Eur J Med Chem (1996) 31, 469–478

Antibacterial activity and structure–activity relationships of berberine analogs

K Iwasa[1], M Kamigauchi[1], M Ueki[2], M Taniguchi[2],

[1]*Kobe Pharmaceutical University, 4-19-1 Motoyamakita, Higashinada-ku, Kobe 658;*
[2]*Faculty of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558, Japan*

(Received 21 September 1995; accepted 22 January 1996)

Summary — Analogs of berberine **1** and related compounds were prepared to evaluate structure–activity relationships. Among the 13-alkyl-substituted and the13-unsubstituted protoberberinium salts, the 13-ethyl-9-ethoxyl homolog **30**, the 13-ethyl analog **29**, and the 13-methyl derivative **3** showed an increase in antibacterial activity against *Staphylococcus aureus* by eight-, four- and twofold respectively over the parent base berberine **1**; this is suggestive that steric effects play a significant role in the antibacterial activity. Reduction of the protoberberinium salts yielding the tetrahydro derivatives greatly reduced the antibacterial activity. Replacement of methoxyl groups at the C-2 and the C-3 of ring A by a methylenedioxy group resulted in increased antibacterial activity. These data strongly suggest that the quaternary nitrogen atom such as in protoberberinium salts, an alkylsubstituent at C-13, and a methylenedioxy function at C-2 and C-3 are required for enhanced activity. Tetrahydroprotoberberine α-*N*-metho salts showed higher activity than tetrahydroprotoberberine hydrochlorides, but appreciably lower activity than protoberberinium salts. The effects of substitution at C-13 and on ring A in the α-*N*-metho salt were similar to those in protoberberinium salts. Stereochemical changes of the B/C ring juncture from *trans* to *cis*, and of the methyl group at C-13 from α to β, had, respectively, marked and slight effects on the activity. The tested compounds were less active against *Escherichia coli* (Gram-negative bacterium) and *Candida albicans* (fungus) than *S aureus* (Gram-positive bacterium).

antibacterial activity / 13-methylberberine / 13-ethyl analog / 13-ethyl-9-ethoxyl homolog

Introduction

Berberine **1**, a member of the protoberberine class of isoquinoline alkaloids, is found in a variety of plant tissues [1]. In the past, berberine-containing plants have been used in Chinese medicine and in folk medicine. Berberine has antibacterial activity [2] and a stomachic effect. In Japan, berberine chloride is presently used as an antidiarrhetic agent and berberine tannate as a medicine for intestinal bacterial disorders. The anti-inflammatory activity of berberine has already been reported [3].

The purpose of the present study was to search for related compounds which might have antibacterial activity superior to berberine, and to establish structure–activity relationships among berberine analogs.

Chemistry

Berberine and related compounds (**1–30** and **35–43**) prepared previously or presently for structure–activity investigations are shown in figures 1–3. Analysis data for these compounds are shown in table I. Structural modifications included introduction of methyl and ethyl at C-13 and substitution of ethoxyl and hydroxyl for the methoxyl at C-9.

Reaction of acetoneberberine with ethyl iodide in acetone at 100 or 140 °C, followed by treatment with AgCl, gave rise to 13-ethylberberine chloride **29** and 9-*O*-ethyl-13-ethylberberrubine chloride **30** respectively (scheme 1). The latter compound can be prepared via 13-ethylberberrubine which is obtained from pyrolysis of 13-ethylberberine iodide. The mass spectrum of **29** showed the molecular ion at *m/z* 364 (M-Cl, secondary ion mass spectrometry (SIMS)) which is 28 mass units higher than that of berberine. The ^{1}H NMR spectrum showed a three-proton triplet at δ 1.57 and a two-proton quartet at δ 3.45, representing the ethyl group. The mass spectrum of **30** showed a molecular ion at *m/z* 378 (M-Cl, SIMS) which is 14 mass units higher than that of **29**. The ^{1}H NMR spectrum of **30** exhibited a three-proton triplet at δ 1.51 and two-proton quartet at δ 4.49, indicating the presence of an *O*-ethyl group, instead of an *O*-methyl group at C-9 as in **29**.

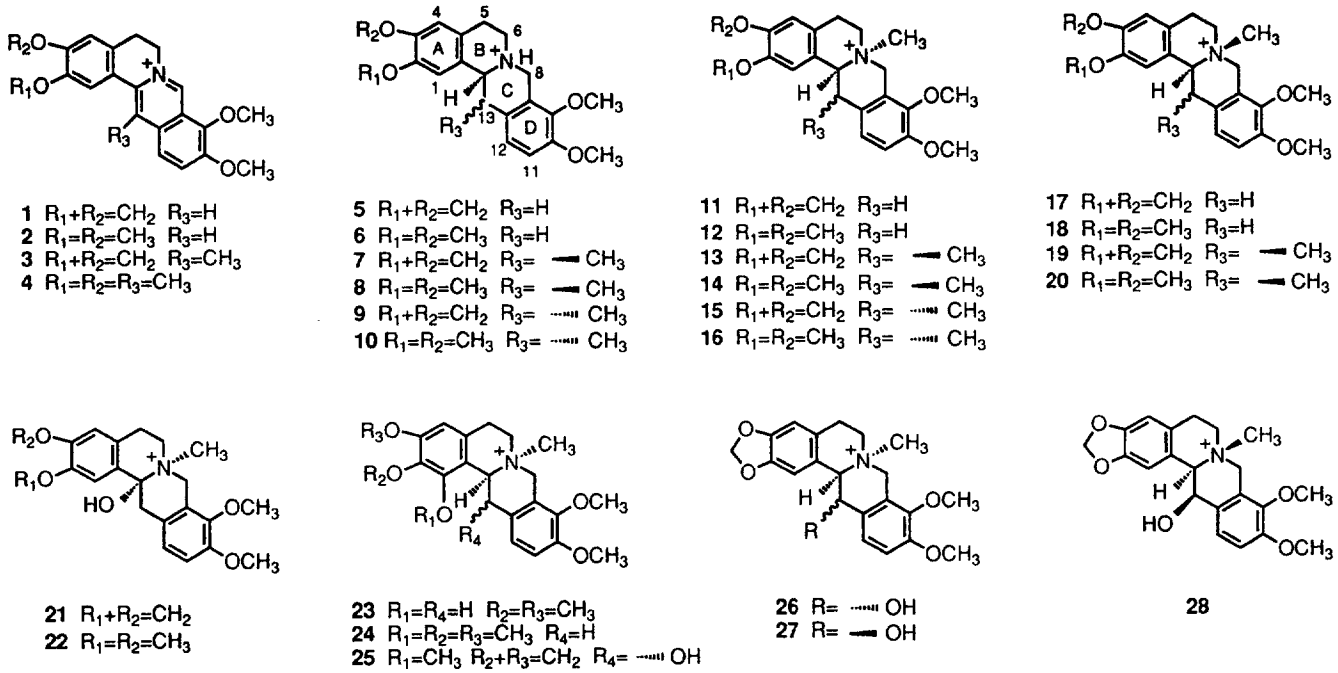


Fig 1. Berberine **1** and related compounds **2–28**.

