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Potential cancer chemopreventive activity of simple isoquinolines, 1-benzylisoquinolines, and protoberberines

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Abstract

Seventeen simple isoquinolines, 15 1-benzylisoquinolines, and 19 protoberberines were tested for their inhibitory activities against Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells. Among the tested alkaloids, the inhibitory activity of all 1-benzylisoquinolines and 11 protoberberines was higher than that of β-carotene. The 1-benzylisoquinolines **19**, **21**, **22**, **29**, and **34** and protoberberines **41**, **47–49**, **51, 52**, and **55** showed potent inhibitory effects on EBV-EA induction (96–100% inhibition at 1×10^3 mol ratio/TPA). These alkaloids were more active than the naturally occurring alkaloids, **23**, **25**, **33**, **53**, and **54**. In addition, fifteen simple isoquinolines, eighteen 1-benzylisoquinolines and eight protoberberines were evaluated with respect to their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. Nine simple isoquinolines, ten 1-benzylisoquinolines, and four protoberberines were more potent than α-tocopherol, and four 1-benzylisoquinolines, **20** and **28–30**, exhibited potent activities (SC_{50} 4.5–5.8 μM). Their activities were higher than the naturally occurring alkaloids, **23**, **25**, and **33**. Therefore, some of the isoquinoline alkaloids indicating the high activity on both assays may be potentially valuable cancer chemopreventive agents. Structure–activity relationships are discussed for both tests.

Keywords: Isoquinoline alkaloids; Chemopreventive effects; Anti-tumor promoter; Epstein–Barr virus early antigen; Free radical scavenging activity; Structure–activity relationships

1. Introduction

Several plant and mammalian species contain simple isoquinolines, such as salsolinol and its *O*-and *N*-methyl analogues, 1-benzylisoquinolines, and protoberberines (Lundström, 1983; Preininger, 1986; Brossi, 1993). The 1-benzylisoquinolines are related structurally to reticuline, which is an intermediate in the biosynthesis of morphine in the opium poppy, and many types of isoquinoline alkaloids including protoberberines (Brossi, 1993; Szantay et al., 1994).

We have previously described the antimicrobial, antimalarial, cytotoxic, and anti-HIV activities of isoquinoline alkaloids (Iwasa et al., 2001a). Some of the tested compounds proved to be significantly active in these assays. The cytotoxic activities of several isoquinoline alkaloids have already been reported (Iwasa et al., 2001a,b) and the anti-tumor-promoting activities of some of isoquinoline-type alkaloids have been published (Yasukawa et al., 1991;

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Ito et al., 2001). The development of anti-tumor-promoters has been regarded as the most effective method for the prevention of carcinogenesis (Yamamoto et al., 1979; Verma et al., 1980). The Epstein–Barr virus early antigen (EBV-EA) activation assay has been considered to be an effective indicator for the evaluation of anti-tumor-promoting activity (Ito et al., 1981). Compounds which inhibit EBV-EA activation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), act as inhibitors of tumor promotion in vivo (Konoshima et al., 1995). A series of human illnesses such as cancer, diabetes, atherosclerosis, etc., can be linked to the damaging action of reactive free radicals (Konoshima et al., 1995). Thus, scavenging of free radicals seems to play a considerable part in the pharmacological activity of antioxidative compounds.

To look for possible chemopreventive anti-tumor-promoters, we carried out a primary screening of naturally occurring isoquinolines [simple isoquinolines (**1**, **7** and **8**), 1-benzylisoquinolines (**23**, **25**, and **33**), and protoberberines (**53**, **54**)] and related synthetic compounds using their inhibitory effects on the EBV-EA activation, induced by TPA, as well as tests of their radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The structure–activity relationship is discussed for different alkyl substituents on aliphatic or aromatic carbon atoms, the position or number of the hydroxyl groups and the oxidation state of the pyridine ring.

2. Results and discussion

2.1. Synthesis

The 6,7-dihydroxytetrahydroisoquinolines **1**–**5** had been prepared previously (Iwasa et al., 2001a). 1-Methyl- (**8**), 1-ethyl-, 1-propyl-, l-butyl-6,7-dimethoxydihydroisoquinolines were prepared by Bischler–Napieralski cyclization of the corresponding amides which were prepared from 3,4-dimethoxyphenylethylamine and the appropriate anhydrides (acetic, propionic, *n*-butyric, and valeric anhydrides). The l-alkyl-6,7-dimethoxydihydroisoquinolines (e.g. **8**) were *N*-methylated to give the *N*-methyl derivatives (e.g. **9**), and acid-catalyzed ether cleavage gave the 6,7-dihydroxy-*N*-methyldihydroisoquinolinium salts (**10**–**13**). Reduction of **9** afforded l,2-dimethyl-6,7-dimethoxytetrahydroisoquinoline (**7**) which was converted to its 6,7-dihydroxy derivative (**6**). The isoquinolines (**14**–**18**) had been prepared previously (Iwasa et al., 2001a). Tetrahydropapaveroline (**19**) was purchased. Commercially available papaverine was methylated to give *N*-methylpapaverinium salt which was reduced by $NaBH_4$ to give *N*-methyltetrahydropapaverine. *N*-Methyltetrahydropapaveroline (**20**) was prepared by acid-catalyzed ether cleavage of *N*-methyltetrahydropapaverine. The 1-benzyl-*N*-methyltetrahydroisoquinolines (**21**–**27**) and 1-benzylisoquinoline (**30**–**33**) were prepared by partial acid-catalyzed ether cleavage of *N*-methyltetrahydropapaverine and papaverine, respectively, and separation by preparative HPLC. On the other hand, 1-benzylisoquinolines (**28** and **29**) were prepared by complete acid-catalyzed ether cleavage of l-benzyl-5,6,3′,4′-tetramethoxyisoquinoline (Iwasa et al., 2001a) and papaverine, respectively. *N*-methylpapaveroline (**34**) was produced by the complete acid-catalyzed ether cleavage of *N*-methylpapaverinium salt. 1-Benzyl-*N*-methylisoquinolinium salts **35** and **36** (Cassels and Deulofeu, 1966) and protoberberinium salts, **37**–**40** (Iwasa et al., 1997), **41**–**50** (Iwasa et al., 1998a), and **53**–**55** and **60** (Iwasa et al., 1998b, 2003) had been prepared as described. The 2,3,9,10- or 2,3,10,11-tetrahydroxyprotoberberines (**51**, **52**, **57**, and **58**) were prepared by complete acid-catalyzed ether cleavage of the corresponding 2,3,9,10-or 2,3,10,11-tetramethoxy protoberberines. The structures of the synthetic compounds were determined by analysis of their MS, HRMS, ^{1}H NMR, and NOESY spectra. Some compounds (**1**, **7**, **8**, **23**, **25**, **33**, **53**–**56**, **59** and **60**) have previously been identified from plant materials (Lundström, 1983; Preininger, 1986; Brossi, 1993; Claude et al., 1978; Patra et al., 1987).

