ethylamine (1.09 g) in ethyl formate (6 ml), sodium hydroxide (200 mg) was added and the mixture was refluxed for 1 h. H_2O (10 ml) was added to this mixture and the aqueous solution was extracted with three portions of $CHCl_3$ (10 ml). The chloroform layers were combined and concentrated to give 1.28 g of *N*-[2-(4-nitrophenyl)ethyl]formamide. To a solution of *N*-[2-(4-nitrophenyl)ethyl]formamide (1.28 g), triphenylphosphine (2.03 g) and tetrachloromethane (1.0 ml) in $CHCl_3$ (10 ml), triethylamine (1.0 ml) was added. The mixture was stirred at room temperature for 12 h. H_2O (10 ml) was added to this mixture and the aqueous solution was extracted with three portions of $CHCl_3$ (10 ml). The chloroform layers were combined, concentrated and purified by SiO_2 column chromatography ($CHCl_3$) to give 681.9 mg of 2-(4-nitrophenyl)ethyl isocyanide as yellow crystals.[20] ^{1}H NMR ($CDCl_3$, ppm) $\delta=3.10$ (2H, br. t, $J=6.5$ Hz), 3.69 (2H, br. t, $J=6.5$ Hz), 7.43 (2H, d, $J=8.6$ Hz), 8.23 (2H, d, $J=8.6$ Hz); ^{13}C NMR ($CDCl_3$, ppm) $\delta=35.0$, 42.1, 123.8, 123.8, 129.6, 129.6, 144.0, 147.2, 157.7. EIMS: $m/z=176$ (M^+).

Synthesis of 2-(2,4-Dichlorophenyl)ethyl Isocyanide (17). To a solution of 2-(2,4-dichlorophenyl)ethylamine (1.26 g) in ethyl formate (6 ml), sodium hydroxide (200 mg) was added; the mixture was then refluxed for 1 h. H_2O (10 ml) was added to this mixture and the aqueous solution was extracted with three portions of $CHCl_3$ (10 ml). The chloroform layers were combined and concentrated to give 1.45 g of *N*-[2-(2,4-dichlorophenyl)ethyl]formamide. To a solution of *N*-[2-(2,4-dichlorophenyl)ethyl]formamide (1.45 g), triphenylphosphine (2.03 g) and tetrachloromethane (1.0 ml) in $CHCl_3$ (10 ml), triethylamine (1.0 ml) was added. The mixture was stirred at room temperature for 12 h. H_2O (10 ml) was added to this mixture and the aqueous solution was extracted with three portions of $CHCl_3$ (10 ml). The chloroform layers were combined, concentrated and purified by SiO_2 column chromatography ($CHCl_3$) to give 931.6 mg of 2-(2,4-dichlorophenyl)ethyl isocyanide as a yellow oil.[20] ^{1}H NMR ($CDCl_3$, ppm) $\delta=3.08$ (2H, br. t, $J=6.7$ Hz), 3.65 (2H, br. t, $J=6.7$ Hz), 7.24 (2H, br.), 7.41 (1H, br.); ^{13}C NMR ($CDCl_3$, ppm) $\delta=33.0$, 40.9, 127.5, 129.6, 132.1, 132.7, 134.1, 134.6, 157.2. EIMS: $m/z=199$ (M^+), 201, 203.

Synthesis of 2-(4-Nitrophenyl)ethyl Isothiocyanate (18). To a solution of 2-(4-nitrophenyl)ethylamine (590.0 mg) in diethyl ether (4 ml), CS_2 (1 ml) was added at 0 °C. Dicyclohexylcarbodiimide (900 mg) was added to this mixture at 0 °C. The mixture was stirred for 4 h at 0 °C, and then stirred for 12 h at room temperature. H_2O (10 ml) was added to this mixture and the aqueous solution was extracted with three portions of diethyl ether (10 ml). The diethyl ether layers were combined, concentrated and purified by SiO_2 thin-layer chromatography (benzene/hexane = 7/3) to give 558.8 mg of 2-(4-nitrophenyl)ethyl isothiocyanate as a yellow powder.[21] ^{1}H NMR ($CDCl_3$, ppm) $\delta=3.06$ (2H, t, $J=7$ Hz), 3.78 (2H, t, $J=7$ Hz), 7.38 (2H, d, $J=8$ Hz), 8.14 (2H, d, $J=8$ Hz); ^{13}C NMR ($CDCl_3$, ppm) $\delta=35.8$, 45.4, 123.7, 123.7, 128.1, 129.6, 129.6, 144.6, 146.9. EIMS: $m/z=208$ (M^+).

Synthesis of 2-(2,4-Dichlorophenyl)ethyl Isothiocyanate (19). To a solution of 2-(2,4-dichlorophenyl)ethylamine (630.0 mg, 500 µl) in diethyl ether (4 ml), CS_2 (1 ml) was added at 0 °C. Dicyclohexylcarbodiimide (730 mg) was added to this mixture at 0 °C. The mixture was stirred for 1 h at 0 °C, and precipitated thiourea was then removed by filtration. H_2O (10 ml) was added to the filtrate and the aqueous solution was extracted with three portions of diethyl ether (10 ml). The diethyl ether layers were combined and concentrated to give an oily material. Benzene and hexane were added to this oily material. The mixture was left overnight and deposited crystals were collected by filtration. The hexane-soluble part of the filtrate was collected and concentrated to give a yellow oily material. This oily material was purified by SiO_2 column chromatography (hexane) to give 553.2 mg of 2-(2,4-dichlorophenyl)ethyl isothiocyanate as a colorless oily material.[21] ^{1}H NMR ($CDCl_3$, ppm) $\delta=2.98$ (2H, t, $J=7$ Hz), 3.64 (2H, t, $J=7$ Hz), 7.12 (2H, m), 7.30 (1H, d, $J=2$ Hz); ^{13}C NMR ($CDCl_3$, ppm) $\delta=33.8$, 44.2, 127.4, 128.3, 129.6, 132.0, 133.1, 133.9, 134.6. EIMS: $m/z=231$ (M^+), 233, 235.

Synthesis of Triethylammonium *N*-[2-(4-Nitrophenyl)ethyl]-dithiocarbamate (20). To a solution of sodium hydroxide (4 g) in

water (50 ml) and chloroform (50 ml), 2-(4-nitrophenyl)ethylamine hydrochloride (3.2 g) was added and partitioned. The chloroform layer was dried with sodium sulfate and concentrated to give salt-free 2-(4-nitrophenyl)ethylamine. To a cooled solution of carbon disulfide (30 ml) and triethylamine (50 ml), 2-(4-nitrophenyl)ethylamine was added with stirring. The mixture was stirred for 24 h at r.t. The mixture was filtered, washed with absolute ether and dried in vacuo to give 4.4 g of triethylammonium *N*-[2-(4-nitrophenyl)-ethyl]dithiocarbamate as a yellow solid.[22] ^{1}H NMR ($CDCl_3$, ppm) δ = 1.36 (9H, t, J = 7.3 Hz), 3.02 (2H, t, J = 7.2 Hz), 3.25 (6H, q, J = 7.3 Hz), 3.83 (2H, br. q, J = 6.8 Hz), 7.38 (2H, d, J = 8.7 Hz), 7.63 (1H, br. s,), 8.09 (2H, d, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$, ppm) δ = 8.4, 34.8, 45.7, 47.8, 123.6, 129.6, 146.5, 147.2, 214.1.

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