Phytochemistry, Vol. 22, No. 11, pp. 2509–2512, 1983.
Printed in Great Britain.

0031–9422/83 $3.00+0.00

N^6-(2-HYDROXYETHYL)ADENOSINE, A BIOLOGICALLY ACTIVE COMPOUND FROM CULTURED MYCELIA OF *CORDYCEPS* AND *ISARIA* SPECIES*

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(*Revised received* 4 *March* 1983)

Key Word Index—*Cordyceps pruinosa*; Ascomycotiana; vegetable wasps; higher fungi; N^6-(2-hydroxyethyl)adenosine; cordycepin; adenosine; Ca^{2+} antagonist; inotropic response.

Abstract—The water–ethanol extracts of the cultured mycelia of 17 species of *Cordyceps* and six species of *Isaria* were each examined for the presence of Ca^{2+} antagonists and inotropic agents using the left atrium of the guinea pig *in vitro* system. The extracts from two of the *Cordyceps* species and four of the *Isaria* species showed strong activity in both tests. The active compound was isolated from the extracts and identified as N^6-(2-hydroxyethyl)adenosine. This is the first time that synthetically known compound has been isolated from biological sources. At the same time, the extracts of these *Cordyceps* and *Isaria* species were examined for the presence of cordycepin and adenosine.

INTRODUCTION

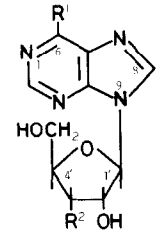
Cordyceps is an interesting genus of fungus because of its characteristic parasitic habit on larvae and pupae of insects and even on fully grown insects. *Cordyceps sinensis* (Chinese name: Tochu-Kaso) has long been regarded as a celebrated drug in the Chinese Pharmacopeia and is used as a tonic. The first report of a metabolite of *C. sinensis* is due to Chatterjee *et al.* [1], who isolated cordycepic acid. Miyazaki *et al.* [2] investigated the water-soluble mycelial polysaccharide of these fungi. Reports of the isolation of the antibacterial compound cordycepin from the culture broth of *C. militaris* have been made by Spring *et al.* [3, 4] and Frederiksen *et al.* [5]. Recently, Kneifel *et al.* [6, 7] isolated and characterized the hitherto unknown antibiotic ophiocordin from submerged cultures of *C. ophioglossoides*. Although many studies have been done, they have not revealed the true active component of *Cordyceps* species.

In the hope of finding the true tonic principle, we have screened the extracts of a large number of *Cordyceps* and *Isaria* species for the presence of Ca^{2+} antagonists and inotropic agents using the left atrium of the guinea pig heart *in vitro* system. In this paper, we report the development of a culture system for *Cordyceps* and *Isaria* species and the isolation of a new fungal metabolite, N^6-(2-hydroxyethyl)adenosine, which behaves as a Ca^{2+} antagonist and an inotropic agent.

RESULTS AND DISCUSSION

In some cases, the ascospores of *Cordyceps* do not germinate. The results of a germination test are summarized in Table 1. The ascospores of *C. prolifica* (H99) and *Cordyceps* species H63 and H75 germinated easily on all media tested. The ascospores of *C. gracilioides* (H64) germinated only on No. 6 medium whilst *C. nutans* did not germinate on any of the test media. From the results shown in Table 1, the medium containing many kinds of natural extracts was the best for ascospore germination.

