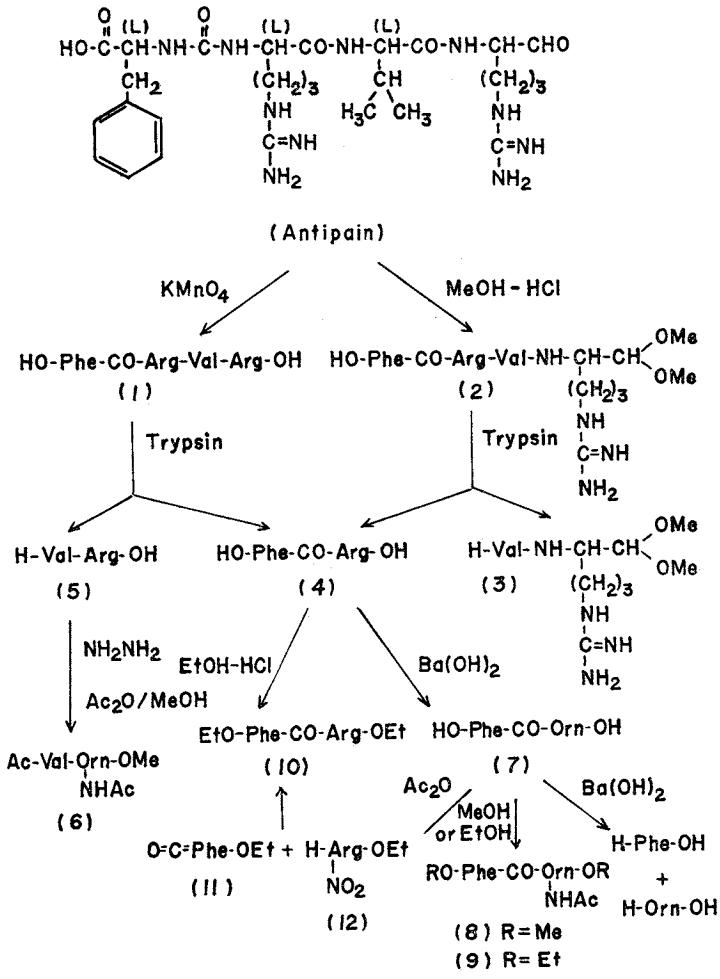
STRUCTURE OF ANTIPAIN, A NEW SAKAGUCHI-POSITIVE PRODUCT OF STREPTOMYCES

Sir:

In the course of our chemical screening of culture filtrates of microorganisms[1], a new SAKAGUCHI-positive metabolite was discovered in culture filtrates of the strain KC 84-AG 13, closely related to *Actinomyces violascens* and *Streptomyces mauvecolor*. This metabolite was found to be identical with antipain which was independently obtained by testing for anti-papain activity of culture filtrates[2]. The present paper will describe the isolation and the structural elucidation of antipain.

