NOTES

Isolation and Structural Elucidation of Antioxidative Substances, Carbazoquinocins A to F

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(Received for publication January 18, 1995)

Interests in active oxygen species have grown with experimental evidences that free radicals play important roles in a variety of pathological conditions such as ischemia-reperfusion, autoimmune diseases, cardiovascular diseases, cancer-initiation and aging processes[1~3]. As a consequence, antioxidative substances are now considered to be prospects as protective agents against these diseases.

In the course of our screening program for novel compounds from microorganisms showing antioxidative activity[4~11], we discovered carbazoquinocins A to F from *Streptomyces violaceus* 2448-SVT2. They showed strong inhibitory activity against lipid peroxidation induced by free radicals in rat liver microsome preparations free from vitamin E[12]. In this paper, we report the fermentation, isolation and structural studies on carbazoquinocins A to F.

A well grown agar slant of *Streptomyces violaceus* 2448-SVT2 was used to inoculate a vegetative medium (15 ml in a 50 ml tube) consisting of starch 1%, polypepton 1%, molasses 1% and beef extract 1%, the pH being adjusted to 7.2 before sterilization. Incubation was carried out at 27°C for 2 days with shaking and 2 ml of the fermentation broth were transferred into six 500-ml Erlenmeyer flasks each containing 100 ml of the production medium consisting of glycerol 2%, molasses 1%, casein 0.5%, polypepton 0.1% and $CaCO_3$ 0.4% (pH 7.2). The flasks were incubated at 27°C for 2 days on a rotary shaker. The fermentation broth (600 ml) was then transferred into a 50-liter jar fermenter containing 30 liters of the same medium, and cultivation was carried out at 30°C for 3 days with agitation at 200 rpm and aeration at 30 liters/minute.

The active materials were isolated according to the scheme shown in Fig. 1. The acetone extract of the mycelium and solvent extract of the broth filtrate were separately purified by silica gel column chromatography and the combined active fraction was finally purified by reversed phase HPLC (YMC-Pack ODS column) monitored by its inhibitory activity against lipid peroxidation and by TLC (SiO_2, $CHCl_3$ - MeOH (50 : 1), detected by UV absorption at 254 nm). Then carbazoquinocins A to F were obtained as dark green powders. Since all compounds showed similar physico-chemical properties, their structural studies were carried out using the main components C and D.

Carbazoquinocin C: HRFAB-MS (*m/z*) 310.1814 $(M+H)^+$, calcd for $C_{20}H_{24}NO_2$, 310.1807 (matrix: *m*-nitrobenzyl alcohol); mp 210~212°C; UV λ_{max} (MeOH) 228 nm (ε 14,200), 264 (11,200), 398 (2,700), λ_{max} (0.01 N NaOH - MeOH) 240 nm (ε 13,500), 284 (11,500), 453 (3,500); IR (KBr) 3440, 1640 (sh), 1625, 1245, 750 cm^{-1}.

Carbazoquinocin D: HRFAB-MS (*m/z*) 324.1961 $(M+H)^+$, calcd for $C_{21}H_{26}NO_2$, 324.1963 (matrix: *m*-nitrobenzyl alcohol); mp 208~210°C; UV λ_{max} (MeOH) 228 nm (ε 12,700), 264 (10,600), 398 (2,400), λ_{max} (0.01 N NaOH - MeOH) 240 nm (ε 12,100), 284 (10,700), 453 (3,200); IR (KBr) 3440, 1640 (sh), 1625, 1245, 750 cm^{-1}.

The carbazoquinocins exhibited characteristic UV absorptions at 228, 264 and 398 nm in MeOH, the last one shifted to 453 nm in alkaline solution. The IR spectrum displayed absorptions due to a quinone at 1640 cm^{-1} and 1625 cm^{-1}, and a 1,2-disubstituted benzene ring at 750 cm^{-1}. The structures of carbazoquinocins were determined by NMR spectral analysis. The ^1H and ^{13}C NMR data of Carbazoquinocins C and D are summarized in Table 1. The ^1H, ^{13}C NMR and DQF-COSY of carbazoquinocin C indicated the presence of partial structures of **1**, **2**, **3** and an aromatic methyl group together with an unassignable methylene group due to signal overlapping at 1.25 ppm in carbazoquinocin C as shown in Fig. 2.