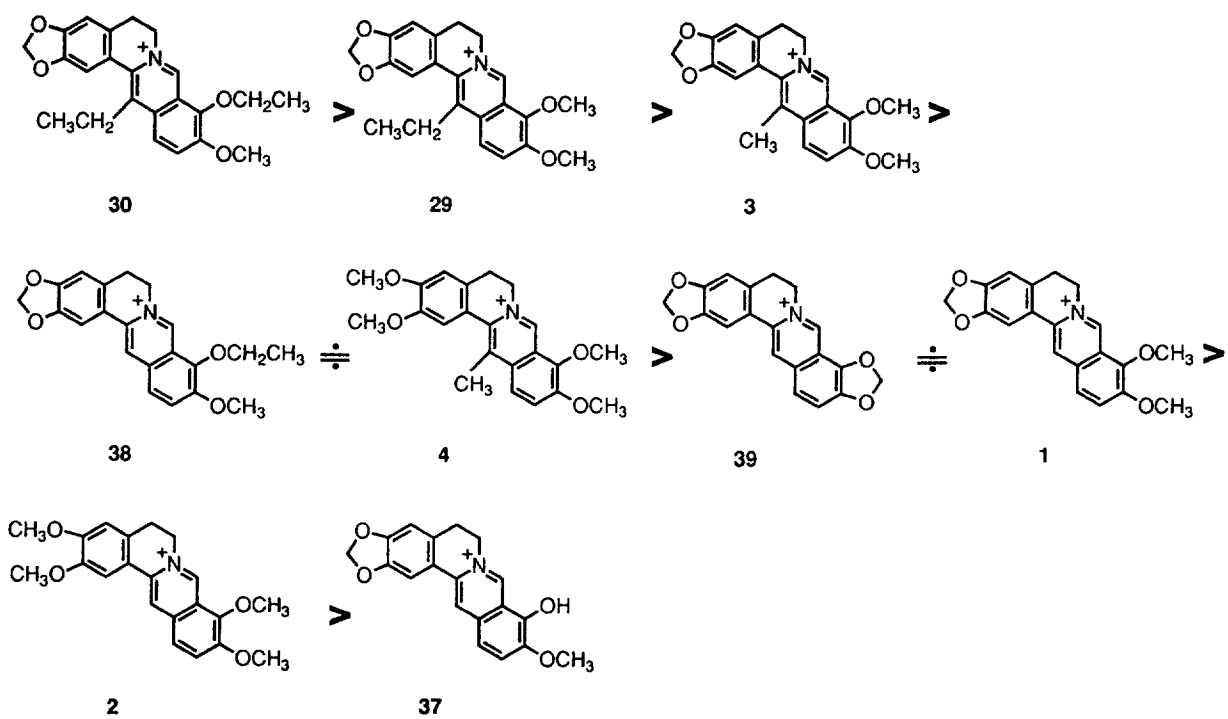


Fig 2. Comparison of antibacterial activity of berberine and structural isomers against *Staphylococcus aureus*.

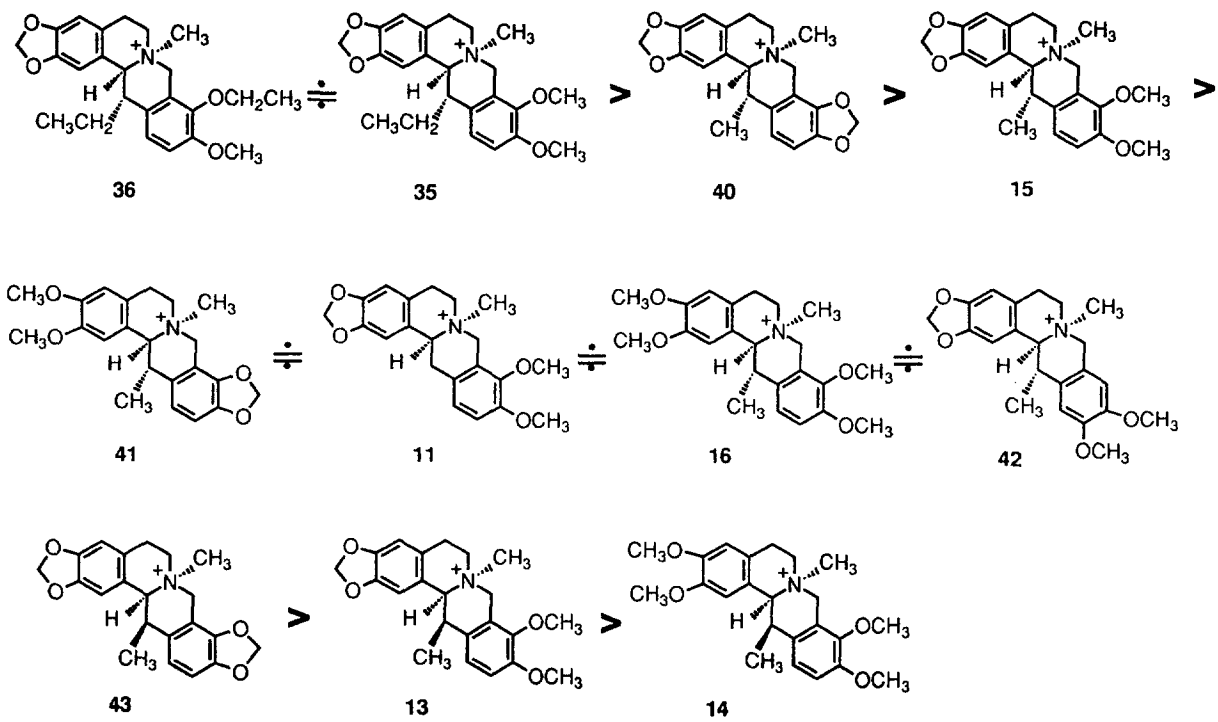


Fig 3. Comparison of antibacterial activity of tetrahydroprotoberberine α-*N*-metho salts against *Staphylococcus aureus*.

