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# The Biosynthesis of Conium Alkaloids. Identification of a Novel Nonnitrogenous Base from *Conium maculatum* as 3-Formyl-4-hydroxy-2H-pyran*

Olajide A. Koleoso, Sonia M. C. Dietrich,† and R. O. Martin

ABSTRACT: The rapidly turning over compound D, detected in the alkaloid extracts of *Conium maculatum*, *Sedum sarmentosum*, and *Punica grantum* during our studies of the kinetics of $^{14}C$ incorporation into the known propylpiperidine alkaloids, has been isolated by ion exchange and paper chromatography. Its behavior is consistent with that of a weak base (p$K$ = <0.1) bearing no charged groups in the pH range 2–10.

The nuclear magnetic resonance spectrum showed an aldehyde proton at $\tau$ 0.55, two vinyl protons (conjugated) at $\tau$ 2.82 and 3.56, a pair of methylene protons at $\tau$ 5.38, and an exchangeable proton at $\tau$ 5.76. The high-resolution mass spectrum showed a molecular ion peak at 126 ($C_6H_6O_3$), an $(M - 17)^+$ at 109 ($C_6H_5O_2$), and an $(M - 29)$ at 97 ($C_5H_5O_2$) (base peak). Mass fragmentation after $D_2O$ exchange and oxime formation confirmed the compound to be 3-formyl-4-hydroxy-2H-pyran, final choice among the alternate *ortho* and two *para* isomers being made on the basis of the ultraviolet absorption ($\lambda_{max}$ 280 m$\mu$ ($\epsilon$ 5100)) as well as the nuclear magnetic resonance spectrum. Though this compound appears unrelated to the known propylpiperidine bases, its possible role in the formation of *N*-heterocycles is discussed.

Several biogenetic pathways have been proposed for the formation of propylpiperidine alkaloids of the *Conium* and *Sedum* type (Leete, 1968). Feeding experiments using either lysine-$^{14}C$ or acetate-$^{14}C$ have shown that both can serve as *precursors* of the *Conium* alkaloids. However no evidence has ever been obtained for the existence of any *intermediate*[1] prior to the already known alkaloids.

During the course of our studies on the biosynthesis of the alkaloids of *Conium maculatum* (poisonous hemlock) using the $^{14}CO_2$ kinetic approach, several unidentified, highly radioactive, minor bases were detected (Dietrich and Martin, 1968, 1969). The most intriguing of these was the earliest labeled one which contained more than 90% of the $^{14}C$ in the total alkaloid extract after only 1-hr photosynthesis in

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[1] The distinction proposed by (Davis, 1954) is made between the terms precursor as "any substance whether endogenous or exogenous that can be converted by an organism into some product" and *intermediate* as "a compound formed and converted by the organism into a product."

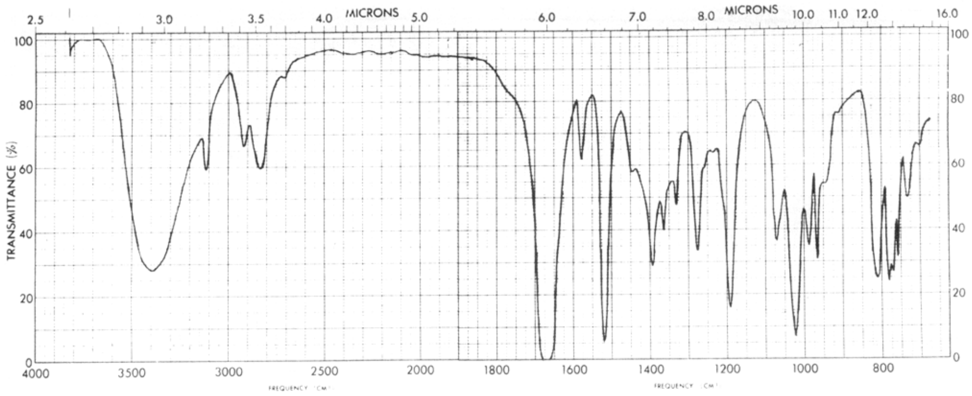

FIGURE 1: Infrared spectrum of compound D. Film on NaCl disk. NaCl disk in reference beam.

an atmosphere of $^{14}CO_2$. After return to normal air the $^{14}C$ rapidly disappeared from this same compound, while the known alkaloid $\gamma$-coniceine became more heavily labeled. Feeding experiments showed $^{14}C$ from this compound to be incorporated into $\gamma$-coniceine; 0.2% compared with 0.01% for a control feeding using acetate-1-$^{14}C$.