A new metabolite, compound **1**, was isolated from cultured mycelia of *C. pruinosa*. The isolation procedure and the activity of each purification step are summarized in Table 2. The specific activity was increased some 500-fold on purification. Finally, compound **1** (76.3 mg from 1.8 kg fr. wt mycelia) was isolated as colourless needles, mp 193°, $C_{12}H_{17}N_5O_5$. It showed UV maxima at 213.5 and 267 nm (log ε 4.19 and 4.20) and IR bands at 3300 (OH, NH) and 1625 cm^{-1} (–C=N–). These spectral data closely resembled those of purine nucleosides. The ^1H NMR spectrum (DMSO-d_6) of compound **1** exhibited two triplets at δ 4.73 (1H, J = 4.5 Hz) and 5.37 (1H, J = 7.5 Hz), two doublets at δ 5.14 (1H, J = 4.5 Hz) and 5.40 (1H, J = 6 Hz), and a broad singlet at δ 7.61 (1H). These signals disappeared on addition of D_2O. These data indicated the presence of four hydroxyl groups and one secondary amine. The ^{13}C NMR spectrum of compound **1** (Table 3) provided further evidence for the structure elucidation. Twelve signals were observed in the proton noise decoupling spectrum. These were assigned with the aid of the off-resonance decoupled spectrum and reported



1 $R^1 = NH_2$, $R^2 = OH$ adenosine

2 $R^1 = NH_2$, $R^2 = H$ cordycepin

3 $R^1 = HNCH_2CH_2OH$, $R^2 = OH$ N^6-(2-hydroxyethyl)-adenosine

*Part 1 in the series "Studies on the Metabolites of Higher Fungi".

Table 1. Percentage germination of the ascospores of *Cordyceps* species in various media*

Species		Media†					
		No. 2	No. 3	No.4	No. 5	No. 6	No. 7
Cordyceps prolifica	(H 99)	40	50	67	60	17	—
Cordyceps sp.	(H 63)	100	—	—	—	80	—
C. gracilioides	(H 64)	0	0	0	0	29	0
Cordyceps sp.	(H 75)	89	60	—	100	100	83
C. tricentri	(H 76)	0	25	0	17	42	29
C. nutans		0	0	0	0	0	0

* % Germination = [number of germination ascospores ÷ total number of inoculating asci and/or ascospores (measured by micromanuplator)] × 100.

†The composition of each medium is given in Experimental.

Table 2. Isolation of N^6-(2-hydroxyethyl)adenosine

Purification step	Activity* (%)	Yield (%)
Lyophilized powder	73.5 ± 5.2(60 μg/ml)	100.00
Butanol extract	80.1 ± 7.4(10 μg/ml)	13.51
HPLC-C	70.5 ± 3.3(1 μg/ml)	1.59
HPLC-M	87.6 ± 2.7(0.5 μg/ml)	0.32
HPLC-IV	87.3 ± 1.9(0.3 μg/ml)	0.18
Compound **1**	67.9 ± 8.6(0.1 μg/ml)	0.10

*Negative inotropic response expressed as the percentage of initial level before addition of samples in physiological salt solution

Table 3. ^{13}C NMR chemical shift data of N^6-(2-hydroxyethyl)adenosine (HEA) and cordycepin (25 MHz, DMSO-d_6)

C	HEA	Cordycepin
2	153.4(*d*)	153.6(*d*)
4	149.1(*s*)	150.1(*s*)
5	120.7(*s*)	120.2(*s*)
6	155.9(*s*)	157.2(*s*)
8	140.9(*d*)	140.3(*d*)
10	43.6(*t*)	—
11	60.7(*t*)	—
1′	89.0(*d*)	91.9(*d*)
2′	74.5(*d*)	75.6(*d*)
3′	71.6(*d*)	35.0(*t*)
4′	86.9(*d*)	81.7(*d*)
5′	62.6(*t*)	63.6(*t*)

literature data of adenosine [8]. The chemical and physical data of compound **1** were completely identical with those of N^6-(2-hydroxyethyl)adenosine (HEA). The biological effect of natural HEA closely parallels that of the synthetic compound. Takagi *et al.* [9] reported the synthesis of HEA and showed that it provided radioprotection as strong as that attainable with mercaptoethylguanidine.

All the spectroscopic data of compound **2** were identical with those of adenosine. Adenosine showed a Ca^{2+} antagonistic effect and negative inotropic response (56.8 ± 7.2%; 10 μg/ml). Thus this purine seems to be a second active compound among the metabolites of *Cordyceps* and *Isaria*.