Further evidence for the structure of **29** and **30** was provided by the ^{1}H NMR and mass spectral data of their reduction products. Reduction of compound **29** with zinc in methanol and hydrochloric acid generated isomeric compounds **31** and **32** which were separated by preparative TLC (scheme 2). Reduction of **30** by the same method furnished the epimeric tetrahydro derivatives **33** and **34** (scheme 2). The mass spectrum of **31** displayed a molecular peak at *m/z* 367 (M^+, 57%) and a fragment peak at *m/z* 192 (100%) assignable to ion A, arising by way of retro-Diels–Alder cleavage of ring C. The high-resolution mass spectrum of **31** had a molecular ion at *m/z* 367.1774, consistent with the molecular formula $C_{22}H_{25}NO_4$. The peak at *m/z* 192.1156 was in accord with the formula $C_{12}H_{16}O_2$ The ^{1}H NMR spectrum of **31** exhibited the methyl protons and the methylene protons of the ethyl group at δ 0.80 and 1.38 respectively. The ^{13}C NMR spectrum of **31** displayed peaks corresponding to the methyl and methylene carbons of the ethyl group at C-13 at δ 12.79 and 24.55 respectively. The mass spectrum of **33** showed a molecular peak at *m/z* 381 (M^+, 100%) and a fragment peak at *m/z* 206 (89%) assignable to ion B. The formulae $C_{23}H_{27}NO_4$ and $C_{13}H_{18}O_2$ of the molecular ion at *m/z* 381.1922 and the fragment ion at *m/z* 206.1297, established by high-resolution mass spectrum of **33**, indicated the presence of an *O*-ethyl group, instead of an methyl group at C-9 as in **31**. The ^{1}H NMR spectrum of **33** included signals due to the methyl and methylene protons of the *O*-ethyl group at δ 1.39 and 4.7 respectively, together with signals of the ethyl group at C-13. The ^{13}C NMR spectrum of **33** showed peaks due to methyl and methylene carbons of the *O*-ethyl group, at δ 15.95 and 68.14 respectively, in addition to signals due to the ethyl group at C-13. The ^{1}H NMR peaks of H-14 in **31** and **33** appeared as broad singlets indicating the *cis* arrangement of protons at H-13 and H-14 in these compounds, and thus the axial orientation of the ethyl groups at C-13.

Compounds **32** and **34** were separated by preparative TLC and, without further purification, were *N*-methylated to furnish the *N*-metho salts **35** and **36** respectively (scheme 3). The mass spectra of **35** and **36** showed the molecular ion at *m/z* 368 (M-Cl, SIMS) and 382 (M-Cl, SIMS) respectively. The ^{1}H NMR spectrum of **35** exhibited signals for the *N*-methyl group at δ 3.22 (3H) and the ethyl group at δ 0.85 (3H), 1.88 (1H), and 2.14 (1H). The ^{1}H NMR spectrum of **36** showed absorptions of the *N*-methyl group

Table I. Analysis data on the protoberberine alkaloids **1–30** and **35–43**.

Compd	*LC/APC-MS*[a] *data*	
	RT (min)	*observed ions (m/z)* $[M]^+$ $[M + H]$ $[M + CHF_3]^+$
1	18.7[b]	406
2	18.9[b]	422
3	19.9[b]	420
4	19.6[b]	436
5	35.0[b]	340
6	28.0[b]	356
7	40.0[b]	354
8	33.7[b]	370
9	32.7[b]	354
10	26.3[b]	370
11	15.0[b]	354
12	13.0[b]	370
13	18.1[b]	369
14	17.1[b]	385
15	18.7[b]	369
16	18.1[b]	385
17	11.3[b]	354
18	9.5[b]	370
19	16.6[b]	369
20	15.3[b]	385
21	12.8[b]	370
22	11.0[b]	386
23	9.4[c]	386
24	11.9[c]	400
25	6.0[c]	400
26	5.0[c]	370
27	3.4[c]	370
28	4.4[c]	370
29	14.6[c]	434
30	16.1[c]	448
35	13.8[c]	382
36	15.9[c]	396
37	14.8[c]	392
38	12.8[c]	420
39	26.6[d]	390
40	28.8[d]	353
41	13.8[c]	369
42	9.0[c]	369
43	28.2[d]	353

[a]LC/APCI-MS (liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry): Hitachi M-1000H (nebulizer and vaporizer temps 300–340 and 399 °C, drift voltage 20–30 V); Hitachi M-6200 inteligent pump (1 mL/min); Hitachi L-4000 UV detector (280 nm); Cosmosil 5 C_{18}-AR (4.6 id × 150 mm); [b]0.1 M NH_4OAc (0.05% TFA, A)-MeOH (B): initial (0% of B), 10 min (30% of B), 15 min (50% of B), 20–25 min (60% of B), 30 min (70% of B), 35 min (80% of B), 40 min (30% of B); [c]0.1 M NH_4OAc (0.05% TFA, A)-MeOH (0.05% TFA, B): initial (30% of B), 10 min (50% of B), 20 min (80% of B), 30 min (80% of B); [d]H_2O (0.1% TFA, A)-MeOH (B): initial (25% of B), 25 min (25% of B), 30 min (75% of B).

at δ 3.23 (3H) and the ethoxyl group at δ 1.38 (3H) and 4.15 (2H) together with those of the ethyl group at C-13. The coupling constants (10 Hz) between the H-13 and H-14 protons in the ^1H NMR spectra of **35** and **36** indicate the diaxial configuration of these protons. It follows that the ethyl group at C-13 is in an equatorial orientation. The B/C ring juncture of **35** and **36** was determined to be *cis* by comparison of the chemical shifts of the *N*-methyl group (δ 3.22 and 3.23) with those of the α- and β-*N*-metho salts of thalictricavine (**13** and **19**; δ 3.26 and 3.06), which have B/C-*cis* and -*trans* ring juntures respectively.

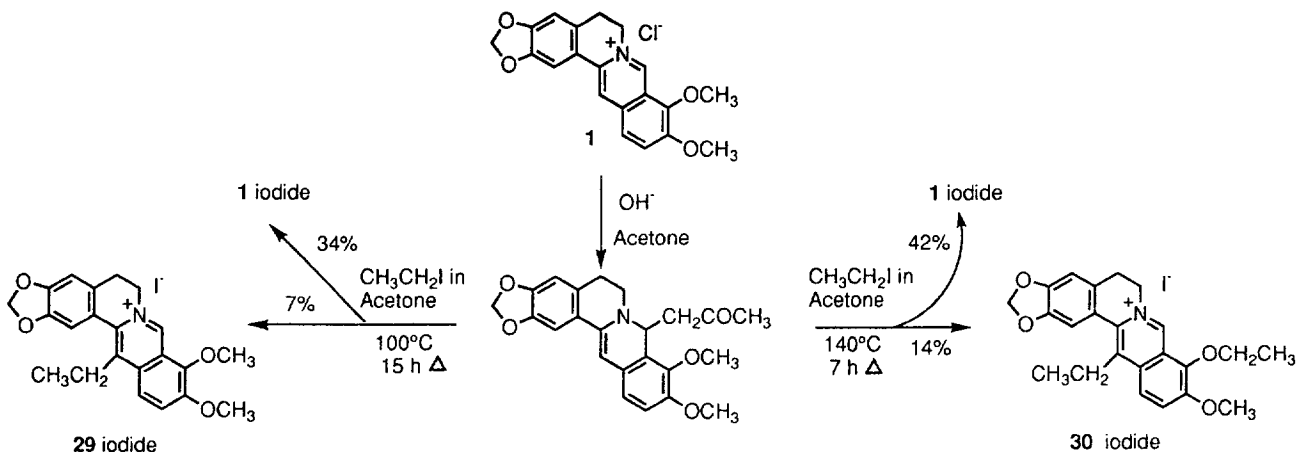
Berberrubine **37** obtained by pyrolysis of berberine was refluxed with ethyl iodide in DMF at 120 °C to furnish 9-*O*-ethylberberrubine **38** (scheme 4). The mass spectrum of **38** (chloride) had a peak at *m/z* 350 (M-Cl, SIMS). In the ^1H NMR spectrum of **38**, the methyl and methylene protons corresponding to the ethoxyl group at C-9 appeared respectively as a triplet at δ 1.51 and a quartet at δ 4.48.