A compound showing the same $^{14}C$ kinetic behavior and identical chromatographic properties was also detected in *Sedum sarmentosum*, an unrelated plant which elaborates propylpiperidine bases similar to those of *Conium*, thus strengthening our interest in this compound as a possible early biosynthetic intermediate in the natural *formation* of these alkaloids. Using $^{14}C$-labeled material from photosynthesis experiments as a marker we have isolated and purified sufficient amounts of this compound to permit a structure assignment based largely on physical measurements.

## Methods

*Alkaloid Standards and Reagents.* As described in the preceeding paper (Dietrich and Martin, 1969).

*Plant Material.* For large-scale isolation of alkaloids, fresh 3-month-old plants were harvested from an outdoor summer garden, sown with seeds of the same "California variety" of *C. maculatum* described in the preceeding paper.

*Isolation and Purification of Radioactive Alkaloids.* Radioactive material, used for preliminary studies and as a marker for large-scale preparations, was obtained from the alkaloid extract from a 1-hr photosynthesis experiment as described in the preceeding paper. Preparative descending paper chromatography (no overrun) was carried out on acid-washed Whatman No. 3MM paper using *t*-pentyl alcohol–*t*-butyl alcohol–1 N HCl (9:3:2, v/v) (Cromwell, 1956).

$\gamma$-Coniceine, the major known alkaloid, moved with an $R_F$ of 0.4. The major radioactive band, D, located by autoradiography, had an $R_F$ of 0.90. Elution with 0.01 N HCl followed by rechromatography and elution gave a single, radioactive compound by the criteria below and referred to hereafter as compound D.

*Large-Scale Isolation of Alkaloids.* Fresh 3-month-old plants (2 kg, vegetative stage) were extracted for alkaloids as described in the preceeding paper. The final methylene chloride extract after solvent removal *in vacuo* gave 1 g of a pale yellow syrup (total alkaloids). This syrup was dissolved in excess 0.01 N HCl, $5 \times 10^5$ dpm of pure radioactive compound D was added as a marker, and the solution was passed through a $1 \times 30$ cm column of Dowex 50-X12 (AG) ($H^+$) (50 mesh) followed by water. All the known alkaloids were retained on the column. The radioactive effluent was reduced *in vacuo* to 1 ml and purified by paper chromatography as described above. Extraction, with methylene chloride at pH 10, of the eluate from the chromatography, followed by drying over anhydrous sodium sulfate, then solvent removal *in vacuo* gave 16–18 mg of a pale yellow gum. The latter crystallized on standing at 5° for 1 week.

*Chromatographic and Electrophoretic Methods.* These were identical with those described in our preceeding paper.

Autoradiographic and scintillation counting methods were described in the previous paper. Ultraviolet spectra were taken in methanol using a Cary 14 recording spectrophotometer. Infrared spectra were taken as a film on NaCl plates using a Perkin-Elmer 237B instrument. Nuclear magnetic resonance spectra were carried out in carbon tetrachloride with tetramethylsilane as an internal standard, using a Varian HA-100 spectrometer.[2]

Low-resolution mass spectra were obtained on an AEI MS-12 instrument using a direct probe all glass inlet and high-resolution mass spectra on an AEI-MS902.[3]

## Results

*Large-Scale Isolation.* YIELD. From 2 kg of fresh plants we obtained about 16–18 mg of a pale yellow gum which crystallized on standing in the refrigerator over a period of 1 week. About 900 mg of $\gamma$-coniceine was isolated at the same time.

