# Notes

## (1-Amino-2-propenyl)phosphonic Acid, an Inhibitor of Alanine Racemase and D-Alanine:D-Alanine Ligase†

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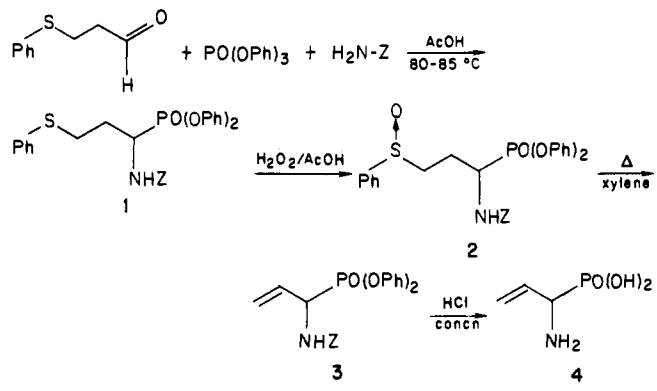
DL-(1-Amino-2-propenyl)phosphonic acid was synthesized through the sequential oxidation, sulfoxide elimination, and deprotection of diphenyl [1-[(benzyloxycarbonyl)amino]-3-(phenylthio)propyl]phosphonate. This analogue of vinylglycine is a strong inhibitor of the alanine racemases from *Pseudomonas aeruginosa* and *Streptococcus faecalis* and of the D-Ala:D-Ala ligase from this latter species. This molecule is ineffective against the whole bacterial cells. Unlike vinylglycine, this unsaturated phosphonate does not inhibit the following mammalian enzymes: aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase, which indicates its specificity. Thus, its incorporation in a peptide structure could induce interesting antimicrobial properties.

Many antibacterial agents owe their selective toxicity to inhibiting enzymic reactions involved in the biosynthesis of the peptidoglycan. Among the several alanine analogues described as antagonists of alanine racemase and D-alanine:D-alanine ligase,[1] two key enzymes implicated in the synthesis of the peptidoglycan precursor, we have taken a particular interest in the amino phosphonic acid series. One of them, (1-aminoethyl)phosphonate, was found to be a potent inhibitor of alanine racemase[2-6] and of D-Ala:D-Ala ligase[5,7] of several Gram-positive and Gram-negative bacteria.

During the past decade much effort has been directed toward the generalization of the new concept of mechanism-based inactivators.[8-10] The most successful approach to design enzyme-activated inhibitors for pyridoxal phosphate dependent enzymes consists in incorporating olefinic, acetylenic, or halogen functional groups in the substrate of the target enzymes. Thus, β-haloalanines have been described as causing reversible or irreversible inactivation of some bacterial PLP-dependent enzymes.[11-16] Recently we have shown that the mono- and dichloro derivatives of Ala-P exhibit a strong inhibition against the alanine racemases of *Pseudomonas aeruginosa* and *Streptococcus faecalis*[17] as well as D-Ala:D-Ala ligase of this latter species. Several examples of enzymic inactivation by compounds containing olefinic groups were also reported.[8] Among them, 2-amino-3-butenoate (vinylglycine) has been recognized as a suicide substrate of D-amino acid transaminases from *Bacillus subtilis* and *Bacillus sphaericus*[18] as well as of cytosolic and mitochondrial aspartate aminotransferases from pig heart.[19]

In this paper, we report the synthesis DL-(1-amino-2-propenyl)phosphonic acid, the phosphonic analogue of DL-vinylglycine, and the investigation of its potential inhibitory properties on the alanine racemases and the D-Ala:D-Ala ligases from *P. aeruginosa* and *S. faecalis*. Furthermore, we examine the effect of this new compound on alanine and aspartate transaminases and on D-amino acid oxidase of mammalian tissues.

**Scheme I**[a]




[a] Z = $C(O)OCH_2Ph$.

†The abbreviations used are as follows: VGP, (1-amino-2-propenyl)phosphonic acid; Ala-P, (1-aminoethyl)phosphonic acid; PLP, pyridoxal 5′-phosphate; D-Ala:D-Ala ligase, D-alanine:D-alanine ligase.