Biological activity and discussion

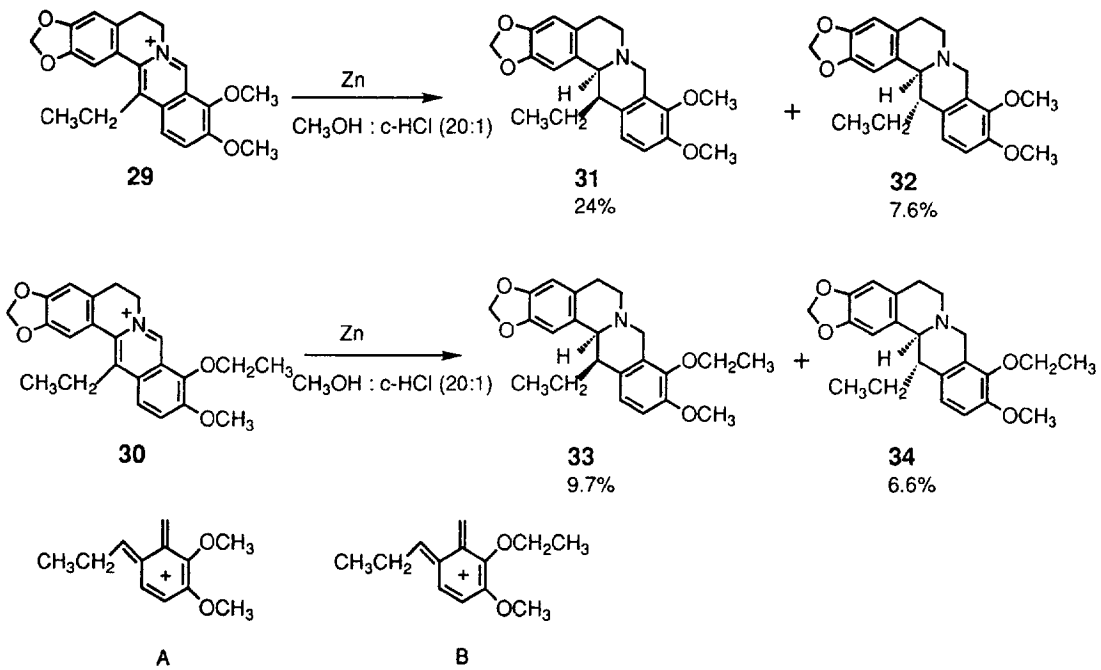
Antimicrobial activity of protoberberine alkaloids **1–22** against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* was determined by the paper disc method and the results are summarized in table II.

Methylation at C-13 of protoberberinium salts or tetrahydroprotoberberine *N*-metho salts increased the activity against *S aureus* (comparison of **3** with **1**, **4** with **2**, **13** or **15** with **11**, and **14** or **16** with **12**). Reduction of ring C of protoberberinium salts decreased the activity, suggesting that the quaternary nitrogen atom is required for the activity (comparison of **1** with **5**, **2** with **6**, **3** with **7** or **9**, and **4** with **8** or **10**). The activity of the α-*N*-metho salts **13**, **14** (B/C-*cis* ring juncture) was stronger than that of the β-*N*-metho salts **19**, **20**. The α-*N*-metho salts **11**, **12** without the methyl group at C-13 showed no inhibitory zone against *S aureus*, and neither did the β-*N*-metho salts **17**, **18**. It is of interest to note that the compounds **1–4** and **13–15** with a clear inhibitory zone against *S aureus* exhibited an ill-defined inhibitory zone against *E coli*, and that alkaloids **11**, **12**, **17** and **18** with no inhibitory zone against *S aureus* exhibited an inhibitory zone against *E coli*. These results might suggest that the mode of action in *S aureus* (Gram-positive bacterium) is different from that that in *E coli* (Gram-negative bacterium). The tested protoberberines **1–10**, **13–16**, **19** and **20** showed no inhibitory zone against *C albicans*.

Active protoberberinium salts **1–4** and tetrahydroprotoberberine *N*-metho salts **11–18**, together with inactive alkaloids **9**, **19**, and **20** (shown in table II), and the *N*-metho salts **21–28** with oxygen function at

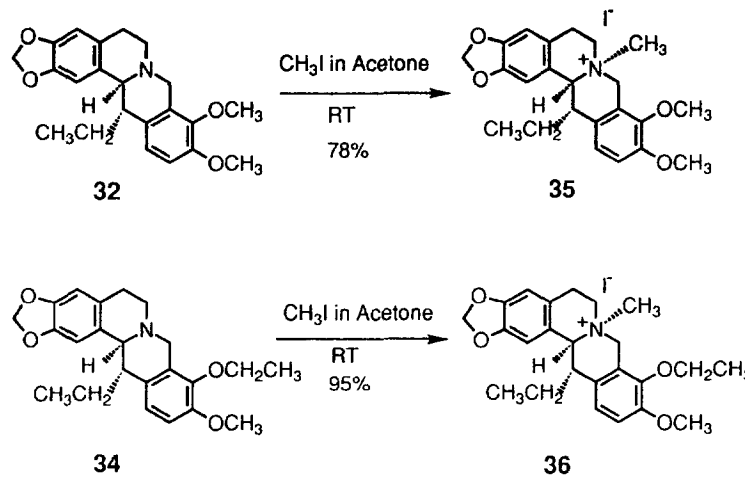


Scheme 1.



