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A NEW MICROBIAL METABOLITE PHOSPHORAMIDON
(ISOLATION AND STRUCTURE)

Sumio Umezawa, Kuniaki Tatsuta, Osamu Izawa and Tsutomu Tsuchiya
Department of Applied Chemistry, Keio University, Yokohama-shi, Japan
Hamao Umezawa
Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

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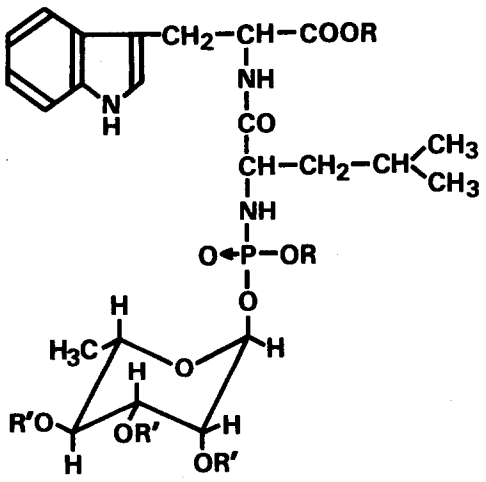
As the results of our screening of culture filtrates of microorganisms for chemical color reactions, dienomycins (1), sphydrofuran (2) and arglecin (3) had already been found as metabolites of *Streptomyces*. As the continuation of this work, we now report the isolation and the structural elucidation of a new metabolite phosphoramidon which is produced by the strain of *Streptomyces tanashiensis* (4) and found by the color screening with Ehrlich reagent.

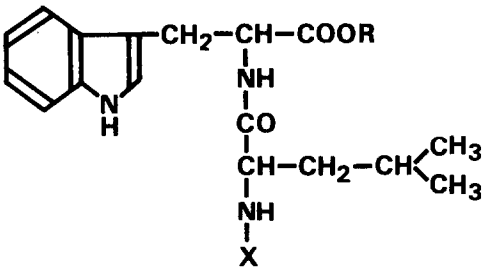
Phosphoramidon in the culture filtrate was adsorbed on charcoal, eluted with methanol at pH 8.0, and isolated by column chromatography with cellulose and then with Sephadex LH-20. Phosphoramidon was crystallized as ammonium, cyclohexylammonium or dicyclohexylammonium salts. Di-cyclohexylammonium salt of phosphoramidon (from isopropanol-isopropyl ether): mp 130∿148°C (dec.); $[\alpha]_D^{21}$ -21° (*c* 1.0, H_2O); Found: C 56.53, H 8.07, N 9.22, P 4.0%; Calcd. for $C_{23}H_{34}N_3O_{10}P \cdot 2C_6H_{13}N$: C 56.67, H 8.15, N 9.44, P 4.1%; IR(KBr): 3500∿3200(OH, NH), 2900(CH), ∿1640(amide I), 1590(phenyl), 1525(amide II), 1450(phenyl), 1400(CH), 1190(P→O), 1175(P-O-C), ∿1070(C-O), 980 (P-N ?), 740 cm^{-1}(phenyl); UV: $\lambda_{max}^{MeOH}(\varepsilon)$ 291(4,400), 282(5,000), 276mμ(sh., 4,700); NMR[ammonium salt of phosphoramidon in D_2O; δ value (ppm)]: 0.79, 0.83[each 3H, d, J ∿6Hz, CH-($\underline{CH_3}$)$_2$], 1.30(3H, d, J 6Hz, CH-$\underline{CH_3}$), 0.7∿1.7(3H, m), 3.0∿4.2(7H, m), ∿4.6(1H, m), 5.33(1H, q, $J_{1,2}$ ∿1.5Hz, $J_{P,CH}$ ∿8 Hz, anomeric proton), 7.1∿7.8(5H, m, indolyl protons); pka 3.6 and <3; Rf 0.68[cellulose, n-BuOH-AcOH-H_2O (12 : 3 : 5)]; Rf 0.34[silica gel, n-BuOH-EtOH-$CHCl_3$-17%NH_4OH (4 : 4 : 2 : 3)]. Phosphoramidon gave positive reactions to Ehrlich, Tollens and ammonium molybdate-perchloric acid (for phosphoric acid) reagents, negative to ninhydrin reagent. On paper electrophoresis (3500V, Toyo Roshi paper No.51) using a buffer solution of formic acid-acetic acid-water (1 : 3 : 36), phosphoramidon moved toward the anode from the origin.