PURITY. The total alkaloid extract, after passage through Dowex 50 ($H^+$) resin, was free of all but traces of $\gamma$-coniceine or any of the other known alkaloids which would react with Dragendorff's reagent (bismuth iodide complex; *cf.* Farnsworth *et al.* (1962) for a discussion of this widely used if not well-understood reaction). Final purification by preparative paper chromatography gave a single radioactive compound

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[2] Our thanks to Mr. M. Mazurek of the Prairie Regional Laboratory for the nuclear magnetic resonance spectrum.

[3] High resolution mass spectra before and after $D_2O$ exchange and after reaction with hydroxylamine. HCl were very generously carried out by Professor J. Majer of the Department of Chemistry, University of Birmingham.

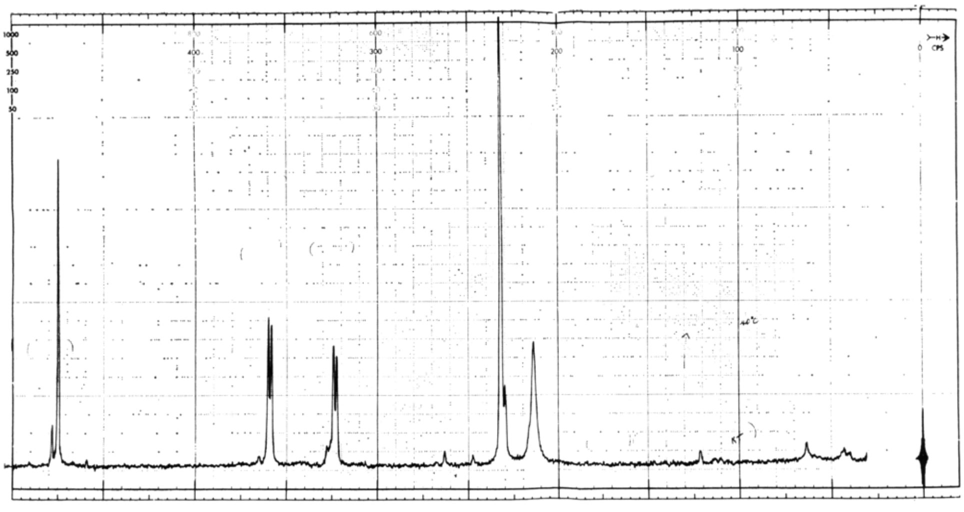

FIGURE 2: Nuclear magnetic resonance spectrum of compound D in $CCl_4$ in a microtube; 100 Mc. Sample concentration approximately 7 mg/ml. Internal standard: tetramethylsilane (0 ppm).

as revealed by its behavior in the three thin-layer chromatography systems, on gas–liquid partition chromatography, and on electrophoresis. When larger amounts of pure material were analyzed by thin-layer chromatography (system A), only a single major spot upon charring having an $R_F$ of 0.85, now giving a Dragendorff's reaction and staining with iodine vapors was detectable, corresponding to the radioactively marked spot ($R_F$ 0.85). The only detectable impurity (<3%) was a weakly radioactive area at a slightly higher $R_F$.

ELECTROPHORETIC AND CHROMATOGRAPHIC BEHAVIOR were identical with that previously reported for the original radioactive compound "D" isolated on a small scale (Dietrich and Martin, 1969).

ULTRAVIOLET ABSORPTION SPECTRUM with rather narrow, single absorption bands were observed as follows: $\lambda_{\max}$ 280 mμ ($\epsilon$ 5100) (in methanol), $\lambda_{\max}$ 276 mμ ($\epsilon$ 5400) (in 0.1 N HCl, 10% methanol), $\lambda_{\max}$ 276 mμ ($\epsilon$ 5600) (in 1.0 N HCl, 10% methanol), and $\lambda_{\max}$ 284 mμ ($\epsilon$ 4700) (in 1.0 N NaOH, 10% methanol).

The last three values are corrected for the presence of water (Dyer, 1965).