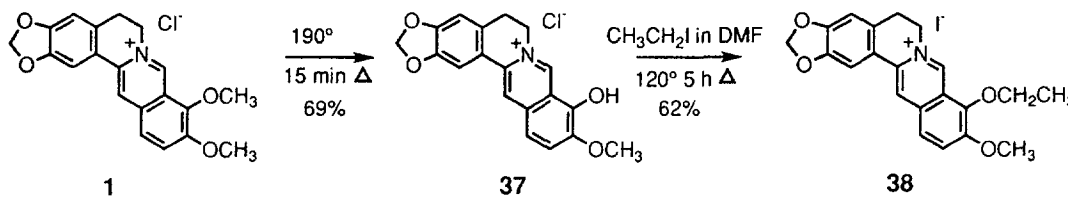
Scheme 2.

C-1 and/or C-13 or C-14, were tested against *S aureus*, *E coli* and *C albicans* by the liquid dilution method. The minimmum inhibitory concentrations (MIC) of the tested alkaloids are presented in table III. The 13-methyl-substituted derivatives **3**, **4**, and **13** of protoberberinium salts of tetrahydroprotoberberine *N*-metho salts demonstrated stronger activity against *S aureus* than parent compounds **1**, **2**, and **11** respectively (table III). Reduction of ring C in protoberberinium salt **3** to give rise to tetrahydro derivative **9** reduced the activity. Stereochemical change of the B/C ring juncture from the α-*N*-metho salts (**13**, B/C-*cis*) to the β-*N*-metho salts (**19**, B/C-*trans*) resulted in approximately twofold decrease in the activity. The above results on *S aureus* obtained by the liquid dilution method are in accord with those obtained by paper



32 35 34 36

Scheme 3.



1 37 38

Scheme 4.

Table II. Diameter of inhibitory zone formed by protoberberine alkaloids.

Sample (500 μg/disc)	*Diameter of inhibitory zone (mm)*			
	Staphylococcus aureus		*Escherichia coli*	*Candida albicans*
	ATCC 25922	*NCTC 8530*	*IFO 3545*	*IFO 1061*
1	11.0	12.0	(10.5)[a]	[b]
2	14.0	16.0	(10.0)[a]	[b]
3	22.0	22.0	(12.0)[a]	[b]
4	24.0	25.0	(12.3)[a]	[b]
5, 6	[b]	[b]	[b]	[b]
7–10	[b]	[b]	(91–9.5)[a]	[b]
11	[b]	[b]	11.3	[c]
12	[b]	[b]	10.5	[c]
13[d]	12.5	12.5	(< 9.0)[a]	[b]
14[d]	13.0	11.0	(9.5)[a]	[b]
15[d]	11.0	12.0	(< 9.0)[a]	[b]
16[d]	11.0	12.5	[b]	[b]
17	[b]	[b]	9.5	[c]
18	[b]	[b]	9.0	[c]
19[d], **20**[d]	[b]	[b]	[b]	[b]
21	[b]	[b]	< 9.0	[c]
22	[b]	[b]	[b]	[c]

[a]Inhibitory zone is not clear; [b]diameter of inhibitory zone < 8.5 mm (control disc 8.0 mm); [c]not tested; [d][*N*-$^{13}CH_3$]-derivative.

Table III. Minimum inhibitory concentration (MIC) of protoberberine alkaloids.

Sample	*MIC (μg/mL)*			
	Staphylococcus aureus		*Escherichia coli*	*Candida albicans*
	ATCC 25922	*NCTC 8530*	*IFO 3545*	*IFO 1061*
1	250	250	[a]	1000
2	1000	1000	[a]	[a]
3	125	125	[a]	[a]
4	250	250	[a]	[a]
9	[a]	[a]	[c]	[c]
11, 12	[a]	[a]	[a]	[a]
13[d]	500	500	[a]	[a]
14[d]	[a]	[a]	[a]	[c]
15[d]	1000	1000	[a]	[a]
16[d]	[a]	[a]	[a]	[a]
17, 18	[a]	[a]	[a]	[a]
19[d], **20**[d]	[a]	[a]	[a]	[c]
21-28	[a]	[a]	ab	[c]

[a]MIC >1000; [b]except for compounds **22**, **25**, and **26**; [c]not tested; [d][*N*-$^{13}CH_3$]-derivative.

disc method. Replacement of the methylenedioxy group at C-2 and C-3 by the methoxyl groups reduced the activity (comparison of **1** with **2**, **3** with **4**, **13** with **14**, and **15** with **16**). It could not be expected from the MIC of the compounds **21–28** that replacement of the hydrogen at C-1 and/or C-13 or C-14 of tetrahydroprotoberberine α-*N*-metho salts by an oxygen function would lead to an increase in the activity. It was found that the substitution of the alkyl group at C-13 and of the oxygen function on ring A of berberine-like compounds and the quaternary nitrogen atom seems to play an important role in antibacterial activity against *S aureus*. The MIC values of the compounds tested (**1–4**, **11–21**, **23**, **24**, **27** and **28**) against *E coli* were over 1000 μg/mL. Apart from compound **1** (1000 μg/mL), the MIC values of the tested akaloids (**2–4**, **11–13**, and **15–18**) against *C albicans* were over 1000 μg/mL. These results indicate that the antibacterial activities of the tested compounds against *E coli* and *C albicans* are weak. Even if the inhibitory zone is not clear in the case of *E coli* (**1–4**, **7–10**, **14** and **15**) or is low against *E coli* (**11**, **12**, **17**, **18** and **21**) or *S aureus* (**14** and **16**), these compounds probably have some antibacterial activity which is not detected until a concentration of 1000 μg/mL when the liquid dilution method is used (tables II and III).

The effects on the activity of substitution at C-13 on rings A or D, and the configuration of the alkyl group at C-13, were further examined. The MIC values of the protoberberinium salts (**1–4**, **29**, **30** and **37–39**) (fig 2) and the tetrahydroprotoberberine α-*N*-metho salts (**13–16**, **35**, **36** and **40–43**) (fig 3) against *S aureus*, and the bacterial growth below the MIC estimated by measuring the optical density (OD) at 655 nm, are given in table IV. The most important factor in the activity of berberine analogs is the substituent at C-13 (table V). Analog **29** with an ethyl groug at C-13, bulkier than the methyl groupof **3**, displayed activity against *S aureus* twofold stronger than by **3**. Analog **3** showed twofold higher activity than the parent base. Similarly replacement of the hydrogen by a methyl group at C-13 increased the activity by fourfold (comparison of **4** with **2**). Replacement of the hydrogen at C-13 of 9-ethylberberrubine **38** by an ethyl group to give rise to **30** markedly increased the activity (eightfold). The second important factor for the activity is the nature of the substituents at C-2 and C-3 on ring A (table V). Replacement of two methoxyl groups at the 2,3 position on ring A by one methylenedioxy function increased the activity by two- to fourfold. The effect of a similar type of replacement on ring D on the activity may be small. The third important factor for the activity is the substituent at C-9 (table V). The activity of 9-*O*-ethyl derivative **30** of 13-ethylberberrubine is stronger than that of the 9-*O*-methyl homolog **29**. However, the equivalent conversion of **1** to **38** slightly increased the activity. Replacement of the hydroxyl group by a methoxyl group at C-9 enhanced the activity by more than fourfold.