The heteronuclear multiple bond correlation (HMBC) spectrum of carbazoquinocin C showed long range couplings from 14-H and 17-H to C-15. Therefore, both the partial structures **1** and **2** are linked to the methylene (C-15) revealing the presence of an *n*-heptyl residue. The

Fig. 1. Isolation of carbazoquinocins.

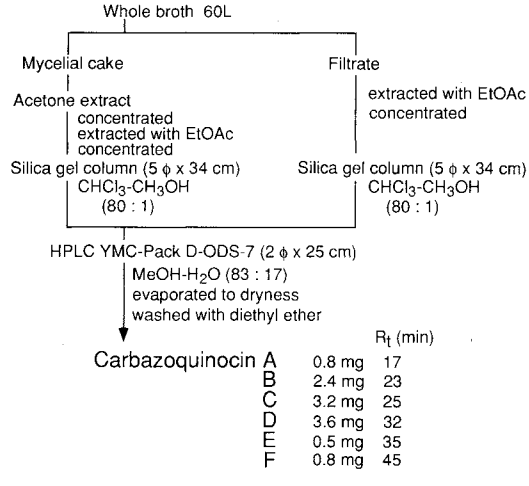
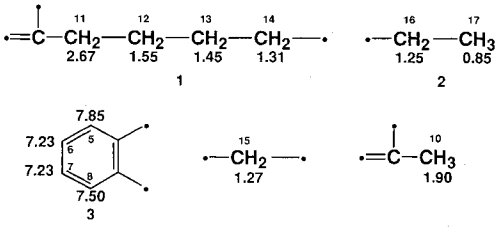


Table 1. ^1H and ^{13}C chemical shifts of carbazoquinocins C and D.

No.	C		D	
	δ_C	δ_H	δ_C	δ_H
1	142.2 (s)		142.2 (s)	
2	133.1 (s)		133.1 (s)	
3	183.5 (s)		183.5 (s)	
4	172.7 (s)		172.7 (s)	
4a	111.0 (s)		111.0 (s)	
4b	125.7 (s)		125.6 (s)	
5	120.2 (d)	7.85 (1H, m)	120.2 (d)	7.85 (1H, m)
6	123.9 (d)*	7.23 (1H, m)	123.9 (d)*	7.23 (1H, m)
7	124.1 (d)*	7.23 (1H, m)	124.1 (d)*	7.23 (1H, m)
8	113.4 (d)	7.50 (1H, m)	113.4 (d)	7.51 (1H, m)
8a	137.2 (s)		137.1 (s)	
9a	145.7 (s)		145.6 (s)	
10	11.4 (q)	1.90 (3H, s)	11.4 (q)	1.90 (3H, s)
11	28.0 (t)	2.67 (2H, t, 7.5 Hz)	28.1 (t)	2.66 (2H, t, 7.5 Hz)
12	28.5 (t)	1.55 (2H, m)	28.8 (t)	1.53 (2H, m)
13	29.0 (t)	1.45 (2H, m)	26.6 (t)	1.46 (2H, m)
14	28.6 (t)	1.31 (2H, m)	35.8 (t)	1.35 (1H, m), 1.14 (1H, m)
15	31.2 (t)	1.27 (2H, m)	33.8 (d)	1.30 (1H, m)
16	22.0 (t)	1.25 (2H, m)	28.9 (t)	1.30 (1H, m), 1.10 (1H, m)
17	13.9 (q)	0.85 (3H, t, 8 Hz)	11.2 (q)	0.82 (3H, t, 7 Hz)
18			19.1 (q)	0.83 (3H, d, 6.5 Hz)

ppm from internal TMS in DMSO-d_6.
* Assignments are interchangeable.

Fig. 2. Partial structures of carbazoquinocin C.



terminal methylene protons 11-H of this alkyl residue were long-range coupled to C-1 (δ 142.2), C-2 (δ 133.1) and C-9a (δ 145.7), the methylene protons 12-H to C-1 and C-11 (δ 28.0) as shown in Fig. 3. In addition, the aromatic methyl protons 10-H were long-range coupled to C-1, C-2 and a carbonyl carbon C-3 (δ 183.5). Thus, the *n*-heptyl residue and the aromatic methyl group are located at C-1 and C-2 respectively as shown in Fig. 3.

As mentioned above, carbazoquinocin C contains a quinone system which must be *o*-benzoquinone because C-9a in Fig. 3 cannot be assigned to a quinone carbonyl carbon due to its chemical shift (δ 145.7). The presence of this functional group was confirmed by a chemical method (see later). This *o*-benzoquinone system with methyl and heptyl substituents is linked to the remaining unit to form the final structure of carbazoquinocin C as shown in Fig. 4.