Cordycepin was isolated from the culture broth of *C. militaris* and was identified as synthetic 3-deoxyadenosine. In our experiment, the yield of cordycepin was better than that reported in a previous study [3, 4].

The growth, biological activity and metabolites of the mycelia and culture broth of 17 *Cordyceps* and 6 *Isaria* species are summarized in Table 4. The results show that the growth of the hyphae of *Isaria* spp. was better than that of *Cordyceps* spp. Inotropic response activity was found in mycelial extracts of five *Cordyceps* (Cp, Cc, H31, H45, H47) and four *Isaria* (Ij, H55, Is-1, H40) species, and at the same time HEA was detected by HPLC in the extracts of six species. These results indicate that the Ca^{2+} antagonistic effect and inotropic responses are mainly due to HEA produced by these fungi. Although an inotropic response was given by the extract of *C. sinensis*, HEA could not be detected on HPLC. In view of the presence of adenosine in the extract of *C. sinensis*, it seems likely that adenosine is one of the biologically active components produced by this fungus.

Cordycepin, a metabolite of *C. militaris*, was not detected in the cultured broth of any other species of *Cordyceps* and *Isaria*. However, adenosine was detected in all species of *Cordyceps* and *Isaria*.

EXPERIMENTAL

Mps (Büchi apparatus), uncorr; ^1H and ^{13}C NMR: DMSO-d_6, TMS as int. standard; MS: direct insertion probe.

Establishment and culture conditions of Cordyceps *and* Isaria *species.* Each *Cordyceps* species was established in culture from an ascus and/or an ascospore isolated from perithecial stroma using a micromanipulator. *Isaria* species were established in culture from either a single or several conidiospores by the same method. In other cases, each culture was established and the hyphae were isolated from the fruit body, stipe or sclerothium. Developing colonies of the new germinating hyphae from ascospores and conidiospores were transferred to malt agar, Bacto (Difco), containing 0.5% yeast extract in Petri dishes. Pure cultures were used for expts. The media used for the isolation of the hyphae (Nos. 2–7), subculture (No. 2) and experimental

Table 4. Species of *Cordyceps* and *Isaria* examined, the growth, negative inotropic agents and metabolites

Species	Culture symbol	Growth* medium No. 1	Growth* medium No. 2	Negative inotropic response† (%)	Metabolites‡ HEA	Metabolites‡ Adenosine	Metabolites‡ Cordycepin
Cordyceps militaris	Cm	37	48	—	−	+	+
C. pruinosa	Cp	45	48	73.5±5.2(60 μg/ml)	+	+	−
C. coccidiocola	Cc	50	46	56.7±1.5(60 μg/ml)	+	+	−
C. takaomontana	H 31	25	37	29.7±9.8(60 μg/ml)	±	+	−
C. crinalis	H 30	30	25	0 (60 μg/ml)	−	+	−
C. clavata	H 93	32	34	—	−	+	−
C. ophioglossoides	H 102	12	18	0 (60 μg/ml)	−	+	−
C. prolifica	H 99	18	25	0 (60 μg/ml)	−	+	−
C. longissima	H 22	6	15	0 (60 μg/ml)	−	±	−
C. kanzashiana	H 45	10	19	38.7±6.3(60 μg/ml)	−	+	−
C. pleuricapitata	H 47	11	19	23.2±7.2(60 μg/ml)	−	+	−
C. termitophila	H 48	30	34	0 (60 μg/ml)	−	+	−
C. macularis	C-1	28	36	0 (30 μg/ml)	−	+	−
C. michiganensis	H 67	22	23	—	−	+	−
C. alboperitheciata	H 68	9	24	0 (60 μg/ml)	−	+	−
Cordyceps sp.	H 63	37	43	0 (100 μg/ml)	−	+	−
Cordyceps sp.	H 75	10	15	—	−	+	−
Isaria farinosa	If	36	39	0 (30 μg/ml)	−	+	−
I. japonica	Ij	49	52	82.8±3.7(60 μg/ml)	+	+	−
Isaria sp.	H 55	—	—	86.1±4.0(10 μg/ml)	+	+	−
Isaria sp.	Is-1	60	63	54.5±4.9(10 μg/ml)	+	+	−
Isaria sp.	H 40	57	—	77.1±11.7(10 μg/ml)	+	+	−
Isaria sp.	H 60	—	—	0 (60 μg/ml)	−	+	−
Cordyceps sinensis	Not cultured			39.6±4.5(60 μg/ml)	−	+	−