Substitution at C-13 or on ring A of tetrahydroprotoberberine α-*N*-metho salts affects the activity in a similar way to that of protoberberinium salts (table V). However, the antibacterial properties of the α-*N*-metho salts are rather low. This suggests that the quaternary nitrogen atom of the protoberberinium salts plays a greater role in increasing the antibacterial activity than

Table IV. Antibacterial activity of protoberberinium salts and tetrahydroprotoberberine α-*N*-metho salts against *Staphylococcus aureus* ATCC 25922.

Sample	*OD at 655 nm*		
	MIC (μg/mL)	*MIC*	*< MIC*
Protoberberinium salts			
30	31.3	0.021	
29	62.9	0.013	
3	125	0.026	0.069[a]
38	250	0.019	0.083[b]
4	250	0.031	0.105[b]
39	250	0.040	0.148[b]
1	250	0.014	0.177[b]
2	1000	0.041	0.210[c]
37	>1000	0.30	0.405[c]
Tetrahydroprotoberberine α-*N*-metho salts			
36	500	0.022	0.963[b]
35	500	0.036	1.090[b]
43[e]	1000	0.024	0.088[c]
40[e]	1000	0.023	0.252[c]
13[b]	1000	0.029	0.372[c]
15[e]	2000	0.021	0.086[d]
41[e]	2000	0.026	0.760[d]
14[e]	> 2000	0.125	0 956[d]
11	> 2000	0.441	0.982[d]
16[e]	> 2000	0.461	1.019[d]
42[e]	> 2000	0.470	1.139[d]

[a]62.5 μg/mL; [b]125 μg/mL; [c]500 μg/mL; [d]1000 μg/mL; [e][*N*-$^{13}CH_3$]-derivative.

that of the α-*N*-metho salts. Replacement of the hydrogen by a methoxyl group and the methoxyl group by an ethoxyl group at C-9 of tetrahydroberberine α-*N*-metho salts did not display any increase in the activity, in contrast to protoberberinium salts. The change of configuration of the methyl group at C-13 from α to β slightly increased the activity (table V).

It was found that synthetic compounds **30**, **29** and **3** possessed antibacterial activity against *S aureus* respectively eight-, four- and two times that of berberine **1**. The alkyl substitution at C-13 enhanced the antibacterial activity and consequently the MIC values (31.3 to 125 μg/mL) of **3**, **29** and **30** are closer to values usually expected for putative antibacterial substances. These compounds could have more important clinical applications than berberine. The new results (tables II and III) strongly suggest that the quaternary nitrogen atom of protoberberinium salts is required for increasing antibacterial activity, while the bulk of the alkoxy- and alkyl-substituents at C-9 and C-13 respectively, and the nature of the *O*-substituent at C-2 and 3, also influence the activity.

Experimental protocols

Chemistry

Melting points were determined on a Yanako Micromelting Point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR-500 (500 and 125 MHz) spectrometer and a Varian Gemini-300 (300 MHz) using tetramethylsilane as an internal standard in CD_3OD or $CDCl_3$. Mass spectra were determined on Hitachi M-4100. The secondary ion mass spectra (SIMS) were measured using glycelol as matrix. Preparative TLC was performed on Merck silica gel 60F-254 plates using C_6H_6/Et_2O (4:1).

Berberine **1** and palmatine **2** were purchased. 13-Methylprotoberberinium salts **3** and **4** were previously prepared [4]. Tetrahydroprotoberberines **5** and **6** were prepared by reduction of protoberberinium salts **1** and **2**, respectively. *cis*- and *trans*-13-Methyltetrahydroprotoberberines **7**, **8** and **9**, **10** respectively were prepared according to the method of Takao et al [5]. The α- and β-*N*-metho salts **11**, **12** and **17**, **18** of **5** and **6** were prepared by previously described methods [6]. The α- and /or β-*N*-metho salts **13–16** [7], **19** [7], **20** [17], **23** [8], **24** [8], **25** [9], and **26–28** [10] were previously prepared. Protopine **21** and allocryptopine **22** were natural products from *Macleaya cor-*

Table V. Structure–antibacterial activity relationship of structural isomers of protoberberinium salts and tetrahydroprotoberberine α-*N*-metho salts.

Substituent	*Antibacterial activity against Staphylococcus aureus (magnitude of the increase in MIC)*	
	Protoberberinium salts	*Tetrahydroprotoberberine α-N-metho salts*
13-CH_2CH_3 > 13-CH_3	**29** (2) > **3**	**35** (4) > **15**
13-CH_2CH_3 > 13-H	**30** (8) > **38**, **29** (4) > **1**	**35** (4) > **11**
13-CH_3, 13-H	**4** (4) > **2**, **3** (2) > **1**	**13** (2), **11**, **15** > **11**
Ring A 2,3-OCH_2O > 2,3-di-OCH_3	**1** (4) > **2**, **3** (2) > **4**	**40** (2), **41**, **13** (2) > **14**
Ring D 9,10-OCH_2O ~ 9,10-di-OCH_3	**39** ~ **1**	
Rings A and D 2,3- and 9,10-OCH_2O > 2,3,9,10-OCH_3		**40** (2) > **16**, **43** (2) > **14**
9-OCH_2CH_3 > 9-OCH_3	**30** (2) > **29**, **38** > **1**	**36** ~ **35**
9-OCH_3 > 9-OH	**1** (4) > **37**	
β 13-CH_3 > α 13-CH_3		**13** (2) > **15**, **14** > **16**, **43** > **40**

data. For each compound elemental analysis (results within ± 0.4% of theoretical values) or high resolution mass spectrometry (HRMS) was performed.

Preparation of 13-ethylberberine ***29***
A mixture of acetoneberberine (1 g) [11] prepared from berberine **1** and EtI (2 mL) in Me_2CO (100 mL) was placed in a glass-stoppered bottle and heated for 15 h at 100 °C. After the mixture cooled, the resulting crystals were filtered to supply berberine iodide (419 mg, 34%). The filtrate was concentrated to give crude crystals (140 mg) which were filtered and recrystallized from EtOH/$CHCl_3$ to provide 94 mg (7%) of 13-ethylberberine iodide **29**: mp 199–207 °C dec. The iodide **29** (18 mg) was treated with AgCl in MeOH to convert it to the chloride (10 mg): mp 197–207 °C dec. SIMS: 364 (M-Cl, 100). ^{1}H NMR (CD_3OD): δ 1.57 (t, 3H, *J* = 7.5, CH_2CH_3), 3.13 (t, 2H, *J* = 7.0, H5), 3.45 (q, 2H, *J* = 7.5, CH_2CH_3), 4.13 and 4.22 (s, each 3H, OCH_3), 6.12 (s, 2H, OCH_2O), 7.03 and 7.33 (s, each 1H, ArH4 and 1), 8,15 and 8.22 (d, each 1H, *J* = 9.5, ArH11 and 12), 9.79 (s, 1H, ArH8).