2.2. Inhibitory effects on EBV-EA induction

The inhibitory effects of seventeen simple isoquinolines (**1**–**11** and **13**–**18**), fifteen 1-benzylisoquinolies (**19**, **21**–**27**, **29**–**34** and **36**), and nineteen protoberberines (**37**–**55**) on EBV-EA activation induced by TPA were examined as a primary screening of anti-tumor-promoting activities.

The inhibitory effects of 6,7-dihydroxytetrahydroisoquinolines, their *N*- or *O*-methyl derivatives, and their 3,4-dihydro and dehydro analogues bearing an 1-alkyl side chain (**1**–**11**, **13**–**18**) on TPA-induced EBV-EA activation, their effects on the viability of Raji cells, and the 50% inhibitory concentration (IC_{50}) values are shown in Table 1. All tested compounds displayed the same or lower inhibition (IC_{50} 388–447) than that of the reference compound, β-carotene (IC_{50} 400) that has been studied extensively in cancer chemoprevention using animal models (Murakami et al., 1996). The inhibitory activity of the tetrahydroisoquinolines, isoquinolines, and *N*-methylisoquinolinium salts was stronger than that of dihydroisoquinolines (compare **1**, **14**, and **17** with **10**). The inhibition decreased as the number of CH_2 units in the side chain lengthened (**1**–**4**, **10**–**13**, and **14**–**16**), thus suggesting that extension of the alkyl function, and therefore relative lipophilicity of the C-1 substituent, may contribute to the inhibitory effect. There is some increase or decrease of the inhibition by replacement of the hydroxyl groups at C-6 and C-7 with dimethoxyl groups (compare **6** with **7** and **10** with **9**).

The inhibitory effects of the 1-benzyltetrahydroisoquinolines and their dehydro analogues on activation induced by TPA and the viability of Raji cells were examined. The results are shown in Table 2. All tested 1-benzylisoquinolines displayed stronger inhibition (IC_{50} 324–367) than β-carotene (IC_{50} 400). Among them, **19**, **21**, **22**, **29**, and **34** showed at 5×10^2 and 1×10^2 mol ratio/TPA higher inhibitory activity than that of ginsenoside-Rg1 which is known as a strong

Table 1
Inhibitory effects of simple isoquinoline alkaloids on TPA induced EBV-EA activation (100%)[a]

Compounds	Concentration (mol ratio/32 pmol TPA)				IC_{50}[b](mol ratio/32 pmol TPA)
	1000	500	100	10	
TIQ					
1	11.3 (60)	38.0	79.0	100	390
2	12.6 (60)	40.1	81.0	100	398
3	15.4 (60)	41.3	83.2	100	409
4	17.6 (60)	42.7	84.1	100	412
5	16.0 (60)	41.9	84.3	100	411
6	12.1 (60)	39.6	80.0	100	395
7	13.9 (60)	41.2	81.0	100	399
2HIQ					
8	14.3 (60)	43.0	82.0	100	408
9	15.2 (60)	43.2	81.9	100	408
10	18.4 (60)	41.6	85.3	100	434
11	19.3 (60)	43.7	86.6	100	438
13	21.4 (50)	46.8	87.6	100	447
IQ					
14	10.4 (60)	38.3	78.1	100	389
15	12.3 (60)	40.6	80.0	100	397
16	15.9 (60)	42.3	83.6	100	410
17	10.3 (60)	37.4	79.0	100	388
18	12.4 (60)	39.5	81.0	100	397
Ginsenoside -Rg1	0 (80)	32.5	72.6	91.0	310
β-carotene	9.1 (60)	34.3	82.7	100	400

[a] Values represent percentages relative to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cell.
[b] IC_{50} represents the mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol of TPA.

Table 2
Inhibitory effects of 1-benzylisoquinoline alkaloids on TPA induced EBV-EA activation(100%)[a]

Compounds	Concentration (mol ratio/32 pmol TPA)				IC_{50}[b](mol ratio/32 pmol TPA)
	1000	500	100	10	
1-BnTIQ					
19	3.9(60)	26.4	73.4	96.8	349
21	4.0(60)	25.2	72.2	93.0	344
22	4.2(60)	26.1	73.0	94.1	348
23	6.2(60)	29.4	75.7	100	359
24	5.0(60)	27.6	73.8	95.3	350
25	7.7(60)	29.1	75.4	100	360
26	7.9(60)	30.2	76.4	100	362
27	7.0(60)	28.3	72.4	100	358
1-BnIQ					
29	3.1(60)	25.1	71.6	95.3	348
30	6.3(60)	29.1	75.7	100	360
31	7.8(60)	31.4	76.7	100	361
32	8.3(60)	33.7	78.0	100	367
33	8.2(60)	33.5	78.8	100	367
34	2.5(60)	22.3	69.4	91.3	324
36	6.2 (60)	28.0	75.1	100	358
Ginsenoside-Rg1	0 (80)	32.5	72.6	91.0	310
β-carotene	9.1 (60)	34.3	82.7	100	400

[a] Values represent percentages relative to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cell.
[b] IC_{50} represents the mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol of TPA.

anti-tumor-promoter. Compound **34**, bearing four hydroxyl groups, displayed the strongest inhibition and its activity is comparable to that of ginsenoside-Rg1.

From a structure–activity point of view, some trends were observed. The inhibitory activity of the 1-benzylisoquinolines increased as the number of hydroxyl groups on the aromatic ring increased (compare **21** or **22** with **23**–**27** and **29** with **30**–**33**). The 1-benzyltetrahydroisoquinolines having more than three hydroxyl groups on the aromatic rings displayed significant inhibitory activity (**19**–**22**). Among the 1-benzylisoquinolines (**19**, **29**, and **34**) bearing four hydroxyl groups, the 1-benzyl-*N*-methylisoquinolinium salt (**34**) showed the strongest inhibition, indicating a contribution of quaternarization.

The inhibition by 2,3,9,10-oxygenated protoberberinium salts (**37**–**51**) and by 2,3,10,11-oxygenated protoberberinium salts (**52**–**55**) was examined (Table 3). Compounds **41**–**44**, **46**–**52**, and **55** displayed higher activity than β-carotene. The inhibitory activities at 5×10^2 and 1×10^2 mol ratio/TPA of seven compounds (**41**–**43**, **48**–**50**, and **55**) were comparable with ginsenoside-Rg1. The inhibitory activities at 1×10^3, 5×10^2 and 1×10^2 mole ratio/TPA of **51** and **52** bearing four hydroxyl groups were higher than that of ginsenoside-Rg1.

From a structure–activity point of view, some features can be pointed out. The inhibitory activity of 13-alkyl and 8-alkyl-12-bromo derivatives decreased as the C-13 or C-8 alkyl chain was extended by one carbon (compare **37**–**39**, and **42**–**44**). Replacement of the methoxyl or hydroxyl groups on the aromatic rings with methylenedioxy groups decreased the inhibitory activity (compare **39** with **40** and **53** with **54** and **55**).