*The growth of the hyphae was measured as the diameter (mm) of the hyphae after culture for 14 days following inoculation of the centre of a 9 cm Petri dish containing No. 1 or No. 2 medium with a 5 mm hyphal disc. Medium No. 1: malt extract 30 g, agar 15 g, distilled water 1 l.; medium No. 2: medium No. 1 with 0.5% yeast extract. —, Unable to measure because many conidiospores germinated on the whole surface of the dish at the same time.

†Expressed as the percentage of the initial level before application of the samples. The samples were dissolved in a physiological salt solution. The concentrations used are shown in parentheses. —, Not examined.

‡+, Detected clearly; ±, detected slightly; −, not detected.

ture (No. 8) were as follows (g/l. distilled H_2O); No. 1: malt ract 30, agar 15; No. 2: medium No. 1 with yeast extract 5; No. medium No. 1 with peptone 5; No. 4: medium No. 2 with ɔtone 5; No. 5: medium No. 4 with brain heart infusion 5; No. 6: dium No. 5 with tryptone 5; No. 7: diced potatoes 250, yeast ract 5, glucose 20, agar 18 (finely diced potatoes boiled in)ml H_2O until thoroughly cooked; filtered through four layers gauze and the filtrate made up to 1 l. with H_2O. The agar was solved in the filtrate by heating, and the yeast extract and ıcose were added prior to sterilization); No. 8: peptone 5, yeast ract 3, KH_2PO_4 0.3, K_2HPO_4 0.3, $MgSO_4 \cdot 7H_2O$ 0.3, glucose Isolation media were used in slant, and subculture was carried t in 9 cm disposable Petri dishes. The experimental cultures re grown in 1 l. Roux flask containing 200 ml medium. All ltures were grown in the dark at 25°.

Germination test of Cordyceps *species.* Media Nos. 2–7 were ɔd for the test of germination of six species of *Cordyceps*. The ts used an isolated ascus and/or ascospore (method as above), ıng from 7 to 10 slants for each examination.

Ca^{2+} *antagonistic effect and inotropic response* [10]. These re measured in the following way. The left atrium from male ırtley strain guinea pigs (300–400 g) was suspended in organ th (20 ml) containing a physiological salt soln (NaCl 135; KCl $CaCl_2$ 2; $MgCl_2$ 1; $NaHCO_3$ 15; glucose 5.5 mM) aerated with % O_2 + 5% CO_2 at 37°. Isometric contractions were measured with a force displacement transducer (Nihon Kohden) and recorded on an ink-writing oscillograph. The resting tension was kept at 0.5–1 g at which the developed tension was maximum. Electrical field stimulation of 3 msec duration was applied to the left atrial muscles through bipolar platinum plate electrodes at a frequency of 1 Hz with voltage *ca* 2.5 times greater than the threshold. Samples were applied cumulatively and inotropic responses after each dose were expressed as the percentage of initial level before application of the samples. The calcium antagonistic effect of the samples was determined as follows: the second dose–response curve for $CaCl_2$ in the left atrium was expressed as a percentage of the maximum response in the first cumulative application in the absence and in the presence of sample and was compared.