Preparation of 9-O-ethyl-13-ethylberberrubine ***30***
Through a similar procedure as outlined for **29**, acetoneberberine and EtI in Me_2CO were heated at 140 °C for 7 h. Berberine iodide (524 mg, 42%) and other crude iodides (307 mg) were collected. The crude iodide was recrystallized from EtOH yielding 189 mg (14%) of **30** iodide: mp 214–219 °C dec. The iodide (33 mg) was converted by treatment with AgCl in MeOH to the chloride of **30** (23 mg): mp 218–224 °C dec. SIMS: 378 (M-Cl, 100). ^{1}H NMR (CD_3OD): δ 1.51 (t, 3H, *J* = 7.5, OCH_2CH_3), 1.57 (t, 3H, *J* = 7.5, CH_2CH_3), 3.13 (t, 2H, *J* = 7.0, H5), 3.45 (q, 2H, *J* = 7.5, CH_2CH_3), 4.12 (s, 3H, OCH_3), 4.49 (q, 2H, *J* = 7.5, OCH_2CH_3), 6.12 (s, 2H, OCH_2O), 7.03 and 7.33 (s, each 1 H, ArH4 and 1), 8.14 and 8.21 (d, each 1H, *J* = 9.5, ArH11 and 12), 9.75 (s, 1 H, ArH8).

Reduction of 29 and 30 to form 31–34
To a solution of 50 mg of the iodide (**29** or **30**) dissolved in MeOH (50 mL) and concentrated HCl (2.5 mL) was added 1 g of zinc dust. The zinc was removed by suction filtration. The filtrates were diluted with water (20 mL) and concentrated. The solution was cooled in an ice bath, basified with concentrated NH_4OH, and extracted with $CHCl_3$. The organic extract was dried with $MgSO_4$, filtered and evaporated. The residue was subjected to preparative TLC (C_6H_6/Et_2O, 4:1) to obtain **31** (8.9 mg) and **32** (2.8 mg) as oils in yields of 24 and 7.6% respectively from **29**. By the same procedure, **33** (3.7 mg) and **34** (2.5 mg) were obtained as oils in yields of 9.7 and 6.6% respectively from **30**. Products **32** and **34** were used in the next step (*N*-methylation). **31**: EIMS: 367 (M^+, 57), 192 (100). HRMS: $C_{22}H_{25}NO_4$ calc 367.1785; found 367.1774, $C_{20}H_{20}NO_4$ (M-C_2H_5) calc 338.1393, found 338.1376; $C_{12}H_{16}O_4$ calc 192.1151, found 192.1156. ^{1}H NMR ($CDCl_3$): δ 0.80 (t, 3H, *J* = 7.5, CH_2CH_3, 1.38 (m, 2H, CH_2CH_3), 2.51–2.6 (m, 2H, H5 or H6), 2.86 (dt, 1H, *J* = 9.0, 3.0, H13), 3.02–3.14 (m, 2H, H5 or H6), 3.50 and 4.22 (d, each 1H, *J* = 16.0, H8), 3.67 (br s, 1H, H14), 3.86 and 3.87 (s, each 3H, OCH_3), 5.92 (dd, 2H, *J* = 12.0, 1.0, OCH_2O), 6.59 and 6.70 (s, each 1H, ArH4 and 1), 6.79 and 6.86 (d, each 1H, *J* = 8.5, ArH11 and 12). ^{13}C NMR ($CDCl_3$): δ 12.79 (CH_2CH_3), 24.55 (CH_2CH_3), 29.86 (C5). 45.63 (C13), 51.51 (C6), 54.40 (C8), 55.82 (OCH_3), 60.04 (OCH_3), 63.91 (C14), 100.70 (OCH_2O), 105.55 (C1), 108.30 (C4), 110.01 (C12), 124.72 (C11), 128.44, 129.54, and 129.85 (C1a or C4a, or C8a), 133.02 (C12a), 145.16, 145.63, and 146.26 (C2 or C3 or C9), 150.31 (C10). **33**: EIMS: 381 (M^+, 100), 206 (89). HRMS: $C_{23}H_{27}NO_4$ calc 381.1941, found 381.1922; $C_{21}H_{22}NO_4$ (M-C_2H_5) calc 352.1550, found 352.1529; $C_{13}H_{18}O_2$ calc 206.1307, found 206.1297. ^{1}H NMR ($CDCl_3$): δ 0.80 (t, 3H, *J* = 7.5, CH_2CH_3), 1.38 (m, 2H, CH_2CH_3), 1.39 (t, 3H, *J* = 7.5, OCH_2CH_3), 2.5–2.6 (m, 2H, H5 or H6), 2.86 (dt, 2H, *J* = 9.0, 3.0, H13), 3.1–3.17 (m, 2H, H5 or H6), 3.49 and 4.21 (d, each 1 H, *J* = 16.0, H8), 3.67 (br s, 1H, H14), 3.84 (s, 3H, OCH_3), 4.7 (m, 2H, OCH_2CH_3), 5.91 (dd, 2H, *J* = 12.0, 1.5, OCH_2O), 6.58 and 6.69 (s, each 1H, ArH4 and 1), 6.78 and 6.86 (d, each 1H, *J* = 8.5, ArH11 and 12). ^{13}C NMR ($CDCl_3$): δ 12.81 (CH_2CH_3), 15.95 (OCH_2CH_3), 24.59 (CH_2CH_3), 29.87 (C5), 45.62 (C13), 51.46 (C6), 54.66 (C8), 55.84 (OCH_3), 63.91 (C14), 68.14 (OCH_2CH_3), 100.69 (OCH_2O), 105.56 (C1), 108.30 (C4), 110.0 (C12), 124.46 (C11), 128.65, 129.54, and 129.92 (C1a or C4a or C8a), 133.01 (C12a), 144.0, 145.61, and 146.25 (C2 or C3 or C9), 150.37 (C10).