Antipain in a culture filtrate was adsorbed on charcoal and eluted with methanol at pH 4.0. It was purified by successive column chromatographic procedures: acidic alumina column and methanol; cellulose column and *n*-butanol-ethanol-water (4 : 1 : 2); Dowex 1×2 (OH^- form) column and water. Antipain was obtained as its dipicrate or dihydrochloride. Dipicrate·monohydrate (from water): mp 140~144°C; $[\alpha]_D^{23}$ −0.5° (*c* 0.5, water); Found: C 43.27, H 5.21, N 20.33 %; Calcd. for $C_{27}H_{44}N_{10}O_6 \cdot 2C_6H_3N_3O_7 \cdot H_2O$: C 43.33, H 4.85, N 20.73 %. Dihydrochloride·monohydrate (from water-acetone): mp 170~177°C(dec.); $[\alpha]_D^{20}$ −10° (*c* 1.0, water), $[\alpha]_D^{18}$ −17° (*c* 1.0, 1 N HCl); Found: C 46.44, H 6.94, N 19.49, Cl 10.32 %; Calcd. for $C_{27}H_{44}N_{10}O_6 \cdot 2HCl \cdot H_2O$: C 46.62, H 6.95, N 20.13, Cl 10.19 %; IR (KBr): 3350, 3150 (NH, OH), 2950 (CH), 1730 (sh., aldehyde?), 1695 (sh., COOH), ~1650 (amide I, guanidinium), 1550 (amide II), 1450 (phenyl), 1395, 1230, 1175 (CH); 1100, 1000 (C-O, C-N); 750, 700 cm^{-1} (phenyl), UV, $\lambda_{max}^{H_2O}$ (ε) 247 (490), 252 (475), 257 (435), 263 (320), 267 mμ (240); NMR [100 MHz; in D_2O; δ value (ppm)]: 0.94 [6 H, d, J~6 Hz, CH-($C\underline{H}_3$)$_2$], 1.3~2.4 (9 H, m), 2.9~3.7 (6 H, m), 3.8~4.3 (3 H, m), ~4.5 (1 H, m), 4.98 (approximately 0.5 H, d, J~4 Hz), 5.41 and 5.45 (approximately 0.5 H in total, each d, J~3 Hz), ~7.3 (5 H, m, phenyl protons); pKa 3.3, >10.5; Rf 0.42 [cellulose, *n*-BuOH - EtOH - 17 % aq. NH_4OH (3 : 1 : 2) (Solvent A)]; Rf 0.45 [cellulose, *n*-BuOH - AcOH - H_2O (12 : 3 : 5)]; Rf 0.33 [silica gel, *n*-BuOH - EtOH - H_2O (4 : 1 : 2)]. Antipain gave positive reactions to SAKAGUCHI, diacetyl, WOOD, TOLLENS and triphenyl tetrazolium chloride reagents, negative to ninhydrin, ferric chloride and EHRLICH reagents.

Treatment of antipain with potassium permanganate gave an oxidation product (1)



which was obtained as its dihydrochloride: mp 202~211°C (dec.); $[\alpha]_D^{19}$ −10° (*c* 1.0, water); $[\alpha]_D^{20}$ −15° (*c* 1.0, 1 N HCl); pKa 2.9, 3.8, >11; Rf 0.38 (cellulose, Solvent A). On treatment with 6 N hydrochloric acid (in sealed tube, 105°C, 72 hours), **1** gave arginine (1.3 mol.), valine (1.0 mol.) and phenylalanine (0.3 mol.), while antipain, on the same treatment, gave arginine (0.3 mol.), valine (1.0 mol.) and phenylalanine (0.3 mol.). On drastic hydrazinolysis (in sealed tube, 120°C, overnight), **1** gave phenylalanine and ornithine, though antipain gave no ornithine using the same treatment. These results suggested that phenylalanine and argininal would be the C-terminal residues of antipain.

Treatment of antipain with 0.1 N methanolic hydrochloric acid gave its dimethyl acetal (**2**); dihydrochloride·monohydrate: mp 175~180°C (dec.); $[\alpha]_D^{20}$ −5° (*c* 1.0, water); Rf 0.53 (cellulose, Solvent A); UV, $\lambda_{max}^{H_2O}$ (ε) 247 (155), 252 (185), 258 (215), 263 (170), 267 mμ (120). Since phenylalanine in 0.1 N hydrochloric acid has the absorption maxima[3] at 247 (ε 115), 252 (154), 258 (196), 263 (152) and 267 mμ (92), the absorption peak of **2** could be ascribed solely to the phenylalanine moiety, and the enhanced absorption strength at the maxima in antipain in comparison with that of **2** is ascribed to the overlapping of the high-intensity end-absorption of an aldehyde group in antipain. The NMR spectrum of **2** (in D_2O) showed a six-proton multiplet* centered at δ 3.45 due to O-methyl groups. On treatment with 0.1 N hydrochloric acid, **2** was converted to antipain.