Extraction procedure. The mycelia were harvested with nylon cloth and homogenized with H_2O–EtOH (2:1) in a Waring blender and allowed to stand for 1 week at room temp. The homogenate was then filtered and the residue re-extracted with the same solvent, followed by MeOH (×2). The organic solns were combined and evapd to remove the organic solvent. The aq. soln was extracted with $CHCl_3$ and the aq. fraction from H_2O–EtOH (2:1) extraction was lyophilized and used (a) in the tests for Ca^{2+} antagonistic effect and inotropic response, and (b) for the detection of HEA and adenosine by HPLC. The culture broth was extracted with $CHCl_3$–MeOH (2:1) and the

$CHCl_3$–MeOH fraction evapd to a small vol. and examined for the presence of cordycepin with TLC using $CHCl_3$–MeOH (6:1, R_f 0.16).

Isolation and identification of HEA and adenosine. After 3 (20 Roux flasks) and 4 (19 Roux flasks) weeks culture, the mycelia (total 1.8 kg fr. wt) of *C. pruinosa* were harvested, homogenized with 9.1 l. of H_2O–EtOH (2:1) and allowed to stand for 1 week at room temp. The homogenate was then filtered and the residue re-extracted with 3.0 l. of the same solvent. The filtrates were combined and evapd under red. pres. The residue was extracted with $CHCl_3$ and the aq. soln lyophilized (total 65.4 g). The lyophilization powder (CpHEFd) was dissolved in H_2O and extracted with H_2O–saturated BuOH. After evapn, the lyophilized H_2O–saturated BuOH fraction gave a brown powder [(CpHEBuFd) total 9.64 g]. Further separation was achieved by HPLC. In the first step, an aliquot of the H_2O–saturated BuOH fraction (1200 mg) was run on a Unisil Q C18 packed column (7.6 mm × 300 mm) eluted with aq. 13% MeOH (4 ml/min). Each fraction was evapd and the residue was dissolved in H_2O and lyophilized: A (R_t 0–3.3 min, 586.7 mg), B (R_t 3.3–5.5 min, 320.6 mg) and C (R_t 5.5–21 min, 141.1 mg). Fraction C (HPLC-C), the most biologically active fraction, was separated on Unisil Q C18 eluted with aq. 15% MeOH (4 ml/min) to give: Y (R_t 5.6–6.9 min, 14.7 mg), Z (R_t 6.9–10.2 min, 41.1 mg) and M (R_t 10.2–21.0 min, 28.7 mg). Fraction M (HPLC-M) showed strong activity but was a mixture of two components on HPLC (R_t 11.0 and 14.1 min). Fraction Z was less active than fraction M. In the final chromatographic step, fraction M was separated into 2 fractions corresponding to the peak areas of R_t 11.0 (II) and 14.1 (IV, HPLC-IV, 16.4 mg) min by HPLC (conditions as above). The separation process and the activities are summarized in Table 2. Finally, fraction IV which contained the most active compound, **1**, was recrystallized from MeOH to yield colourless needles (9.5 mg), mp 193°, $C_{12}H_{17}N_5O_5$ (required 311.1230; M^+: 311.1208). UV λ_{max}^{EtOH} nm (log ε): 213.5 (4.19), 267 (4.20); IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1625 (–C=N–); MS (probe) 20 eV *m/z* (rel. int.): 311 $[M]^+$ (15), 281 (19), 222 (30), 208 (47), 149 (88), 148 (100); ^1H NMR (90 MHz, DMSO-d_6): δ 3.60 (6H, *s (br)*, NH–C<u>H</u>$_2$–C<u>H</u>$_2$–OH, C<u>H</u>$_2$OH), 3.97 (1H, *m*, H-4′), 4.16 (1H, *m*, H-3′), 4.60 (1H, *t*, J = 6 Hz, H-2′), 4.73 (1H, *t*, J = 4.5 Hz, lost with D_2O, $CH_2CH_2O\underline{H}$), 5.14 (1H, *d*, J = 4.5 Hz, lost with D_2O, OH-3′), 5.37 (1H, *t*, J = 7.5 Hz, lost with D_2O, OH-5′), 5.40 (1H, *d*, J = 6 Hz, lost with D_2O, OH-2′), 5.92 (1H, *d*, J = 6 Hz, H-1′), 7.61 (1H, *s(br)*, lost with D_2O, NH–), 8.25 (1H, *s*, H-2), 8.40 (1H, *s*, H-8). The chemical shifts and assignment of ^{13}C NMR of **1** are summarized in Table 3. **1** was identified by IR, NMR, MS and mmp comparison with an authentic sample. Compound **2** showing weak activity was isolated from fraction Z and recrystallized from H_2O, mp 225–226.5°, $C_{10}H_{13}N_5O_4$ (required 267.0967; M^+: 267.0971). IR ν_{max}^{KBr} cm^{-1}: 3360, 1690, 1340, 1310. The mmp of **2** and adenosine was not depressed. Therefore, **2** was determined to be adenosine.