Preparation of ***35*** *(N-methylation of* ***32****)*
MeI (2 mL) was added to a solution of **32** (230 mg) in Me_2CO (4 mL), and the mixture was allowed to stand for 30 min at room temperature. The resulting crystals were filtered to produce the α-*N*-methyl iodide **35** (250 mg, 78%): mp 269–271 °C dec. The iodide (240 mg) was treated with AgCl in MeOH to convert it to the α-*N*-methyl chloride (143 mg): mp 248–250 °C dec. SIMS: 382 (M-Cl). ^{1}H NMR (CD_3OD): δ 0.85 (t, 3H, *J* = 7.5, CH_2CH_3), 1.88 and 2.14 (m, each 1H, CH_2CH_3), 3.17 (dt, 1H, *J* = 10, 4.5, H13), 3.22 (s, 3, NCH_3), 3.23, 3.39, 3.70, and 3.88 (m, each 1H, H5 and H6), 3.89 and 3.91 (s, each 3H, OCH_3), 4.51 (d, 1H, *J* = 10.0, H14), 4.72 and 4.93 (d, each 1H, *J* = 16.0, H8), 6.02 (s, 2H, OCH_2O), 6.79 and 6.81 (s, each 1H, ArH1 and 4), 7.10 and 7.12 (d, each 1H, 9.0, ArH11 and 12).

Preparation of ***36*** *(N-methylation of* ***34****)*
A mixture of **34** (60 mg) in Me_2CO (1 mL) and MeI (1 mL) was allowed to stand for 30 min at room temperature. The resulting crystals were filtered to supply the α-*N*-methyl iodide **36** (79 mg, 95%): mp 273–275 °C dec. The iodide (60 mg) was treated with AgCl in MeOH to convert it to the α-*N*-methyl chloride (42 mg): mp 259–264 °C dec. SIMS: 396 (M-Cl, 100). ^{1}H NMR (CD_3OD): δ 0.85 (t, 3H, *J* = 7.5, CH_2CH_3), 1.38 (t, 3H, *J* = 7.0, CH_2CH_3), 1.88 and 2.14 (m, each 1H, CH_2CH_3), 3.17 (dt, 1H, *J* = 10.0, 4.5, H13), 3.23 (s, 3H, N-CH_3), 3.22, 3.38, 3.70, and 3.88 (m, each 1H, H5 and H6), 3.88 (s, 3H, OCH_3), 4.15 (m, 2H, OCH_2CH_3), 4.51 (dd, 1H, *J* = 10.0, 1.5, H14), 6.01 and 6.02 (d, each 1H, *J* = 1.5, OCH_2O), 6.80 and 6.81 (s, each 1H, H1 and H4), 7.09 (dd, 1H, *J* = 8.5, 1.0, H11), 7.13 (d, 1H, *J* = 8.5, H12).

Preparation of berberrubine ***37***
Berberine (1 g) was heated at 190 °C in a dry oven under vacuum (20–30 mmHg) for 15 min. The crude product was recrystallized from EtOH/MeOH to provide 660 mg (69%) of **37**: mp 270–285 °C dec. SIMS: 322 (M-Cl, 100). ^{1}H NMR (CD_3OD, 300 MHz): δ 3.23 (t, 2H, *J* = 6.3, H6), 4.06 (s, 3H, OCH_3), 4.86 (t, 2H, *J* = 6.3, H5), 6.09 (s, 2H, OCH_2O), 6.94 and 7.62 (s, each 1H, H4 and H1), 7.68 and 7.97 (d, each 1H, *J* = 9.0, H12 and H11), 8.56 (s, 1H, H13), 9.74 (s, 1H, H8).

Preparation of 9-O-ethylbeberrubine ***38***
A mixture of berberrubine (770 mg) and EtI (5 mL) in DMF (50 mL) was placed in a glass-stoppered bottle and heated for 5 h at 120 °C. After the mixture cooled, the resulting crystals were filtered to give rise to 321 mg (31%) of **38** iodide. After concentration of the filtrate and addition of Et_2O, the resulting solid was filtered and recrystallized from EtOH to generate 317

mg (31%) of **38** iodide. The iodide (68 mg) was treated with AgCl in MeOH to convert it to the chloride (28 mg) of **38**: mp 223–229 °C dec. SIMS: 350 (M-Cl, 100). ^{1}H NMR (CD_3OD): 1.51 (t, 3H, J = 7.0, OCH_2CH_3), 3.26 (m, 2H, H5), 4.10 (s, 3H, OCH_3), 4.48 (q, 2H, J = 7.0, CH_2CH_3), 4.94 (m, 2H, H6), 6.96 and 7.66 (s, each 1H, ArH4 and 1), 8.0 and 8.11 (d, each 1H, J = 9.0, ArH12 and 11), 8.71 (s, 1H, H13), 9.72 (s, 1H, H8).

Antimicrobial activity

S aureus ATCC 25922 or NCTC 8530 and *E coli* IFO 3545 were cultivated in 3% nutrient broth ('Nissui') at 35 °C, while *C albicans* IFO 1061 was cultivated in 3% malt extract powder ('Oriental') at 25 °C. Antimicrobial assay was carried out using two different methods.

Paper disc method

The test compound was dissolved in MeOH containing 1% DMF at a concentration of 10 mg/mL and 50 μL of the solution was applied to a paper disc (8 mm). The disc was dried overnight at 60 °C and placed on an agar plate seeded with the test organism. After incubation for 24 h, the diameter of the inhibitory zone arround the disc was measured. A paper disc saturated with MeOH (50 μL) containing 1% DMF was used after drying as a negative control.

Broth dilution method

The test compound was dissolved in H_2O containing 1% DMF and its antimicrobial activity was measured by a twofold serial broth dilution method in 24- or 96-well test plates. After incubation for 24 h, the microbial growth was examined with the naked eye or by measuring the optical density at 655 nm with a Model 450 Microplate Reader (Bio-Rad). The concentrations examined were in the range of 31.25–2000 μg/mL. The MIC of the test compound was defined as the lowest concentration at which there was no visible growth.

References

1 Preininger V (1986) In: *The Alkaloids* (Brossi A, ed) Academic, New York, vol 29, 1–98
2 Hahn FE, Ciak J (1967) *Antibiotics* (Berlin) 3, 577–580
3 Otuka H, Fujimura H, Sawada T, Goto M (1981) *Yakugaku Zasshi* 101, 883–890
4 Iwasa K, Kondoh Y, Kamigauchi M (1995) *J Nat Prod* 58, 379–391
5 Takao N, Iwasa K, Kamigauchi M (1976) *Chem Pharm Bull* 24, 2859–2868
6 Takao N, Kamigauchi M, Okada M (1983) *Helv Chim Acta* 66, 473–484
7 Iwasa K, Kamigauchi M, Takao N (1993) *J Nat Prod* 56, 2053–2067
8 Takao N, Iwasa K (1976) *Chem Pharm Bull* 24, 3185–3194
9 Iwasa K, Kamigauchi M, Takao N (1988) *J Nat Prod* 51, 1232–1235
10 Iwasa K, Sugiura M, Takao N (1982) *J Org Chem* 47, 4275–4280
11 Tani C, Takao N, Takao S (1962) *Yukugaku Zasshi* 82, 748–750