Fig. 3. HMBC experiment of carbazoquinocin C (arrows).

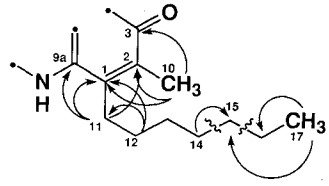
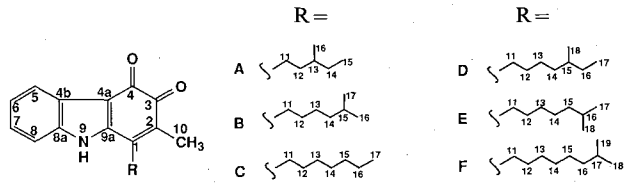


Fig. 4. Structures of carbazoquinocins.



Carbazoquinocin D showed physico-chemical properties similar to C, but contains an additional methyl group, and the ^1H NMR spectrum showed the same signals due to four aromatic protons and an aromatic methyl group (δ 1.90, s) as in carbazoquinocin C. Thus, carbazoquinocin D is different from C only in the alkyl residue. As shown in Table 1, the ^1H NMR spectrum of carbazoquinocin D showed doublet and triplet aliphatic methyl groups, and the chemical shifts of the corresponding aliphatic methyl carbons shifted to higher field (δ 19.1 and 11.2, respectively) due to the γ-effect[13]. Thus, the presence of a 5-methylheptyl residue was confirmed.

The presence of an *o*-benzoquinone system in carbazoquinocin D was confirmed by the preparation of an *o*-phenylenediamine adduct. Its molecular formula was proved as $C_{27}H_{29}N_3$ by HRFAB-MS [$(M+H)^+$, *m/z* 396.2449 (+0.9 mmu error)]. In addition to the signals originating from carbazoquinocin D, the ^1H NMR spectrum of this compound showed four aromatic protons due to the *o*-phenylenediamine moiety [δ 8.39 (1H, d, J=7.5 Hz), 8.30 (1H, d, J=7.5 Hz), 7.83 (1H, dt, J=7.5, 1.5 Hz) and 7.76 (1H, dt, J=7.5, 1.5 Hz)]. Thus, the structure of carbazoquinocin D was determined as shown in Fig. 4. The *o*-phenylenediamine adduct showed no inhibitory activity against lipid peroxidation.

The structures of the remaining components, carbazoquinocins A, B, E and F (Fig. 4) were established by comparison of the HRFAB-MS and ^1H NMR spectral data. Their molecular formulae and mps are as follows; A: $C_{19}H_{21}NO_2$ [$(M+H)^+$, *m/z* 296.1678 (+2.7 mmu error)], 210~212°C; B: $C_{20}H_{23}NO_2$ [$(M+H)^+$, *m/z* 310.1812 (+0.5 mmu error)], 213~217°C; E: $C_{21}H_{25}NO_2$ [$(M+H)^+$, *m/z* 324.1971 (+0.8 mmu error)], 209~210°C; F: $C_{22}H_{27}NO_2$ [$(M+H)^+$, *m/z* 338.2137 (+1.7 mmu error)], 208~210°C.

The ^1H NMR spectrum of carbazoquinocin A showed two aliphatic methyl groups [δ 0.97 (3H, d, J=8 Hz) and 0.87 (3H, t, J=8 Hz)] in addition to the well separated

terminal methylene protons of the alkyl residue [δ 2.64 (1H, ddd, J=13.5, 11, 5.5 Hz) and 2.56 (1H, ddd, J=13.5, 11, 5.5 Hz)]. These data proved the presence of a 3-methylpentyl residue.

The ^{1}H NMR spectrum of carbazoquinocins B, E and F showed doublets of aliphatic methyl groups [B: δ 0.86 (6H, d, J=7 Hz); E: δ 0.81 (6H, d, J=7 Hz); F: δ 0.71 (6H, d, J=7 Hz)]. Therefore, the structures of these components were determined as shown in Fig. 4.

Carbazoquinocins are carbazole derivatives[5,14~17] containing an *o*-quinone. IC_{50} values of carbazoquinocins A, B, C, D, E and F in the assay system employed in this experiment were 0.41, 0.12, 0.22, 0.37, 0.33 and 0.42 mM, respectively, while that of vitamin E was 0.28 mM.

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