INFRARED ABSORPTION SPECTRUM. The following assignments were made from the infrared spectrum shown in Figure 1: 3400 (s) ($\nu_{OH}$), 3100 (s) (olefinic $\nu_{CH}$), 2920 and 2840 (m) (methylene $\nu_{CH}$), 2700 (w) (aldehyde $\nu_{CH}$), 1675 (s) ($\nu_{C=O}$), 1576 (w) and 1520 (s) ($\nu_{C=C}$), 810 (s) and 780 $cm^{-1}$ (s) (out-of-plane CH bending).

NUCLEAR MAGNETIC RESONANCE SPECTRUM. The spectrum in carbon tetrachloride shown in Figure 2 integrated for six protons. The minor peaks indicate a small impurity (<3%). The spectrum shows a 1H proton at $\tau$ 0.55, 1H doublet at $\tau$ 2.82 ($J$ = 4 cps), 1H doublet at $\tau$ 3.56 ($J$ = 4 cps), 2H singlet at $\tau$ 5.38, and a 1H singlet at $\tau$ 5.76 which underwent a diamagnetic shift by 18 cps on raising the probe temperature from 25 to 40°. There were no protons at lower fields than the one at $\tau$ 0.55.

MASS FRAGMENTATION PATTERN. Figure 3 shows the low-resolution mass spectrum for compound D. The molecular ion $(M)^+$ occurred at $m/e$ 126, an $(M + 1)^+$ at $m/e$ 127, an $(M + 2)^+$ at $m/e$ 128, an $(M - 17)^+$ at $m/e$ 109, and an $(M - 29)^+$ at $m/e$ 97 which was also the base peak. Other major peaks occurred at $m/e$ 69, 41, and 39; "Metastables" occurred at $m/e$ 74.6, ($m_1$*) (most intense) 49.1 ($m_2$*), 24.4 ($m_3$*) and 37.1 ($m_4$*).

The high-resolution mass spectra showed the nominal peaks at 126 ($M^+$) 109, 97, and 69 to have the following precise mass and constitutions: 126.030584 ($C_6H_6O_3$), 109.098428 ($C_6H_5O_2$), 97.027028 ($C_5H_5O_2$), and 69.033468 ($C_4H_5O_2$). Addition of $D_2O$ to the sample moved only the peaks at 126, 97, 69, 41, and 39 upward by one mass unit; a "metastable" occurred at 75.6. Addition of hydroxylamine hydrochloride solution to the sample resulted in movement of the peaks at 126 and 109, only, to positions 15 mass units higher.

## Discussion

*The Neutral Character of D.* The electrophoretic behavior of compound D (Figure 4) (Dietrich and Martin, 1969) over the pH range 2–8.5 showed it to be a neutral or at most a very weakly basic substance. Similar inferences were made from its movement on thin-layer chromatography.

Its behavior toward ion-exchange resins again were consistent with the absence of any cationic or anionic groups. The failure of its hydrolysis product to be retained by Dowex 50 ($H^+$) cast strong doubts as to the presence of any nitrogen in the molecule, which was confirmed by microkjeldahl N determination on the unhydrolyzed compound.

*Molecular Weight and Skeletal Structure.* A comparison of the mass spectrum of compound D with those of all the known *Conium* alkaloids showed it to be fundamentally different (R. O. Martin and O. A. Koleoso, unpublished data). The latter (*e.g.*, coniine and conhydrine) show a base peak at $m/e$ 84 due to the loss of the alkyl side chain; γ-coniceine shows a base peak at $m/e$ 96).