On the basis of these results, it appears that in protoberberinium salts, the size of substituents at the C-8 and C-13, and type and position of the oxygenated substituents on rings A and D influence the inhibitory activity. Replacement of the methoxyl or hydroxyl groups by the methylenedioxy group on the rings A and D strongly influences the inhibition.

1-Benzylisoquinolines **19**, **21**, **22**, **29**, and **34** and protoberberines **41**, **47**–**49**, **51**, **52**, and **55** showed potent inhibitory effects on EBV-EA induction (96–100% inhibition at 1×10^3 mol ratio/TPA) as compared with those of the naturally occurring alkaloids **23**, **25**, **33**, **53**, and **54**. These synthetic alkaloids might be valuable antitumor promoters. They appear to be useful leads for further development of potential cancer chemopreventive agents.

2.3. Free radical scavenging activity

Next, the ability of the isoquinoline-type alkaloids to scavenge DPPH free radicals were examined. To evaluate the free radical scavenging activity of the isoquinolines,

Table 3
Inhibitory effects of protoberberine alkaloids on TPA induced EBV-EA activation (100%)[a]

Compounds	Concentration (mol ratio/32 pmol TPA)				IC_{50}[b](mol ratio/32 pmol TPA)
	1000	500	100	10	
PB					
37	9.5 (60)	31.8	74.7	100	361
38	10.1 (60)	34.5	77.7	100	364
39	12.9 (50)	36.3	79.0	100	382
40	9.7 (60)	32.3	75.3	100	363
41	2.5 (60)	23.1	73.1	93.1	327
42	5.0 (60)	27.4	71.5	96.0	350
43	5.9 (60)	28.0	72.7	97.4	353
44	7.0 (50)	29.6	74.8	100	359
45	10.6 (60)	34.9	78.5	100	381
46	8.3 (50)	30.2	75.0	100	363
47	2.7 (60)	25.9	74.3	95.8	346
48	2.0 (60)	23.8	73.5	94.0	325
49	2.4 (60)	24.6	72.8	95.1	343
50	4.8 (60)	26.8	73.5	95.9	352
51	0 (60)	22.7	71.5	93.7	304
52	0 (60)	21.8	69.3	92.5	300
53	15.6 (60)	37.6	80.3	100	394
54	9.4 (60)	31.7	77.8	100	363
55	2.3 (60)	24.5	71.4	94.7	326
Ginsenoside-Rg1	0 (80)	32.5	72.6	91.0	310
β-carotene	9.1 (60)	34.3	82.7	100	400

[a] Values represent percentages relative to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cell.
[b] IC_{50} represents the molratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol of TPA.

the concentration required to scavenge DPPH free radicals by 50% (SC_{50}) were determined. The antioxidant, α-tocopherol was used as reference compound.

1-Alkylated tetrahydroisoquinolines, dihydroisoquinolines, isoquinolines, and their *N*-methyl analogues were tested and the results are presented in Table 4. As shown there, 6,7-dihydroxylated tetrahydroisoquinolines (**1**–**5**) and the *N*-methyldihydroisoquinolines (**10**–**13**) displayed higher radical-scavenging activity than that of α-tocopherol. 6,7-Dimethoxytetrahydroisoquinoline (**7**), 6,7-dihydroxylated isoquinolines (**14** and **15**) and their *N*-methyl analogues (**17** and **18**) were inactive. Oxidation of tetrahydroisoquinoline to the dihydroisoquinolinium salt increased the activity (compare **6** with **10**). Aromatization of the ring B in the isoquinoline molecule led to disappearance of the activity (compare **1** with **14**, **2** with **15**, **10** with **17**, and **11** with **18**) as regardless of the presence of the hydroxyl groups at C-6 and C-7 which may contribute to increase of the activity. The activity was decreased by *N*-methylation (compare **1** with **6**). Replacement of the hydroxyl groups by the methoxyl groups at C-6 and C-7 demolished the activity (compare **6** with **7**). The activity of tetrahydroisoquinolines and *N*-methyldihydroisoquinolines did not always grow with increasing of the aliphatic chain at C-l (compare **1**–**5** and **10**–**13**).

The free radical scavenging activity of 1-benzyltetrahydroisoquinolines, 1-benzylisoquinolines, and their *N*-methyl analogues were examined. The data are shown in Table 5. Among the tested alkaloids, 1-benzyltetrahydroisoquinoline (**19**), 1-benzyl-*N*-methyltetrahydroisoquinolines (**20**–**22**), and 1-benzylisoquinolines (**28**–**31**) bearing more than two hydroxy groups displayed higher activity than α-tocopherol. Among the 1-benzyl-*N*-methyltetrahydroisoquinolines, the activity enhances as the number of the hydroxyl group on the aromatic rings increases (compare **25** and **27** → **23** and **24** → **21** and **22** → **20**) (see Fig. 1).

Table 4
Radical scavenging activity of simple isoquinoline alkaloids

Alkaloids	SC_{50} (μM)[a]
TIQ	
1	14.2
2	16.0
3	13.8
4	19.6
5	15.8
6	26.9
7	>250
2HIQ	
10	13.7
11	15.2
12	17.1
13	12.9
IQ	
14	>250
15	>250
17	>250
18	>250
α-tocopherol	20.3

[a] The compound concentration showing radical scavenging efficacy of 50% was defined as SC_{50}.

Table 5
Radical scavenging activity of 1-benzylisoquinoline alkaloids

Alkaloids	SC_{50}(μM)[a]
1-BnTIQ	
19	7.4
20	4.5
21	7.0
22	7.2
23	21.9
24	17.7
25	31.3
26	23.7
27	27.7
1-BnIQ$^+$	
28	5.8
29	5.4
30	5.0
31	8.2
32	196
33	>250
34	15.4
35	>250
36	>250
α-tocopherol	20.6

[a] The compound concentration showing radical scavenging efficacy of 50% was defined as SC_{50}.