Mild acid hydrolysis of phosphoramidon with 1N HCl at room temperature for 1 day afforded a dipeptide, L-leucyl-L-tryptophan (5)[I, mp 138∿140°C (dec.), $[\alpha]_D^{22}$ -9° (H_2O); Mass spectrum (m/e): 299 (M^+-18)] and a monosaccharide, L-rhamnopyranose (6)[mp 86∿90°C, $[\alpha]_D^{23}$ -6 → +8° (H_2O)]. Carbobenzoxylation of I with carbobenzoxy chloride followed by esterification with diazomethane gave N-carbobenzoxy-L-leucyl-L-tryptophan methyl ester [II, mp 116∿117°C, $[\alpha]_D^{22}$-12.5° (MeOH)], which was identical with an independently synthesized sample. The results described above indicated that phosphoramidon is composed of L-leucyl-L-tryptophan (I), L-rhamnose and probably a phosphono group.

Treatment of phosphoramidon in methanol with ethereal diazomethane at room temperature for 10 min. gave a methyl ester (III), which was detected on thin-layer chromatogram of silica gel as a single spot. Though III was considerably unstable and could not be isolated, its acetyl derivative (IV) was obtained as crystals [mp 76∿78°C, $[\alpha]_D^{20}$ -10° ($CHCl_3$)]. The molecular formula of IV was established by elemental analysis and mass spectrometry (M^+ 697) as $C_{31}H_{44}N_3O_{13}P$; NMR (in DMSO-d_6): a six-proton doublet at δ 0.89 (J ∿6Hz, $\underline{CH_3}$>CH), a three-proton doublet at 1.15 (J 6Hz, $\underline{CH_3}$-CH), three three-proton singlets at 1.95, 2.03 and 2.12 (OAc), two doublets of total three-proton (in the ratio of 1 : 1) at 3.52 and 3.53 (J_{P,CH_3} ∿11Hz, $\underline{CH_3}$O-P), a three-proton singlet at 3.59 ($COO\underline{CH_3}$), a five-proton multiplet at 6.8∿7.7 (indolyl protons).



	R	R′
Phosphoramidon :	H	H
III :	CH_3	H
IV :	CH_3	$COCH_3$



	R	X
I :	H	H
II :	CH_3	$COOCH_2C_6H_5$
V :	CH_3	$O\leftarrow P(=O)(OCH_3)_2$
VI :	CH_3	$O\leftarrow P(OCH_3)(OCH_2CH_3)$

On an elevated temperature, the above signals became sharpened and, in particular, two doublets at δ 3.52 and 3.53 collapsed to a three-proton doublet centered at 3.53 (J_{P,CH_3} ∿11Hz). By analogy with the NMR spectrum of the anomeric mixture of 1,2,3,4-tetra-O-acetyl-L-rhamnopyranoses [$[\alpha]_D^{22}$ -35° ($CHCl_3$)], the above three O-acetyl groups of IV were assigned to those at C-2, 3 and 4 positions of the rhamnopyranose moiety. Mild hydrolysis of IV with 70% aqueous acetic acid at 100°C for 45 *min.* gave 2,3,4-tri-O-acetyl-L-rhamnopyranose (7)[mp 95∿99°C, $[\alpha]_D^{20}$ -17.5° (EtOH)], supporting the above conclusion. Therefore, the L-rhamnose moiety was decided to be connected to the other part through a glycosidic linkage. At the same time, the molecular formula of phosphoramidon was decided to be $C_{23}H_{34}N_3O_{10}P$.