The mass spectrum indicated a molecular weight of 126 for D and precise mass analysis showed it to have the composition $C_6H_6O_3$. The weak absorption at 2700 $cm^{-1}$ and the strong one at 1675 $cm^{-1}$ were indicative of an unsaturated aldehyde group. The proton absorption at $\tau$ 0.55 ($H_a$) in the nuclear magnetic resonance spectrum, the loss of mass 29

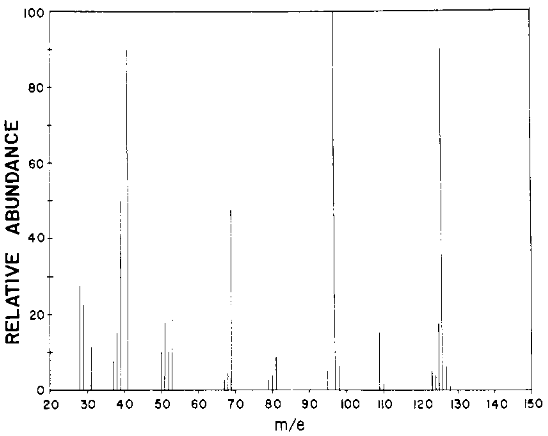

FIGURE 3: Mass spectrum of compound D (low resolution).

(CHO) from the parent ion to give the base peak at *m/e* 97, and the formation of the oxime at *m/e* 141 (126 + 15) ($M^+$) confirmed the presence of an aldehydic group.

The strong absorption band at 3400 $cm^{-1}$ was indicative of a hydroxyl group. Assignment of $H_e$ ($\tau$ 5.76) to this proton was based on its diamagnetic shift on elevating the nuclear magnetic resonance probe temperature. The presence of one active hydrogen was further confirmed by deuterium exchange which increased the parent ion and the base peak by one mass unit. That this hydroxyl was enolic rather than alcoholic was indicated by the loss of an OH (OD) radical from the parent ion to give the peak at *m/e* 109 instead of at 108, expected for loss of $H_2O$.

The possibility of D being a substituted methyl furan were ruled out by the absence of any methyl proton absorption in the nuclear magnetic resonance or any methyl fragment loss in the mass spectrum. A pair of isolated methylene protons ($H_d$) next to an ether oxygen was indicated by the 2H singlet at $\tau$ 5.38. The two vinyl protons $H_b$ ($\tau$ 2.82) and $H_c$ ($\tau$ 3.56) are only split by each other ($J$ = 4 cps) and therefore isolated from all the remaining hydrogen atoms. The accumulated data leave as the only possible basic structure for D, that of a formylhydroxy-$\alpha$-pyran of which four isomeric structures **1**, **2**, **3**, and **4**, may be written consistent with the data discussed. A partial mass fragmentation scheme based on structure **1** is given in Figure 4 to account for major observed mass peaks.[4]

*Final Choice among the Possible Isomers.* The deshielding of the methylene protons ($H_d$) cannot be accounted for by the presence of an $\alpha$-hydroxy group as in **2** or **4** [compare $H_c$ ($\tau$ 6.1) in dihydropyran, **5** (Bhacca *et al.*, 1962a), with $H_a$ ($\tau$ 6.11) and $H_b$ ($\tau$ 6.33) in methylarabinoside, **6** (Bhacca *et al.*, 1962b)] but can through the extended conjugation system of **1**. The strong deshielding of the vinyl protons $H_b$ and $H_c$ in **1** (compare $\tau$ 3.63 and 5.35 for $H_a$ and $H_b$, respectively, in dihydropyran) can be accounted for if they are the terminal protons of the conjugated system of structure **1** but not the cross conjugated **2** or structures **3** or **4**.