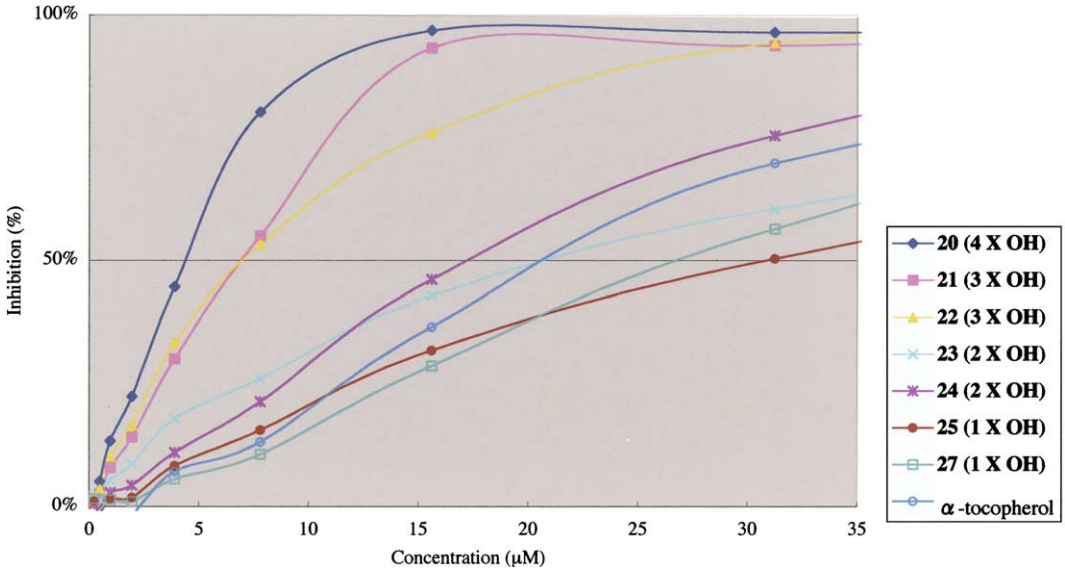


Fig. 1. DPPH radical scavenging activity of 1-benzyl-*N*-methyltetrahydroisoquinolines.

In the 1-benzylisoquinolines, the activity decreased by *N*-methylation (compare **29** with **34**). The activity grows as the number of the hydroxyl groups increase (compare **30** with **31**), and the 1-benzylisoquinolines and 1-benzyl-*N*-methylisoquinolines having a hydroxyl group displayed no activity (**32**, **33**, **35**, and **36**). Shifting hydroxyl groups at C-6 and C-7 to C-5 and C-6 had no influence on the activity (compare **28** with **29**).

Structure-radical scavenging activity relationship study of 1-benzylisoquinolines pointed to the importance of the number of the phenolic hydroxyl groups present for the antiradical efficacy. It appears that the 1-benzylisoquinolines and their tetrahydro derivatives bearing more than three hydroxyl groups on the aromatic rings are the most effective radical scavengers except for the compounds with quinolizidinium structure (Table 5).

The free radical scavenging activity of 2,3,9,10- and 2,3,10,11-oxygenated protoberberinium salts (**51**, **52**, **55**, **56**) and their tetrahydro analogues (**57**–**60**) were examined (Table 6). Compounds **51**, **52**, **57**, and **58** displayed higher activity than that of α-tocopherol. In both the protoberberinium salts and their tetrahydro derivatives, 2,3,9,10-oxygenated alkaloids showed slightly higher activity as compared with that of their 2,3,10,11-oxygenated analogues. Tetramethoxy derivatives (**55**, **56**, **59**, and **60**) are inactive. The phenolic hydroxyl groups on the aromatic rings A and D influence the activity strongly.

Among the isoquinoline alkaloids tested, some 1-benzylisoquinolines (SC_{50} 4.5–5.8 μM for **20**, **28**–**30**, SC_{50} 7.0–8.2 μM for **19**, **21**, **22**, **31**) displayed higher free radical scavenging activity than that of the simple isoquinolines (SC_{50} 12.9–14.2 μM for **1**, **3**, **10**, and **13**) and the protoberberines (SC_{50} 11.5–14.1 μM for **51**, **52**, **57**, and **58**). These alkaloids were more active than naturally occurring alkaloids **23** (SC_{50} 21.9 μM), **33**, **56**, and **59** ($SC_{50} > 250$μM), and the reference α-tocopherol (SC_{50} 20.3–20.6 μM). These

Table 6
Radical scavenging activity of protoberberine alkaloids

Alkaloids	SC_{50} (μM)[a]
Protoberberines (PB)	
51	12.3
52	14.1
55	>250
56	>250
Tetrahydroprotoberberines (TPB)	
57	11.5
58	12.4
59	>250
60	>250
α-tocopherol	20.4

[a] The compound concentration showing radical scavenging efficacy of 50% was defined as SC_{50}.

compounds possessing free radical scavenging activity might be applied to the prevention of carcinogenesis.

Among the three types of isoquinoline alkaloids, 1-benzylisoqinolines indicated the highest activity in both assays. There appears to be a correlation between free radical scavenging and tumor promoter-inhibitory activities within the 1-benzylisoquinolines from the point of view that the activities in both assays depend mainly on the number of hydroxyl groups on the aromatic rings. The high activity of the benzylisoquinolines may be explained by the ability to donate a hydrogen atom from the hydroxyl groups and thereby scavenge free radicals.

Of the test compounds prepared on the basis of the natural products, the synthetic compounds in both assays displayed higher activity in comparison with the corresponding natural products. The results of the present investigation suggest that some 1-benzylisoquinoline and protoberberine alkaloids might be useful leads for the further development of potential cancer chemopreventive agents. A study exam-

ining the tumor-promoting inhibitory activity of some compounds in vivo is in progress.

3. Experimental

3.1. General

Mps: uncorr. Conventional ^{1}H NMR and NOESY spectra were obtained on a Varian VXR-500S spectrometer (^{1}H: 500 MHz) using CD_3OD as the solvent, if not stated otherwise, with TMS as internal standard. Mass spectra were determined on a Hitachi M-4100 instrument at 75 eV. SIMS (glycerol). LC was performed on a Cosmosil 5 C_{18}-AR (4.6 i.d. × 150 mm or 20 i.d. × 250 mm) reversed phase column. The mobile phase was 0.1 M NH_4OAc (0.05% trifluoroacetic acid, TFA, A), to which MeOH (0.05% TFA, B) was added by a linear gradient. The flow rate was 1 ml/min; detection: 280 nm.

3,4-Dimethoxyphenylethylamine, tetrahydropapaveroline hydrochloride (**19**) and palmatine chloride (**56**) were obtained from commercial suppliers. The isoquinolines **1**–**5** and isoquinolines **14**–**18** had been prepared previously (Iwasa et al., 2001a). 1-Benzyl-*N*-methylisoquinolines **35** and **36** had been prepared previously according to Cassels and Deulofeu, 1966. Protoberberinium salts, **37**–**40** (Iwasa et al., 1997), **41**–**50** (Iwasa et al., 1998a), and **53**–**55** and **60** (Iwasa et al., 1998b, 2003), had been prepared previously.