Isolation and identification of cordycepin. After 36 days culture, the culture broth (6.4 l.) of *C. militaris* was extracted with $CHCl_3$–MeOH (2:1). The $CHCl_3$–MeOH fraction was evapd to dryness (*ca* 3.86 g) and a part (334.4 mg) chromatographed on Sephadex LH 20 (27 mm × 370 mm) eluted with MeOH (13 ml fractions). From fractions 13–15, crude cordycepin (67.8 mg) was obtained and recrystallized from EtOH to give colourless needles (31.5 mg). On repeating this process on the rest of fractions 13–15, 487.8 mg cordycepin was obtained. Mp 220–221°, $C_{10}H_{13}N_5O_3$ (required 251.102; M^+: 251.099), IR ν_{max}^{KBr} cm^{-1}: 3300, 1680, 1615, 1570; UV λ_{max}^{MeOH} nm (log ε): 260 (4.16); ^1H NMR (DMSO-d_6): δ 1.93 (1H, *ddd*, J = 12, 6, 3 Hz, Ha-3′), 2.32 (1H, *ddd*, J = 12, 7.5, 6 Hz, Hb-3′), 3.65 (2H, *m*, H_2-5′), 4.38 (1H, *m*, H-4′), 4.62 (1H, *m*, H-2′), 5.15 (1H, *t*, J = 5.6 Hz, lost with D_2O, OH-5′), 5.66 (1H, *d*, J = 4.5 Hz, lost with D_2O, OH-2′), 5.90 (1H, *d*, J = 3.0 Hz, H-1′), 7.27 (2H, *s*, lost with D_2O, NH_2), 8.17 (1H, *s*, H-2), 8.37 (1H, *s*, H-8). The chemical shifts and assignment of the ^{13}C NMR of cordycepin are summarized in Table 3.

HPLC. This was performed on a Waters liquid chromatograph 6000 A instrument fitted with a Waters absorbance detector (model 440) and a differential refractometer. A column (7.6 × 300 mm) packed with Unisil Q C18 was operated under the following conditions: prep. HPLC: either aq. 13 and 15% MeOH (4.0 ml/min) or aq. 20% MeOH and aq. 10% MeCN (2.0 ml/min). R_t (min): HEA: 17.2, 14.0; adenosine 14.5, 14.6; codycepin: 21.5, 21.6, respectively.

Acknowledgements—We are grateful to Dr. S. Tanaka and Miss M. Iijima, Nissan Chemical Industries Ltd., for the bioassay and helpful discussions; Dr. M. Shikita, National Institute of Radiological Sciences, and Professor M. Ikehara, Osaka University, for the authentic samples; Drs. Y. Kobayashi and Y. Doi, National Science Museum, for identification of fruit bodies and the suggestion of the isolation technique for obtaining pure cultures; and the members of the Analytical Center of this University for NMR and MS.

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