Digestion of **2** with trypsin (pH 8.0, 27°C, overnight; enzyme-substrate 1 : 20 in weight), which splits specifically the peptide bond of an arginine residue at the site of its carbonyl, gave two diacetyl positive fragments **3** and **4**, the Rf's of which were 0.61 and 0.22, respectively, on a thin-layer chromatogram of cellulose with a solvent system of *n*-butanol - ethanol - chloroform - 17 % aq. ammonia (4 : 4 : 2 : 3). The product **3** was obtained as a hemicarbonate: mp 134~144 °C (dec.); $[\alpha]_D^{18}$ +10° (*c* 1.0, methanol); Found: C 48.12, H 8.96, N 20.27 %; Calcd. for $C_{13}H_{29}N_5O_3 \cdot {}^1/_2H_2CO_3$: C 48.48, H 9.04, N 20.94 %; positive ninhydrin and SAKAGUCHI. The N-terminal amino acid of **3** was determined to be L-valine. The NMR (in D_2O) of **3** showed a six-proton multiplet* centered at δ 3.47 as was seen in **2**.

On digestion of **1** in a manner similar to that described above, it gave **4** and a ninhydrin-positive product **5**: mp 176~179 °C (dec.); $[\alpha]_D$ +19° (*c* 1.0, water). Mild hydrazine hydrolysis (guanidino group⟶NH_2) of **5** followed by N-acetylation and esterification (NH_2⟶NHAc; COOH⟶COOMe) gave a product **6**: mp 164~166°C; $[\alpha]_D^{16}$ −27.5° (*c* 1.0, methanol), *m/e* 329 (M^+), 142 (Ac-Val) in its mass spectrum. L-Valine and DL-arginine were obtained by hydrolysis of **5**. Thus, **3** was proved to be L-valyl-argininal dimethyl acetal.