3.2. Synthesis of isoquinolines *6*–*9*

A mixture of 3,4-dimethoxyphenylethylamine hydrochloride (20 g) and Ac_2O (15 ml) was allowed to react at room temperature for 2–3 h. Then ice water was added and the mixture was stirred overnight. The solution was extracted with $CHCl_3$, the solvent was removed at reduced pressure and the resulting crystals were washed with Et_2O to give the appropriate acetamide (21.7 g, Yield: 88%), m.p. 85–90 °C. A solution of this amide (5 g) in dry benzene (50 ml) and $POCl_3$ (4 ml) was heated to reflux for 2 h and then allowed to stand at room temperature. The resultant crystals were purified to give **8** (6.89 g, Yield: 99%, m.p. 132–133 °C) as a phosphate (H_3PO_4) which was suspended in $CHCl_3$ and treated with NH_4OH solution. The $CHCl_3$ extracts were dried and evaporated to afford the free base. The resulting free base (100 mg) was dissolved in MeOH–HCl, and the solvent was removed at reduced pressure to give **8** hydrochloride (m.p. 214–215 °C). The free base obtained from **8** phosphate (770 mg) was dissolved in a mixture of MeOH and Me_2CO (1:1) (20 ml), and after addition of CH_3I (1 ml) the mixture was refluxed at room temperature for 30 min. The resulting crystals were recrystallized from MeOH to give **9** iodide (640 mg, 75%, m.p. 169–171 °C), which was converted to the chloride, m.p. 79–80 °C; ^{1}H NMR ($CDCl_3 + CF_3COOD$, 500 MHz) δ 2.79 (3H, *s*, CH_3), 3.13 (2H, *t*, $J = 7.5$ Hz, H-4), 3.74 (3H, *s*, N–CH_3), 3.94 (3H, *s*, 6-OCH_3), 4.00 (2H, *t*, $J = 7.5$ Hz, H-3), 4.01 (3H, *s*, 7-OCH_3), 6.83 (1H, *s*, H-5), 7.22 (1H, *s*, H-8); SIMS *m*/*z* $[M - Cl]^+$ 220; HRMS *m*/*z* $[M - Cl]^+$ 220.1347 ($C_{13}H_{18}NO_2$ requires 220.1336).

To a solution of the methiodide **9** (3 g) in MeOH (50 ml) was added, in portions, $NaBH_4$ (1 g), and the mixture was stirred for 2 h. To the mixture was added H_2O (30 ml). The solution was concentrated and extracted with $CHCl_3$. The extract was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was dissolved in MeOH (50 ml) and conc. HCl (0.5 ml), and the solvent was evaporated in vacuo to give carnegine hydrochloride **7** (2.04 g, 97%): m.p. 210–211 °C (lit. Brown et al., 1972, m.p. 210–211 °C); ^{1}H NMR (CD_3OD, 500 MHz) δ 1.66 (3H, *d*, $J = 7.0$ Hz, CH_3), 2.95 (3H, *s*, N–CH_3), 3.08 (2H, *m*, H-4), 3.42 and 3.64 (each 1H, *m*, H-3), 4.48 (1H, *q*, $J = 7.0$ Hz, H-1), 3.82 (6H, *s*, 6- and 7-OCH_3), 6.78 (1H, *s*, H-8), 6.80 (1H, *s*, H-5); SIMS *m*/*z* $[M - Cl]^+$ 222; HRMS *m*/*z* $[M + H - Cl]^+$ 222.1500 ($C_{13}H_{20}NO_2$ requires 222.1493). Carnegine hydrochloride **7** (100 mg) was heated in 47% HBr (1 ml) at 125° for 20 min. The solvent was evaporated under reduced pressure, and the residue was purified by preparative HPLC [H_2O (0.05% TFA)/MeOH(0.05% TFA)] and converted to the amorphous **6**–HCl (70 mg, 58%): ^{1}H NMR (CD_3OD, 500 MHz) δ 1.61 (3H, *d*, $J = 7.0$ Hz, CH_3), 2.93 (3H, *s*, N–CH_3), 2.99 (2H, *m*, H-3), 3.39 and 3.61 (each 1H, *m*, H-4), 4.41 (1H, *q*, $J = 7.0$ Hz, H-1), 6.60 (1H, *s*, H-5 or H-8) and 6.61 (1H, *s*, H-5 or H-8); SIMS *m*/*z* $[M - Cl]^+$ 194; HRMS *m*/*z* $[M + H - Cl]^+$ 194.1189 ($C_{11}H_{16}NO_2$ requires 194.1180).

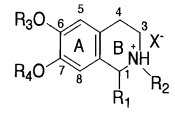
3.3. Preparation of 1-alkyl-6,7-dihydroxy-N-methylisoquinolinium salts *10*–*13*

Dihydroisoquinolinium salt **9** (100 mg) was heated in 47% HBr (1 ml) at 120–123 °C for 2 h. The solvent was evaporated under reduced pressure and the residue was crystallized from acetone to give **10** bromide (88 mg, 73%): m.p. 191–197 °C (dec.); ^{1}H NMR ($CDCl_3 + CF_3COOD$, 500 MHz)δ 2.75 (3H, *s*, CH_3), 3.07 (2H, *t*, $J = 7.5$ Hz, H-4), 3.71 (3H, *s*, N–CH_3), 3.94 (2H, *t*, $J = 7.5$ Hz, H-3), 6.91 (1H, *s*, H-5), 7.56 (1H, *s*, H-8); SIMS *m*/*z* $[M - Br]^+$ 192; HRMS *m*/*z* $[M - Br]^+$ 192.1024 ($C_{11}H_{14}NO_2$ requires 192.1024).

The *N*-methylisoquinolinium salts **11**–**13** were prepared similarly to the synthesis of **10** starting from 3,4-dimethoxyphenethylamine and the appropriate anhydrides (propionic, *n*-butyric, and valeric anhydrides). **11** bromide (57 mg, 72%): m.p. 200–201 °C; ^{1}H NMR (CD_3OD, 500 MHz) δ 1.34 (3H, *t*, $J = 7.5$ Hz, CH_2CH_3), 3.02 (2H, *t*, $J = 7.5$ Hz, H-4), 3.10 (2H, *q*, $J = 7.5$ Hz, CH_2CH_3), 3.71 (3H, *s*, N–CH_3), 3.94 (2H, *t*, $J = 7.5$ Hz, H-3), 6.79 (1H, *s*, H-5), 7.36 (1H, *s*, H-8); SIMS *m*/*z* $[M - Br]^+$ 206; HRMS 206.1180 ($C_{12}H_{16}NO_2$ requires 206.1178); **12** bromide (71 mg, 89%): m.p. 200–203 °C; ^{1}H NMR (CD_3OD, 500 MHz) δ 1.13 (3H, *t*, $J = 7.5$ Hz, $CH_2CH_2CH_3$), 1.72 (2H, *m*, $CH_2C\underline{H}_2CH_3$), 3.01 (2H, *t*, $J = 7.5$ Hz, H-4), 3.05

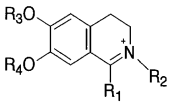
(2H, *m*, C<u>H</u>$_2$CH$_2$CH$_3$), 3.71 (3H, *s*, N–CH$_3$), 3.94 (2H, *t*, *J* = 7.5 Hz, H-3), 6.78 (1H, *s*, H-5), 7.35 (1H, *s*, H-8); SIMS *m*/*z* [M − Br]$^+$ 220; HRMS 220.1351 ($C_{13}H_{18}NO_2$ requires 220.1336); **13** bromide (65 mg, 80%): m.p. 201–204 °C; ^{1}H NMR (CD$_3$OD, 500 MHz) *δ* 1.03 [3H, *t*, *J* = 7.5 Hz, (CH$_2$)$_3$CH$_3$], 1.56 (2H, *m*, CH$_2$C<u>H</u>$_2$CH$_2$CH$_3$), 1.67 (2H, *m*, CH$_2$CH$_2$C<u>H</u>$_2$CH$_3$), 3.01 (2H, *t*, *J* = 7.5 Hz, H-4), 3.07 (2H, *t*, *J* = 7.5 Hz, C<u>H</u>$_2$CH$_2$CH$_2$CH$_3$), 3.70 (3H, *s*, N–CH$_3$), 3.94 (2H, *t*, *J* = 7.5 Hz, H-3), 6.78 (1H, *s*, H-5), 7.35 (1H, *s*, H-8); SIMS *m*/*z* [M − Br]$^+$ 234; HRMS 234.1493 ($C_{14}H_{20}NO_2$ requires 234.1502).