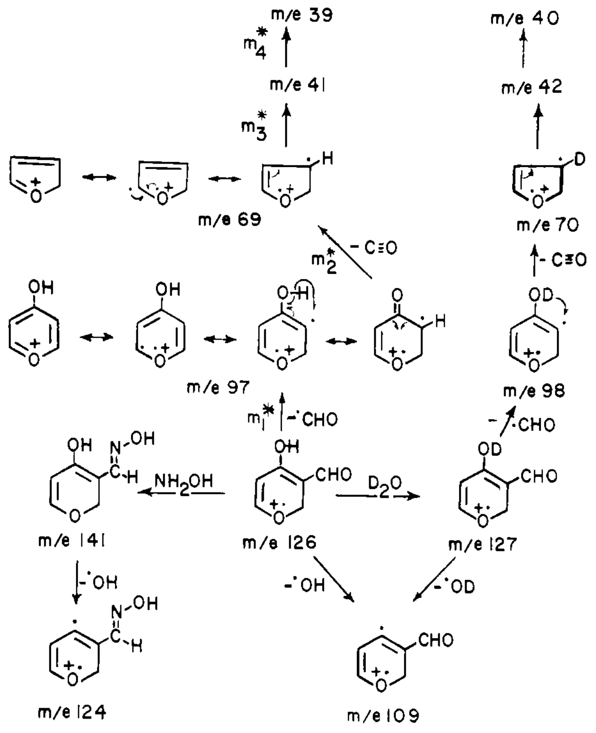

FIGURE 4: Mass fragmentation scheme for compound D.

Calculation of the ultraviolet absorption spectra (Dyer, 1965; Williams and Fleming, 1966) expected for the above structures give a value of 322 $m\mu$ [207 (parent) + 35 ($\alpha$-*O*-alkyl) + 50 ($\delta$-OH) + 30 (C=C extended conjugation)] for **3** or **4**, a value of 252 $m\mu$ [207 (parent) + 10 ($\alpha$-alkyl) + 35 ($\beta$-OH)] for **2** and 282 $m\mu$ [207 (parent) + 10 ($\alpha$-alkyl) + 35 ($\beta$-OH) + 30 (C=C extended conjugation)] for **1**. Comparison with the observed value of 280 $m\mu$ for compound D leaves structure **1** as the final choice.

*Other Properties.* Intermolecular hydrogen bonding between hydroxyl and aldehyde groups in the film infrared spectrum was indicated by the strong, broad absorption at 3400 $cm^{-1}$ and the carbonyl band at 1675 $cm^{-1}$ rather than at the expected 1685 $cm^{-1}$ (Nakanishi, 1962). The absence of deshielding in the nuclear magnetic resonance spectrum expected of strong intramolecular hydrogen bonding was consistent with predictions of only weak interaction made from scale models and the rather dilute sample available. Interestingly enough, the value observed for the exchangeable proton ($H_d$) is much nearer to that of a phenol (*ca.* $\tau$ 5.2) than an enol (*ca.* $\tau$ −4), obviously reflecting the highly delocalized nature of the conjugated system to which the hydroxyl group is attached.

The positive Dragendorff's reaction of this compound is consistent with the nonspecificity of this reagent which will react with many conjugated aldehydes (Farnsworth *et al.*, 1962).

The rather unexpected appearance of this nonnitrogenous compound in our alkaloid extract is deserving of special comment. To be extracted by the procedure used, a compound would be expected to be protonated in 0.5 N HCl to give a charged molecule and deprotonated at pH 10–11 to give a neutral uncharged molecule. Possible protonated forms of **1** in acid solution would be **1a**, **b**, **c**, or **d**. The neutral form being