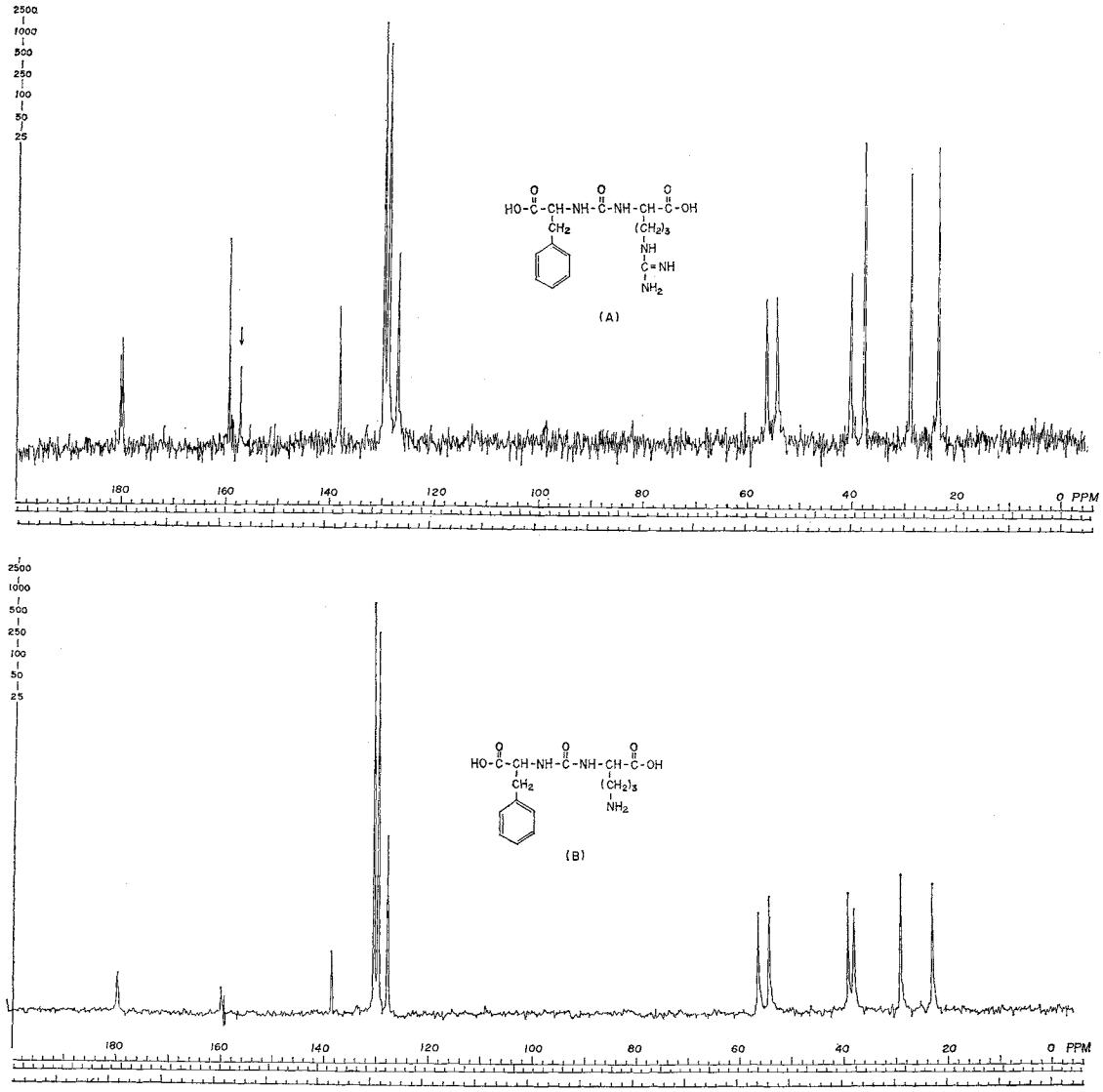
The product **4** was obtained as a monohydrochloride: mp 123~139°C (dec.); $[\alpha]_D^{18}$ +5° (*c* 1.0, water); pKa 3.2, 4.1, >11; SAKAGUCHI positive but ninhydrin negative. A drastic hydrazinolysis (in sealed tube, 120°C, overnight) of **4** gave two C-terminal amino acids, phenylalanine and arginine, the latter being detected as ornithine. Treatment of **4** with barium hydroxide gave a single product (**7**) which was ninhydrin positive and diacetyl negative. **7** was also obtained from antipain by the treatment with barium hydroxide in a high yield; monohydrochloride: mp 126~130°C (dec.); pKa 3.1, 4.1, 10.6. Further alkaline hydrolysis of **7** gave phenylalanine and ornithine. The natural-abundance carbon-13 Fourier transform NMR spectra (in D_2O) of **4** (Fig. 1-A) and **7** (Fig. 1-B) suggested that **4** and **7** had sixteen and fifteen carbon atoms, respectively, and three of their carbon atoms were carbonyl carbon atoms because of their lower field resonance.

Treatment of **7** with acetic anhydride in methanol gave N-acetyl-dimethyl ester **8** and in ethanol gave N-acetyl-diethyl ester **9**. The high resolution mass spectra of **8** and **9** showed molecular ions at *m/e* 393.189 (calcd. for $C_{19}H_{27}N_3O_6$: 393.190) and 421.224 (calcd. for $C_{21}H_{31}N_3O_6$: 421.221), respectively, indica-

* The multiple splittings is ascribed to nonequivalence of the methoxy groups caused by the asymmetric center of argininal, its racemization (the racemization seemed incomplete) and the hindered rotation caused by the amide bond.

Fig. 1. ^{13}C-NMR spectra of **4** (A) and **7** (B)

XL-100-15 ^{13}C-FT spectrum. Sample: about 200 mg/2.5 ml in D_2O. Transients: 10,000. Acquisition time: 0.4 sec. Spectrum width: 5,000 Hz. Proton noise decoupled. δ-Values relative to TMS=0. An arrow in A spectrum indicate a guanidino carbon-13 resonance, which is not discerned in B.



ting the molecular formula of **4** to be $C_{16}H_{23}N_5O_5$.