Simple isoquinoline alkaloids



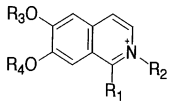
TIQ

	R$_1$	R$_2$	R$_3$	R$_4$	X
TIQ					
1	Me	H	H	H	Cl
2	Et	H	H	H	Cl
3	Pr	H	H	H	Cl
4	Pentyl	H	H	H	Cl
5	Isopropyl	H	H	H	Cl
6	Me	Me	H	H	Cl
7	Me	Me	Me	Me	Cl



2HIQ

2HIQ					
8	Me	H	Me	Me	Br
9	Me	Me	Me	Me	Br
10	Me	Me	H	H	Br
11	Et	Me	H	H	Br
12	Pr	Me	H	H	Br
13	Butyl	Me	H	H	Br



IQ

IQ					
14	Me	H	H	H	Cl
15	Et	H	H	H	Cl
16	Pr	H	H	H	Cl
17	Me	Me	H	H	Cl
18	Et	Me	H	H	Cl

3.4. Preparation of N-methyltetrahydropapaveroline (*20*)

A solution of commercially available papaverine hydrochloride (Sigma, 5 g) and CH$_3$I (5 ml) in MeOH (40 ml) and CHCl$_3$ (20 ml) was placed in a glass-stoppered bottle and heated for 13 h at 110 °C in a oil bath. The solvent was evaporated in vacuo, the residue was dissolved in CHCl$_3$ and washed with 5% Na$_2$S$_2$O$_3$. The CHCl$_3$ solution was dried (Na$_2$SO$_4$) and evaporated in vacuo. The residue (iodide) was converted to the chloride (4.03 g, 78%) by treatment with AgCl in MeOH. *N*-methylpapaverinium chloride: m.p. 145–158 °C (dec.); ^{1}H NMR (CDCl$_3$, 500 MHz) *δ* 3.82 (3H, *s*, 6- or 7-OCH$_3$), 3.84 (3H, *s*, 6- or 7-OCH$_3$), 4.01 (3H, *s*, 3′- or 4′-OCH$_3$), 4.15 (3H, *s*, 3′- or 4′-OCH$_3$), 4.65 (3H, *s*, N–CH$_3$), 5.07 (2H, *s*, H-9), 6.27 (1H, *dd*, *J* = 8.5, 2.0 Hz, H-6′), 6.71 (1H, *d*, *J* = 8.5 Hz, H-5′), 6.88 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.50 (1H, *s*, H-5), 7.53 (1H, *s*, H-8), 8.19 (1H, *d*, *J* = 7.0 Hz, H-4), 9.05 (1H, *d*, *J* = 7.0 Hz, H-3); SIMS *m*/*z* [M − Cl]$^+$ 354; HRMS *m*/*z* [M − Cl]$^+$ 354.1710 ($C_{21}H_{24}NO_4$ requires 354.1704).

To a solution of *N*-methylpapaverinium chloride (519 mg) in MeOH (200 ml) was added NaBH$_4$ (1 g), and the mixture was stirred at room temperature for 2 h. After dilution with water (30 ml), the mixture was concentrated and extracted with CHCl$_3$. The CHCl$_3$ extracts were washed with water, dried, and evaporated to give crystals which were recrystallized from MeOH–Me$_2$CO to give *N*-methyltetrahydropapaverine (200 mg, 42%): mp. 113–113.5°; ^{1}H NMR (CDCl$_3$, 500 MHz) *δ* 2.54 (3H, *s*, N–CH$_3$), 2.56–3.20 (4H, *m*, H-3 and -4), 2.77 (1H, *dd*,*J* = 13.5,7.7 Hz, H-9), 3.14 (1H, *dd*, *J* = 13.5, 5.0 Hz, H-9), 3.57 (3H, *s*, 7-OCH$_3$), 3.70 (1H, *dd*, *J* = 7.7, 5.0 Hz, H-1), 3.79 (3H, *s*, 3′-OCH$_3$), 3.84 (3H, *s*, 6-OCH$_3$), 3.85 (3H, *s*, 4′-OCH$_3$), 6.06 (1H, *s*, H-8), 6.56 (1H, *s*, H-5), 6.60 (1H, *d*, *J* = 2.0 Hz, H-2′), 6.64 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-6′), 6.77 (1H, *d*, *J* = 8.0 Hz, H-5′); SIMS *m*/*z* [M + H]$^+$ 358; HRMS 358.2021 ($C_{21}H_{28}NO_4$ requires 358.2017).

A solution of *N*-methyltetrahydropapaverine (130 mg) in 47% HBr (1 ml) was refluxed for 30 min. The solvent was evaporated in vacuo. The crystalline product was recrystallized from MeOH–Me$_2$CO to give *N*-methyltetrahydropapaveroline hydrobromide **20** (46 mg, 33%): m.p. 226–228 °C (dec.); ^{1}H NMR (DMSO–d$_6$–D$_2$O, 500 MHz) *δ* 2.77 (3H, *s*, N–CH$_3$), 2.8–3.6 (6H, *m*, H-3, −4 and −9), 4.38 (1H, br *s*, H-1), 6.13 (1H, *s*, H-8), 6.47 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-6′), 6.60 (1H, *d*, *J* = 2.0 Hz, H-2′), 6.61 (1H, *s*, H-5), 6.70 (1H, *d*, *J* = 8.0 Hz, H-5′); SIMS *m*/*z* [M + H − Br]$^+$ 302; HRMS 302.1395 ($C_{17}H_{20}NO_4$ requires 302.1391).

3.5. Preparation of phenolic 1-benzyl-N-methyltetrahydroisoquinolines (*21*)–(*27*)

The mixtures of the phenolic compounds prepared by the acid-catalyzed partial ether cleavage of 1-benzyl-*N*-methyltetrahydropapaverine were separated by preparative HPLC [(A) 0.1 M NH$_4$OAc (0.05% TFA)/(B) MeOH (0.05% TFA) initial A/B 85/15, 85 min 0/100] to give **21**–**27**. The ^{1}H NMR and MS spectroscopic data of these compounds will be presented elsewhere.