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(1) Neuhaus, F. C.; Hammes, W. P. *Pharmacol. Ther.* **1981**, *14*, 265.
(2) Lambert, M. P.; Neuhaus, F. C. *J. Bacteriol.* **1972**, *110*, 978.
(3) Adams, E.; Mukherjee, K. L.; Dunathan, H. C. *Arch. Biochem. Biophys.* **1974**, *165*, 126.
(4) Lacoste, A. M.; Cassaigne, A.; Neuzil, E. *C. R. Seances Acad. Sci., Ser. D* **1975**, *280*, 1173.
(5) Atherton, F. R.; Hall, M. J.; Hassal, C. H.; Lambert, R. W.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 677. Atherton, F. R.; Hall, M. J.; Hassal, C. H.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 696.
(6) Badet, B.; Walsh, C. *Biochemistry* **1985**, *24*, 1333.
(7) Lacoste, A. M.; Cassaigne, A.; Neuzil, E. *Curr. Microbiob.* **1979**, *2*, 113.
(8) Walsh, C. *Tetrahedron* **1982**, *18*, 871.
(9) Seiler, N.; Jung, M. J.; Koch-Weser, J.; Eds. "Enzyme Activated Irreversible Inhibitors"; Elsevier/North-Holland: Amsterdam, 1978.
(10) Kalman, T. I. "Drug Action and Design: Mechanism-based Enzyme Inhibitors"; Elsevier/North-Holland: Amsterdam, 1979.
(11) Tate, S. S.; Relya, N. M.; Meister, A. *Biochemistry* **1969**, *8*, 5016.
(12) Arfin, S. M.; Koziell, D. A. *J. Bacteriol.* **1971**, *105*, 519.
(13) Manning, J. M.; Merrifield, N. E.; Jones, W. M.; Gotschlich, E. C. *Proc. Natl. Akad. Sci. U.S.A.* **1974**, *71*, 417.

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## Results and Discussion

**Chemistry.** Recently the sulfoxide[20,21] and the selenoxide[22,23] eliminations have become widely used for the introduction of double bonds into molecules: $\alpha,\beta$-unsaturated amino acid derivatives, dehydropeptides,[24] and even the unconjugated vinylglycine have recently been obtained this way.[25] This reaction was successfully applied to the synthesis of 1-alkenephosphonates with use of oxidative deselenation[26] of $\alpha$-seleno phosphonates or sulfoxide elimination of $\alpha$-sulfenylated alkylphosphonates.[27]

Since the phosphonic analogues of homocysteine derivatives are available,[28-30] the dehydrosulfenylation method provides a promising approach to prepare (1-amino-2-propenyl)phosphonate 3, still unknown.

Thus, we synthesized vinyl-Gly-P (VGP), the phosphonic analogue of vinylglycine, in fair yield through the sequential oxydation, sulfoxide elimination, and deprotection of the homocysteine–phosphonic derivative 1, as outlined in Scheme I.

Diphenyl [1-[(benzyloxycarbonyl)amino]-3-(phenylthio)propyl]phosphonate (1) was obtained from 3-(phenylthio)propanal, benzyl carbamate, and triphenyl phosphite by using the previously described procedure[31] for the efficient synthesis of diphenyl 1-aminoalkanephosphonates. Treatment of 1 with a slight excess of hydrogen peroxide in glacial acetic acid[29] gave the sulfoxide 2 in good yield. The thermolysis of 2 was carried out in refluxing xylene with use of calcium carbonate as a sulfenic acid trapping agent.[24]

Hydrolysis of the resulting diphenyl (1-amino-2-propenyl)phosphonate (3) by hydrochloric acid afforded the desired (1-amino-2-propenyl)phosphonic acid (4) in satisfactory yield.

**Alanine Racemase Assays.** The racemase of *P. aeruginosa* ($K_m$ = 14.2 mM) was competitively inhibited by VGP with a $K_i$ value of 8.5 mM established from Lineweaver–Burk plots. This value is comparable with those previously found for the mono- and dichloro derivatives of Ala-P.[17]

The enzyme of *S. faecalis* ($K_m$ = 7 mM) was strongly inhibited by VGP. The inhibition appears to be competitive from the double-reciprocal plots ($K_i$ = 3.5 mM) when the crude enzyme preparation was preincubated with the cofactor before the addition of VGP.