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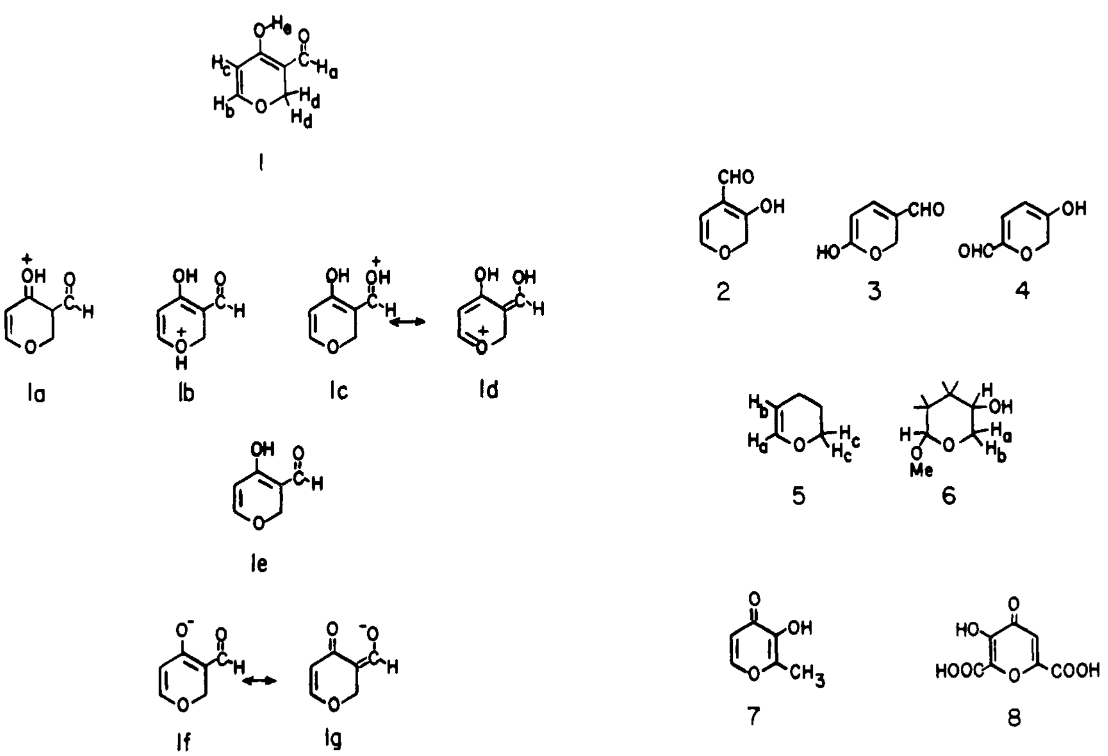
[4] The "metastable" at 74.5 corresponds to that calculated for the fragmentation 126($M^+$) → 97 and indicates this occurred by direct loss of CHO. In line with this view was the observation of a new "metastable" at 75.5 after $D_2O$ exchange and the absence of any after oxime formation.

CHART I



represented by **1e**. A p$K$ value similar to that of 0.1 reported for 4-pyrone (Albert, 1963) would be expected for the above protonation. The electrophoretic mobility of **1** and the fact that it is extracted from $CH_2Cl_2$ with 0.5 N HCl but is not retained by Dowex 50 ($H^+$) in 0.1 N HCl is consistent with such a low basicity. (See Chart I.)

The formation of an enolate anion such as **1f** with contributions from **1g** would account for the small bathochromic shift ($280 \rightarrow 284$ m$\mu$) in 1 N NaOH. As **1** is extracted into methylene chloride at pH 10–11, this p$K$ must be >10–11 which is normal for a phenol.

To our knowledge there are no known naturally occurring monocyclic pyrans, as opposed to dihydro- and tetrahydropyrans or -pyrones. The only related simple monocyclic natural plant compounds are 4-pyrones, such as maltol (**7**), which was reported in the Larch tree (Peratoner and Tamburello, 1903) and in fresh needles of the silver fir tree (Feuerstein, 1901). Meconic acid (**8**) occurs in opium (Gregory, 1837) while chelidonic acid is found in *Chelidonium majus* (Ramstad, 1941) and several other plants of different families. With the exception of coumenic acid being a product of galactose fermentation by *Acetobacter* (Takahashi and Asai, 1934) and Kojic acid, a product of glucose or xylose fermentation by *Aspergillus flavus* (Moyer *et al.*, 1931), little is known of the origin of such ring systems. The rapid high incorporation and turnover of $^{14}C$ in this formylpyran (Dietrich and Martin, 1969) would indicate its mode of biosynthesis should be investigated.

Any justification we might have for proposing a role for this compound in the biosynthesis of the known propylpiperidine alkaloids rests on the circumstantial relation of the $^{14}C$ kinetics observed for these compounds and the occurrence of this pyran in *Sedum*. However, consideration should be given to the ease of amination of pyrones (Bickel, 1947) and $\alpha$-hydroxypyrans (*cf.* the conversion of gentiopicrin into gentianine) (Canonica *et al.*, 1962) with ammonia[5] as well as the occurrence of several unique pyridone and piperidine alkaloids with a single carbon substituent at the $C_2$ position such as ricinine or arecoline. Finally the fact that the best *precursors* of the pyridine ring of nicotinic acid in plant studies have been glycerol and a $C_4$ dicarboxylic acid such as succinate (Spenser, 1968) and the specific incorporation of acetate into coniine (Leete, 1964) are stimulants to further studies to clarify the possible role of oxygen heterocycles in the biosynthesis of *N*-heterocyclic compounds.