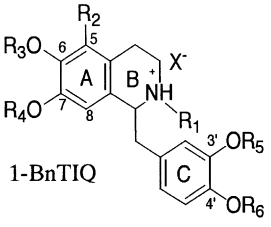
3.6. Preparation of 5,6,3′,4′-tetrahydroxy-1-benzylisoquinoline (*28*)

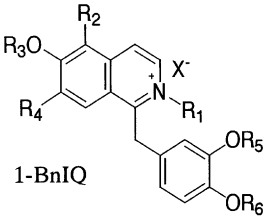
Benzyl-5,6,3′,4′-tetramethoxyisoquinoline hydrobromide (Iwasa et al., 2001a) (100 mg) in 47% HBr (1 ml) was refluxed for 3 h. The solution was evaporated in vacuo. The residue was crystallized from acetone to give **28** hydrobromide (80 mg, 74%): m.p. 223–237 °C; ^{1}H NMR (CD$_3$OD, 500 MHz) *δ* 4.68 (2H, *s*, H-9), 6.59 (1H, *dd*, *J* = 8.0, 1.5 Hz, H-6′), 6.66 (1H, *br d*, H-2′), 6.73 (1H, *d*, *J* = 8.0 Hz, H-5′), 7.55 (1H, *d*, *J* = 9.0 Hz, H-8), 8.10 (1H, *d*, *J* = 9.0 Hz, H-7), 8.12 (1H, *d*, *J* = 6.5 Hz, H-3), 8.33 (1H, *d*, *J* = 6.5 Hz, H-4); SIMS *m*/*z* [M − Br]$^+$ 284; HRMS 284.0916 ($C_{16}H_{14}NO_4$ requires 284.0921).

3.7. Preparation of papaveroline (**29**)

Papaverine hydrochloride (4 g) in 47% HBr (40 ml) was refluxed for 55 h. The resulting crystals were recrystallized from MeOH to give papaveroline hydrobromide **29** (3.27 g, 84%), m.p. 255–260 °C (dec.) which was converted to the trifluoroacetate by HPLC with H_2O–MeOH (0.05% TFA) as the mobile phase. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.63 (2H, *s*, H-9), 6.62 (1H, *dd*, $J = 8.0$, 2.0 Hz, H-6′), 6.72 (1H, *d*, $J = 2.0$ Hz, H-2′), 6.75 (1H, *d*, $J = 8.0$ Hz, H-5′), 7.44 (1H, *s*, H-5), 7.76 (1H, *d*, $J = 1.0$ Hz, H-8), 7.96 (1H, *br d*, $J = 6.5$ Hz, H-4), 8.11 (1H, *d*, $J = 6.5$ Hz, H-3); SIMS *m/z* $[M - Br]^+$ 284; HRMS 284.0936 ($C_{16}H_{14}NO_4$ requires 284.0921).

1-Benzylisoquioline alkaloids





	R_1	R_2	R_3	R_4	R_5	R_6	X
1-BnTIQ							
19	H	H	H	H	H	H	Br
20	Me	H	H	H	H	H	Br
21	Me	H	Me	H	H	H	CF_3COO
22	Me	H	H	H	H	Me	CF_3COO
23	Me	H	Me	H	H	Me	CF_3COO
24	Me	H	H	Me	Me	H	CF_3COO
25	Me	H	Me	H	Me	Me	CF_3COO
26	Me	H	H	Me	Me	Me	CF_3COO
27	Me	H	Me	Me	Me	H	CF_3COO
1-BnIQ							
28	H	OH	H	H	H	H	Br
29	H	H	H	OH	H	H	Br
30	H	H	Me	OH	H	H	CF_3COO
31	H	H	Me	OMe	H	H	CF_3COO
32	H	H	Me	OMe	Me	H	CF_3COO
33	H	H	Me	OMe	H	Me	CF_3COO
34	Me	H	H	OH	H	H	Br
35	Me	H	H	OMe	Me	Me	I
36	Me	H	Me	OH	Me	Me	I

3.8. Preparation of l-benzylisoquinolines (**30–33**)

The mixtures of the phenolic compounds prepared by the acid-catalyzed partial ether cleavage of papaverine were separated by the preparative HPLC [(A) 0.1 M NH_4OAc (0.05% TFA)/(B) MeOH (0.05% TFA) initial A/B 75/25, 10 min 50/50, 20 min 80/20] to give **30–33**. The ^{1}H NMR and MS spectroscopic data of these compounds will be presented elsewhere.

3.9. Preparation of N-methylpapaveroline (**34**)

A solution of *N*-methylpapaverinium chloride (300 mg) in 47% HBr (3 ml) was refluxed for 8 h. The solvent was evaporated in vacuo. The crystalline product was recrystallized from MeOH–Me_2CO to give **34** bromide (240 mg, 64%): m.p. 236–242 °C (dec.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ 4.21 (3H, *s*, N–CH_3), 4.67 (2H, *s*, H-9), 6.34 (1H, *dd*, $J = 8.0$, 2.0 Hz, H-6′), 6.44 (1H, *d*, $J = 2.0$ Hz, H-2′), 6.67 (1H, *d*, $J = 8.0$ Hz, H-5′), 7.44 (1H, *s*, H-5), 7.74 (1H, *s*, H-8), 8.09 (1H, *d*, $J = 6.5$ Hz, H-3), 8.36 (1H, *d*, $J = 6.5$ Hz, H-4); SIMS *m/z* $[M - Br]^+$ 298; HRMS 298.1074 ($C_{17}H_{16}NO_4$ requires 298.1079).

3.10. Preparation of protobererinium salts **51** and **52**

Palmatine (Iwasa et al., 1997) and Pseudopalmatine (Iwasa et al., 2001a) in 47% HBr were refluxed for several hours to give their tetrahydroxy derivatives [**51** bromide: m.p. 239–251 °C (dec.) and **52** bromide: m.p. 254–261 °C (dec.)] in quantitative yield. The bromides were converted to the trifluoroacetates by HPLC [H_2O (0.05% TFA)/MeOH (0.05% TFA)]. **51** trifluoroacetate: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.15 (2H, *t*, $J = 6.5$, H-5), 4.83 (2H, *t*, $J = 6.5$, H-6), 6.81 (1H, *s*, H-4), 7.47 (1H, *s*, H-1), 7.57 (1H, *d*, $J = 8.5$ Hz, H-12), 7.71 (1H, *d*, $J = 8.5$ Hz, H-11), 8.41 (1H, *s*, H-13), 9.64 (1H, *s*, H-8); SIMS *m/z* $[M - CF_3COO]^+$ 296; HRMS 296.0911 ($C_{17}H_{14}NO_4$ requires 296.0921); **52** trifluoroacetate: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.12 (2H, *t*, $J = 6.5$ Hz, H_2-5), 4.69 (2H, *t*, $J = 6.5$ Hz, H_2-6), 6.80 (1H, *s*, H-4), 7.35 (1H, *s*, H-12), 7.42 (1H, *s*, H-9), 7.45 (1H, *s*, H-1), 8.23 (1H, *s*, H-13), 9.11 (1H, *s*, H-8); SIMS

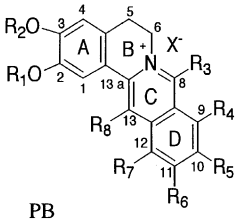
m/z [M – CF_3COO]$^+$ 296; HRMS 296.0931 ($C_{17}H_{14}NO_4$ requires 296.0921).