**D-Alanine:D-Alanine Ligase Assays.** The enzyme from *S. faecalis* ($K_m$ = 7 mM) was competitively inhibited by VGP with an apparent $K_i$ value of 5 mM, slightly lower than the value we have determined for DL-vinylglycine ($K_i$ = 9 mM). All these inhibitory constants were calculated from the graphical method of Lineweaver and Burk.

In contrast, the D-Ala:D-Ala ligase from *P. aeruginosa* was insensitive to these two vinyl derivatives, showing a structural difference between the binding sites of these Gram-positive and Gram-negative enzymes.

**Studies with Glutamate-alanine and Glutamate-aspartate Transaminases.** Since DL-vinylglycine has been found to be a substrate as well as an irreversible inactivator of the cytosolic and mitochondrial aspartate transaminases[19] and a substrate but not an inactivator of the alanine transaminase of pig heart,[19] we have examined the effect of its phosphonic analogue on these enzymes. VGP, at a concentration of 20 mM, inhibits neither alanine nor aspartate transaminases.

**Studies with D-Amino Acid Oxidase.** DL-Vinylglycine was a moderately good substrate of D-amino acid oxidase, and unlike L-amino acid oxidase, D-amino acid oxidase was not inactivated by DL-vinylglycine.[32] We have shown that VGP was neither a substrate nor an inhibitor of D-amino acid oxidase of hog kidney when the reaction was carried out with a ratio inhibitor/substrate = 10. These data allowed us to use D-amino acid oxidase for alanine racemase assays in the presence of VGP but not in that of vinylglycine.

**Antimicrobial Testing.** (1-Amino-2-propenyl)-phosphonic acid did not show antibacterial activity against a series of Gram-positive and Gram-negative microorganisms. The assays were performed on both complete and minimal media with penicillin G and amphotericin as standards. From these results it appears that VGP does not penetrate the cell.

**Conclusion.** Our findings show that the replacement of the carboxylic group of vinylglycine by a phosphonic one produces a molecule that exhibits inhibitory properties on alanine racemase and D-Ala:D-Ala ligase but loses all activity against the mammalian enzymes tested. We will take into account these enzymatic and bacterial investigations to design potentially new antibiotic peptides containing VGP as in the examples of other mimetic peptides containing a bacterial cytotoxic moiety.[5,33,34]

## Experimental Section

**Synthesis.** Melting points were determined on a Mettler FP 61 melting point apparatus. Microanalyses were performed by the Laboratory of Microanalyses, CNRS (Gif-sur-Yvette). IR spectra were taken on a Perkin-Elmer Model A-257 spectrophotometer, and $^{13}C$ and $^{31}P$ NMR spectra were recorded in $CF_3COOD$ on a 80 WP SY Brucker spectrometer equipped with an Heterospin Decoupler at 20.150 MHz for $^{13}C$ ($Me_4Si$ as an external standard) and 32.435 MHz for $^{31}P$ (85% $H_3PO_4$ as an external standard) in the CNRS-SNPE Laboratory (Thiais). $^1H$ NMR were recorded on a Brucker WM 500 spectrometer (Spectro Chemical Center of Pierre et Marie Curie University, Paris). Mass spectra were obtained on a quadrupole RIBER MAG R-10.10 mass