From the above-mentioned results, product **4** was assumed to be an ureido-type derivative composed of phenylalanine and arginine. In order to confirm the suggested structure **4**, its diethyl ester **10**, which was derived from **4** on treatment with 1 N ethanolic hydrogen chloride, was synthesized by condensation of newly synthesized isocyanatophenylalanine ethyl esters [**11**; L-isomer: bp_7 134°C; $[\alpha]_D^{18}$ −82.5° (*c* 1.0, toluene), D-isomer: bp_8 140°C; $[\alpha]_D^{18}$ +82.5° (*c* 1.0, toluene), lit[4]. racemate: bp_{10} 152°C] with nitro-L-arginine ethyl ester [**12**; hydrochloride: mp 94~98°C, $[\alpha]_D^{18}$ +9° (*c* 1.0, ethanol)] in dry benzene, followed by hydrogenation (to liberate a guanidino group). The IR and NMR spectra of natural and synthetic **10** were indistinguishable, respectively: monohydrochloride: IR (KBr): ~3300 (NH), 2960 (CH), 1735 (ester), ~1650 (amide I, guanidinium), 1560 (amide II), 750, 705 cm^{-1} (phenyl); NMR (DMSO-d_6): 1.11 & 1.18 [each 3 H, t, J~7Hz,

Table 1. Melting points and optical rotations of hydrochlorides of the natural **10** and its synthetic products.

	Natural	(S)-Isomer	(R)-Isomer
Consituent amino acids	L-Phe L-Arg	L-Phe L-Arg	D-Phe L-Arg
Mp(℃)	71~76	71~77	72~78
$[\alpha]_{589}^{18}$	+ 7.5°	+ 7.5°	−15.0°
$[\alpha]_{546}$	+11.2°	+11.2°	−17.5°
$[\alpha]_{435}$	+26.2°	+25.5°	−32.5°
$[\alpha]_{405}$	+33.7°	+32.7°	−40.0°
$[\alpha]_{365}$	+52.5°	+52.0°	−55.0°

O-$CH_2\underline{C}H_3$], 1.3~1.9 [4H, m, $C\underline{H}_2$-$C\underline{H}_2$ (Arg)], 2.94 [2H, d, J 7 Hz, CH-$C\underline{H}_2$ (Phe)], ~3.15 [2H, m, $C\underline{H}_2$-NH (Arg)], 4.04 & 4.08 [each 2H, q, J~7 Hz, O-$C\underline{H}_2CH_3$], ~4.1 [1H, m, NH-$C\underline{H}$-CO (Arg)], 4.36 [1H, q, J~7 Hz, NH-$C\underline{H}$-CH_2 (Phe)], 6.44 [1H, d, J~8 Hz, N$\underline{H}$-CH (Phe)], 6.68 [1H, d, J~8 Hz, N$\underline{H}$-CH (Arg)], ~7.2 (8H, m, phenyl protons & C(=N$\underline{H}$)N$\underline{H}_2$ (Arg)], 7.82 [1H, m, $CH_2N\underline{H}$-C(=NH)NH_2 (Arg)]. The optical rotations of the natural product **10** at various wave-lengths agreed with those of that synthesized from L-isomers (Table 1), indicating that the product **10** was [(S)-1-ethoxycarbonyl-2-phenylethyl] carbamoyl-L-arginine ethyl ester.

Based on the aforementioned results, we conclude that antipain is [(S)-1-carboxy-2-phenylethyl] carbamoyl-L-arginyl-L-valyl-argininal.

In the NMR (D_2O) of antipain, the peak due to an aldehyde proton was not discerned clearly, and a doublet (J~4 Hz) of approximately 0.5 proton at δ 4.98 and two doublets (J~3 Hz) of approximately 0.5 proton at δ 5.41 and 5.45 suggested that antipain exists in both hydrate and carbinolamine forms in an aqueous solution as also shown in leupeptins[5].

In so far as we know, antipain is the first reported natural peptide containing a ureylene group.

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