3.11. Preparation of tetrahydroprotoberberines 57 and 58

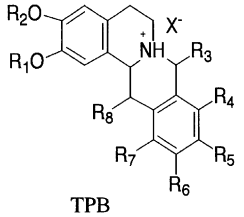
Tetrahydropseudopalmatine (Iwasa et al., 2003) and tetrahydropalmatine in 47% HBr were refluxed for several hours to give their tetrahydroxy derivatives [**57** hydrobromide: m.p. 248–256 °C (dec.) and **58** hydrobromide: m.p. 258–268 °C (dec.)] in quantitative yield.

The hydrobromides were converted to the trifluoroacetates by HPLC [H_2O (0.05% TFA)/MeOH (0.05% TFA)]. **57** trifluoroacetate, ^{1}H NMR (CD_3OD, 500 MHz) δ 2.96 (1H, *m*, H-5), 3.02 (1H, *dd*, $J = 17.0$, 12.0 Hz, H-13), 3.18 (1H, *m*, H-5), 3.52 (1H, *m*, H-6), 3.62 (1H, *m*, H-13), 3.84 (1H, *m*, H-6), 4.32 (1H, *d*, $J = 15.5$ Hz, H-8), 4.64 (1H, *dd*, $J = 12.0$, 4.5 Hz, H-13a), 4.70 (1H, *d*, $J = 15.5$ Hz, H-8), 6.65 (1H, *s*, H-4), 6.66 (1H, *d*, $J = 8.0$ Hz, H-12), 6.78 (1H, *s*, H-1), 6.79 (1H, *d*, $J = 8.0$ Hz, H-11); SIMS *m/z* [M – CF_3COO]$^+$ 300; HRMS 300.1232 ($C_{17}H_{18}NO_4$ requires 300.1235); **58** trifluoroacetate, ^{1}H NMR (CD_3OD, 500 MHz) δ 2.92 (1H, *m*, H-5), 2.99 (1H, *dd*, $J = 17.0$, 12.0 Hz, H- 13), 3.18 (1H, *m*, H-5), 3.47 (1H, *m*, H-6), 3.61 (1H, *m*, H-13), 3.78 (1H, *m*, H-6), 4.43 (2H, *br s*, H-8), 4.65 (1H, *dd*, $J = 12.0$, 4.5 Hz, H-13a), 6.61 (1H, *s*, H-5), 6.64 (1H, *s*, H-4), 6.70 (1H, *s*, H-12), 6.78 (1H, *s*, H-1); SIMS *m/z* [M – CF_3COO]$^+$ 300; HRMS 300.1225 ($C_{17}H_{18}NO_4$ requires 300.1235).

Protoberberine alkaoids



PB



TPB

	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	X
PB									
37	CH_2		H	OMe	OMe	H	H	Pr	Cl
38	CH_2		H	OMe	OMe	H	H	Bu	Cl
39	CH_2		H	OMe	OMe	H	H	Hexyl	Cl
40	Me	Me	H	OMe	OMe	H	H	Hexyl	Cl
41	CH_2		H	OMe	OMe	H	Br	H	Cl
42	CH_2		Et	OMe	OMe	H	Br	H	Cl
43	CH_2		Pr	OMe	OMe	H	Br	H	Cl
44	CH_2		Bu	OMe	OMe	H	Br	H	Cl
45	CH_2		Ph	OMe	OMe	H	Br	H	Cl
46	CH_2		Hexyl	OMe	OMe	H	Br	H	Cl
47	CH_2		Et	OMe	OMe	H	H	H	Cl
48	CH_2		Pr	OMe	OMe	H	H	H	Cl
49	CH_2		Bu	OMe	OMe	H	H	H	Cl
50	CH_2		Ph	OMe	OMe	H	H	H	Cl
51	H	H	H	OH	OH	H	H	H	CF_3COO
52	H	H	H	H	OH	OH	H	H	CF_3COO
53	CH_2		H	H	OCH_2O		H	H	Cl
54	CH_2		H	H	OMe	OMe	H	H	Cl
55	Me	Me	H	H	OMe	OMe	H	H	Cl
56	Me	Me	H	OMe	OMe	H	H	H	Cl
TPB									
57	H	H	H	OH	OH	H	H	H	CF_3COO
58	H	H	H	H	OH	OH	H	H	CF_3COO
59	Me	Me	H	OMe	OMe	H	H	H	Cl
60	Me	Me	H	H	OMe	OMe	H	H	Cl

3.12. In vitro EBV-EA activation experiment (Takemura et al., 1995)

The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer) which were cultivated in 8% FBS RPMI 1640 medium. The indicator Raji cells (1×10^6 cells/ml) were incubated at 37 °C for 48 h in 1 ml of the medium containing *n*-butyric acid (4 mM, inducer), 32 pmol of TPA (20 ng/ml in DMSO), and 32, 16, 3.2, and 0.32 nmol of the test compound (added as a DMSO solution). Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-EA positive sera from nasopharyngeal carcinoma (NPC) patients and detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the experiments were repeated three times. The average extent of EA induction was compared with that of positive control experiments with *n*-butyric acid (4 mM) plus TPA (32 pmol) in which EA induction was ordinarily around 40%. In this screening method, the cell viability required for the judgment of inhibitory effects was more than 60%.

3.13. Determination of the scavenging effect on DPPH radicals (Blois, 1958)

Ethanol (100 μl) was added to individual wells of a 96-well plate. The test compounds were dissolved in DMSO and diluted with EtOH to adjust to 500 μM concentration. The final solvent concentration was 0.25% DMSO (v/v). The sample solution (100 μl) was added to individual wells of a 96-well plate by the twofold dilution, and EtOH solution (100 μl) of DPPH radical (final concentration was 100 μM) was also added. The final concentration of the test compounds was from 0.24 to 250 μM. A control sample containing EtOH solution (100 μl) of DPPH radical and EtOH (100 μl) was prepared in the 96-well plate. The 96-well plate was incubated at 25° for 30 min in the dark. After incubation, the absorbance decrease was determined by measuring the optical density change at 550 nm with a microplate luminescence reader Lucy 2 (ALOKA). The radical-scavenging activity expressed as % inhibition against DPPH radical, was calculated according to Yen and Duh (1994): % Inhibition $= [(A_B - A_A)/A_B] \times 100$, ($A_A$ is the absorbance of the tested sample after 30 min; A_B is the absorbance of blank sample). The data presented are the average from two or three independent experiments.

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