(14) Soper, T. S.; Jones, W. M.; Lerner, B.; Trop, M.; Manning, J. M. *J. Biol. Chem.* **1977**, *252*, 3170.
(15) Wang, E.; Walsh, C. *Biochemistry* **1978**, *17*, 1313.
(16) Wang, E.; Walsh, C. *Biochemistry* **1980**, *20*, 7539.
(17) Vo-Quang, Y.; Carniato, D.; Vo-Quang, L.; Lacoste, A. M.; Neuzil, E.; Le Goffic, F. *J. Med. Chem.* **1986**, *29*, 148.
(18) Soper, T.; Manning, J.; Marcotte, P.; Walsh, C. *J. Biol. Chem.* **1977**, *252*, 1571.
(19) Rando, R. *Biochemistry* **1974**, *13*, 3859 Gehring, H.; Rando, R.; Christen, P. *Biochemistry* **1977**, *16*, 4832.
(20) Field, L. *Synthesis* **1978**, 713.
(21) Trost, B.; Salzmann, T.; Hiroi, K. *J. Am. Chem. Soc.* **1976**, *98*, 4887. Trost, B. *Chem. Rev.* **1978**, *78*, 363.
(22) Sharpless, K. P.; Lauer, R. F.; Teranishi, A. Y. *J. Am. Chem. Soc.* **1973**, *95*, 2697.
(23) Reich, H.; Reich, I.; Renga, J. M. *J. Am. Chem. Soc.* **1973**, *95*, 5813.; **1975**, *97*, 5434.
(24) Rich, D.; Tam, J. *J. Org. Chem.* **1977**, *42*, 3815.
(25) Afzali-Ardakani, A.; Rapoport, H. *J. Org. Chem.* **1980**, *45*, 4817.
(26) Mikolajczyk, M.; Grzejszczak, S.; Korbacz, K. *Tetrahedron Lett.* **1981**, *22*, 3097.
(27) Koizumi, T.; Tanaka, N.; Iwata, M.; Yoshii, E. *Synthesis* **1982**, 917.
(28) Kudzin, Z. H.; Stec, W. J. *Synthesis* **1980**, 1032. Kudzin, Z. H.; Stec, W. J. *Synthesis* **1983**, 812.
(29) Tam, C. C.; Mattocks, K. L.; Tishler, M. *Synthesis* **1982**, 188.
(30) Kowalik, J.; Zygmunt, J.; Mastalerz, P. *Pol. J. Chem.* **1981**, *55*, 713.
(31) Oleksyszyn, J.; Subotkowska, L.; Mastalerz, P. *Synthesis* **1979**, 985.
(32) Marcotte, P.; Walsh, C. *Biochemistry* **1976**, *15*, 3070.
(33) Diddens, H.; Zahner, H.; Kraas, E.; Gohring, W.; Jung, G. *Eur. J. Biochem.* **1976**, *66*, 11.
(34) Cheung, K. S.; Wasserman, S. A.; Dudek, E.; Lerner, A.; Johnston, M. *J. Med. Chem.* **1983**, *26*, 725.

spectrometer operated in an electron ionization made with an electron energy of 70 eV, and data acquisition was performed by a digital PDP 11 computer (Laboratory of Mass Spectroscopy, ENSCP). Analytical thin-layer chromatography was carried out with use of silica gel plates (Merck 60 F-254) with methylene chloride/ethyl acetate (8:2) as an eluting system and 5% ethanolic solution of molybdophosphonic acid as a developing system.

**Diphenyl [1-[(Benzyloxycarbonyl)amino]-3-(phenylthio)propyl]phosphonate (1).** A mixture of triphenyl phosphite (31 g, 0.1 mol), benzyl carbamate (15.1 g, 0.1 mol), 3-(phenylthio)propanal (24.9 g, 0.15 mol), and glacial acetic acid (15 mL) is stirred for 1 h until the exothermic reaction subsides. The mixture is then heated at 80–85 °C for 1 h, and the volatile products are removed in vacuo on a boiling water bath. The oily residue is dissolved in methanol (180 mL) and left at −10 °C for crystallization. After standing overnight, the precipitate is filtered off and recrystallized from a mixture of chloroform and methanol to give 22.9 g (43%) of pure 1 as a white solid: mp 106–107 °C; TLC, $R_f$ 0.94; IR (KBr) $\nu$ 3250 (N–H), 1710 (C=O), 1240 (P=O), 1190 (POEt) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz) $\delta$ 2.03 and 2.27 (2 m, 2 H, H-2 and H-2′), 2.95 and 3.06 (2 m, 2 H, H-3 and H-3′), 4.68 (m, 1 H, H-1, $J_{HH}$ = 3.5 and 10.3 Hz, $J_{HP}$ = 17.6 Hz), 5.11 and 5.08 (dd, 2 H, $CH_2C_6H_5$, $J$ = 12.2 Hz), 5.64 (d, 1 H, NH, $J_{HH}$ = 10.0 Hz), 7.03–7.32 (m, 20 H, $C_6H_5$); MS ($NH_3$), 551 $(M + NH_4)^+$, 534 $(M + H)^+$, 400, 252, 91 $(PhCH_2)^+$ base peak. Anal. ($C_{29}H_{28}NO_5PS$) C, H, N, O, P, S.