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[5] Reaction of 1 with concentrated $NH_4OH$ (sealed tube) for 4 hr at either 100 or 5°, resulted, in both cases in 65% conversion into a new cationic compound, based on its retention by Dowex 50 ($H^+$) and its electrophoretic and thin-layer chromatography mobility.

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# Specificity of Aminoacyl Transfer Ribonucleic Acid Synthetases from *Escherichia coli* K12*

Masatoshi Kondo† and Carl R. Woese

ABSTRACT: The isoleucyl, valyl, and leucyl transfer ribonucleic acid synthetases from *Escherichia coli* K12 are isolated. Their specificity for amino acids is studied by the adenosine triphosphate–inorganic pyrophosphate exchange and aminoacyl transfer ribonucleic acid synthesis reactions. The isoleucyl transfer ribonucleic acid synthetase from strain K12 is found to recognize L-leucine and L-valine as well as L-isoleucine by the adenosine triphosphate–inorganic pyrophosphate-exchange reaction. L-Leucine (and L-valine) competitively inhibits the charging of isoleucyl transfer ribonucleic acid with L-isoleucine. Interestingly, the specificity patterns shown by the aminoacyl transfer ribonucleic acid synthetases isolated from *Escherichia coli* K12 are not in all cases the same as those manifested by *Escherichia coli* B. The isoleucyl transfer ribonucleic acid synthetase from strain K12 is able to form "erroneous enzyme–aminoacyl adenylate complexes" with both L-leucine and L-valine as well as the "correct enzyme complex" with L-isoleucine. However, neither L-leucine nor L-valine can be transferred to any transfer ribonucleic acid species from such erroneous enzyme complexes. The kinetics of [$^{32}$P]inorganic phosphate release from the enzyme–valyl adenylate complex induced by transfer ribonucleic acid was compared with those observed with the enzyme–isoleucyl adenylate complex.

The group of enzymes known as AA-tRNA synthetases is of special interest in that they are major factors in effecting the relationship between the amino acid and its codons. The enzymes are complex and they carry out two successive reactions; the first a recognition of the amino acid and its conversion into the enzyme-bound aminoacyl adenylate, the second a transfer of the amino acid to one of its tRNAs (Hoagland *et al.*, 1958; Berg, 1961). In the specificity or accuracy with which these reactions are accomplished lies the essence of the cell's capacity to synthesize proteins of unique primary structure.

It is likely that the rate of translation mistakes does not exceed more than a few parts in $10^4$/codon per translation in most cases (Loftfield, 1963; Szer and Ochoa, 1964). On the other hand, the mistake level in the formation of the enzyme–aminoacyl adenylate complex has been estimated to be as high as 5%, on the basis of the theoretical calculations (Pauling, 1958) and experimental data (Loftfield and Eigner, 1961, 1965; Bergmann *et al.*, 1961), when the enzyme has to distinguish between very similar amino acids. In fact, Norris and Berg (1964) and Baldwin and Berg (1966) have isolated a valyl adenylate complex bound to the isoleucyl-tRNA synthetase from *Escherichia coli* B. This "erroneous enzyme–valyl adenylate complex" is unable to transfer the valyl moiety to any tRNA species whatsoever and is broken down to enzyme, free valine, and AMP upon contact with tRNA specific for L-isoleucine.

In this report, we should like to present another observation of an erroneous amino acid activation by an AA-tRNA synthetase. In *Escherichia coli* K12 we have found that not only L-isoleucine and L-valine, but also L-leucine can be activated by the isoleucyl-tRNA synthetase. However, neither L-leucine nor L-valine can be transferred to any tRNA species

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