Overnight treatment of phosphoramidon with diazomethane in methanol gave three products, namely, a trimethyl ester [V, mp 64∿67°C, $[\alpha]_D^{21}$ -17.5° (MeOH)] and an anomeric mixture of methyl L-rhamnopyranosides. By elemental analysis and mass spectrometry (M^+ 439), the molecular formula of the former was established to be $C_{20}H_{30}N_3O_6P$. The presence of dimethyl phosphate $-P(O)(OCH_3)_2$ was confirmed by its NMR spectrum (in $CDCl_3$): The two O-methyl protons showed two doublets (J_{P,CH_3} 11Hz) at δ 3.54 and 3.58, which collapsed, on an elevated temperature, to a six-proton doublet (J_{P,CH_3} 11Hz) centered at 3.56.

As soon as the intermediary methyl ester III was detected on a thin-layer chromatogram as described above, the methanol solution was rapidly concentrated *in vacuo* and in the cold to about a half volume to remove residual diazomethane. The ethereal diazoethane was then added and the solution was allowed to stand overnight. The resulting solution contained three degradation products, namely, methyl α- and β-L-rhamnopyranosides and monoethyl-dimethyl ester (VI): $C_{21}H_{32}N_3O_6P$ (M^+ 453); mp 54∿56°C, $[\alpha]_D^{21}$ -17.5° (MeOH); The NMR spectrum (in $CDCl_3$ + D_2O) showed signals due to one ethyl group instead of signals due to one methyl group in V without appreciable changes of other signals: two quintets centered at δ 3.93 and 3.97 (total 2H; J_{CH_2,CH_3} 7Hz, J_{P,CH_2} ∿7Hz; $P-O-\underline{CH_2}CH_3$, collapsed to a two-proton quintet at δ 3.95 on an elevated temperature), two double triplets centered at 1.16 and 1.22 (total 3H; J_{CH_3,CH_2} 7Hz, J_{P,CH_3} ∿1Hz; $P-O-CH_2\underline{CH_3}$). These results and the fact that V and VI had no free amino groups indicate that V and VI are N-(dimethoxyphosphinyl)- and N-(ethoxymethoxyphosphinyl)-L-leucyl-L-tryptophan methyl ester, respectively. This conclusion was further supported by the finding of coupling between P and H in $O\leftarrow\underline{P}(<)-\underline{NH}-CH(CO-)-CH_2CH(CH_3)_2$ ($J_{P,NH}$ ∿10Hz) in the NMR spectrum of V.

The final problem was the assignment of the anomeric configuration. The NMR spectrum (in D_2O) of α-L-rhamnopyranosyl-1-phosphate [di-cyclohexylammonium hemihydrate: mp 193∿194°C,

$[\alpha]_D^{22}$ -21.5° (H_2O)], which was prepared by a method similar to that described by Chatterjee and MacDonald (8), showed that it was in 1C conformation and the anomeric proton resonated at δ 5.31 with couplings of $J_{1,2}$ 1.5Hz and $J_{P,CH}$ 8.5Hz. In phosphoramidon, one-proton quartet ($J_{1,2}$ ∿1.5Hz, $J_{P,CH}$ ∿8Hz) at δ 5.33, therefore, could be assigned to the anomeric proton, indicating that phosphoramidon contains α-L-rhamnopyranosyl phosphate moiety in the 1C conformation. The optical rotation of phosphoramidon also supported this finding. When the structure of phosphoramidon is divided into L-leucyl-L-tryptophan moiety (tentatively named as A) and L-rhamnopyranosyl phosphate moiety (tentatively named as B), the molecular rotation of phosphoramidon will roughly be represented by the sum of the partial molecular rotations of [A] and [B]. Since L-leucyl-L-tryptophan has [M]=-3,000° (H_2O) and L-rhamnopyranosyl phosphate has -9,700° (H_2O)(α-anomer) and +15,700° (H_2O)(β-anomer)(8), the molecular rotation for di-cyclohexylammonium (mol. wt. 741) salt (-15,500°) of phosphoramidon and that for di-dicyclohexylammonium (mol. wt. 906) salt (-15,700°) support the α-anomeric structure of phosphoramidon. Based on the aforementioned results, we conclude that phosphoramidon is N-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan. It is noteworthy that the presence of a significant amount of a metabolite having the labile phosphoramido group was disclosed by this chemical screening procedure.

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