**Diphenyl [1-[(Benzyloxycarbonyl)amino]-3-(phenylsulfinyl)propyl]phosphonate (2).** To a suspension of 1 (21.32 g, 40 mmol) in glacial acetic acid (80 mL) is carefully added aqueous hydrogen peroxide (30%, 4.5 mL, 44 mmol). The mixture is then stirred at room temperature until the reaction is shown to be complete by TLC tests (using 2% methanol in chloroform as an eluent). Acetic acid and water are removed in vacuo on a boiling waterbath and 19.7 g (90%) of the oily residue **2** is obtained. The crude sulfoxide may be used in the next step without further purification. TLC $R_f$ 0.30; IR (film) $\nu$ 3270 (NH), 1750 (C=O), 1245 (P=O), 1190 (POPh) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz) $\delta$ 2.09 and 2.48 (2 m, 2 H, H-2 and H-2′), 2.85 and 3.02 (2 m, 2 H, H-3 and H-3′), 4.57 (m, 1 H, H-1, $J_{HH}$ = 3.8 and 10.4 Hz, $J_{HP}$ = 17.6 Hz), 5.07 (m, 2 H, $CH_2C_6H_5$), 6.08 (d, 1 H, NH, $J_{HH}$ = 9.9 Hz), 7.0–7.6 (m, 20 H, $C_6H_5$); MS ($NH_3$) 567 $(M + NH_4)^+$, 550 $(M + H)^+$, 441, 424, 252, 108 $(PhCH_2OH)^+$ base peak, 91. Anal. ($C_{29}H_{28}NO_6PS$) C, H, N, O, P, S.

**Diphenyl [1-[(Benzyloxycarbonyl)amino]-2-propenyl]phosphonate (3).** A mixture of the crude sulfoxide **2** (5.49 g, 10 mmol), calcium carbonate (2 g, 20 mmol), and xylene (40 mL) is refluxed for 24 h. After cooling, the mixture is filtered, and the filtrate is concentrated in vacuo. The oily residue is dissolved in methanol (20 mL) and left for crystallization at −10 °C. Recrystallization from chloroform–methanol gives 2.98 g (70% yield) of **3**: mp 124 °C; TLC $R_f$ 0.80; IR (KBr) $\nu$ 3280 (NH), 1715 (C=O), 1640 (C=C), 1260 (P=O), 1190 (POPh), 950 ($CH_2$=) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz) $\delta$ 5.36 (m, 1 H, H-3, $J_{2,3(cis)}$ = 10.5 Hz, $J_{3,3'(gem)}$ = 1.7 Hz, $J_{HP}$ = 3.2 Hz), 5.45 (m, 1 H, H-3′, $J_{2,3'(trans)}$ = 17.1 Hz, $J_{3,3'(gem)}$ = 1.7 Hz, $J_{HP}$ = 4.9 Hz), 6.04 (m, 1 H, H-2, $J_{2,3'(trans)}$ = 17.1 Hz, $J_{2,3(cis)}$ = 10.5 Hz, $J_{1,2}$ = 5 Hz, $J_{HP}$ = 5 Hz), 5.12 (m, 3 H, H-1 and $CH_2C_6H_5$), 5.67 (d, 1 H, NH, $J$ = 9 Hz), 7.07–7.32 (m, 15 H, $C_6H_5$).

**(1-Amino-2-propenyl)phosphonic Acid (4, VGP).** Compound **3** (0.01 mol) is dissolved in glacial acetic acid (2 mL) and hydrochloric acid (35%, 20 mL). The mixture is heated under reflux for 7–8 h. The solvents are evaporated under reduced pressure, and the residue is dissolved in ethanol (10 mL). The solution is heated with propylene oxide until pH 6 is reached. The precipitate is filtered, washed with ethanol, and dried. Recrystallization from ethanol–water gives 0.576 g of VGP (4; 42% yield): mp 256 °C; TLC cellulose plates $R_f$ 0.50; eluting system, pyridine–acetic acid–water (10:7:3); developer, 0.5% ethanolic solution of ninhydrin; $^{31}P$ NMR ($CF_3COOD$, 80 MHz) one single signal at 7.17 ppm; $^{13}C$ NMR ($CF_3COOD$, 80 MHz) $\delta$ 54.26 (C-1, $J_{^{13}CP}$ = 148.9 Hz), 125.48 (C-3, $J_{^{13}CP}$ = 10.98), 126.24 (C-2, $J_{^{13}CP}$ = 7.32 Hz).

**Enzymology. A. Source of the Enzymes.** Alanine racemases (EC 5.1.1.1) and D-Ala:D-Ala ligases (EC 6.3.2.4) from *P. aeruginosa* A 237 and *S. faecalis* ATCC 8043 were prepared as described previously.[7,17] All the other enzymes used in this work were purchased from Boehringer-Mannheim: glutamate-aspartate transaminase (EC 2.6.1.1) from pig heart (sp act. 200 unit/mg), glutamate-alanine transaminase (EC 2.6.1.2) from pig heart (sp act. 80 units/mg), D-aminoacid oxidase (EC 1.4.3.3) from hog kidney (sp act. 17 units/mg).

**B. Enzymatic Assays. Alanine racemase** was assayed in the direction L-Ala → D-Ala by monitoring pyruvate lactate formation by coupling D-aminoacid oxidase and LDH and following the oxidation of NADH at 340 nm. The reaction mixture contained L-Ala, 2–40 mM; PLP, 150 $\mu$M; VGP, 0 or 15 mM; enzyme solution, 50 $\mu$L in a final volume of 1 mL of buffer (0.1 M Tris-HCl, pH 8.9 for the enzyme of *P. aeruginosa*, 0.1 M phosphate pH 8 for the enzyme of *S. faecalis*). Unlike the crude preparation of *P. aeruginosa*, the crude extract of *S. faecalis* requires PLP for activity.

**D-Ala:D-Ala ligase** was assayed by determining the formation of radioactive D-Ala-D-Ala from [U-$^{14}$C]-D-Ala (43 mCi/mmol).[6] The assays were carried out in a 50 mM Tris-HCl buffer, pH 7.8, 1 mM KCl, 5 mM ATP, 5 mM $MnCl_2$ with varying concentrations of [$^{14}$C]-D-Ala (2–40 mM) in the presence or absence of 15 mM VGP, and enzyme solution in appropriate amount.

**Glutamate-aspartate transaminase** was assayed spectrophotometrically by coupling oxaloacetate formation to NADH oxidation with malate dehydrogenase.[35] Aspartate transaminase (0.5 $\mu$g) was added to 0.8 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 5 mM L-aspartate; the activity was then determined by adding malate dehydrogenase, NADH, and finally $\alpha$-ketoglutarate (10 mM) to initiate the transaminase reaction. Another assay was performed in the presence of 20 mM VGP.

**Glutamate-alanine transaminase** was assayed spectrophotometrically by coupling pyruvate formation to NADH oxidation with lactate dehydrogenase.[35] Alanine transaminase (1.5 $\mu$g) was added to 0.8 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 5 mM L-alanine; the activity was then determined by adding LDH, NADH, and finally $\alpha$-ketoglutarate (10 mM) to initiate the transaminase reaction. Another assay was performed in the presence of 20 mM VGP.

**D-Amino acid oxidase** was assayed by determining pyruvate formation from D-alanine as described below for the racemase assay, using 0.2 mg of D-Ala in the absence or presence of 2 mM of VGP.

All spectrophotometric measurements were carried out at 25 °C and 340 nm with a Varian spectrophotometer.

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(35) Bergmeyer, H. V. "Methods in Enzymatic Analysis"; 2nd ed.; Academic Press: New York, 1974.