Reaction of Helenalin with Hydrogen Chloride

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Abstract □ Reaction of helenalin (I) with hydrogen chloride–chloroform and deactivated neutral alumina yielded three products: mexicanin A (II), 1-epiallohelenalin (IV), and a new oxide (V). These products were formed *via* intermediates (VII, IX, and VIII, respectively) by the initial addition of hydrogen chloride to the exocyclic methylene grouping of the γ-lactone followed by elimination of hydrogen chloride upon treatment with neutral alumina. Exposure of II to hydrogen chloride–chloroform gave the same intermediates (VII, IX, and VIII) which, upon treatment with the neutral alumina, regenerated II, IV, and V, respectively. Thus, II was the common intermediate for IV and V in the reaction of I with hydrogen chloride–chloroform and neutral alumina. The conversion of IV to its intermediate [11,13-dihydro-13-chloro-1-epiallohelenalin (IX)] with hydrogen chloride–chloroform and the complete recovery of the starting material (IV) with neutral alumina were also observed. Treatment of I with concentrated hydrochloric acid afforded 2,3-dihydro-2-chlorohelenalin (X). Compound X was also readily converted to I upon treatment with either neutral alumina or water. Further reaction of I with deactivated neutral alumina in chloroform led to the direct formation of II. The structures of these compounds were assigned on the basis of chemical as well as spectral evidence. The mode of formation of these compounds was also postulated.

Keyphrases □ Helenalin—reaction with hydrogen chloride–chloroform, identification of products and intermediates, mechanism of formation □ Sesquiterpene lactones—reaction of helenalin with hydrogen chloride–chloroform, identification of products and intermediates □ Hydrogen chloride—reaction with helenalin, identification of products and intermediates, mechanism of formation

The conversion of helenalin (I) to a mixture of mexicanin A (II) and neohelenalin (III) by treatment with hydrogen chloride–chloroform was previously reported (1–3). In connection with the study of the structure–activity relationship among the helenalin-related sesquiterpene lactones for cytotoxic or antitumor activity (4–6), this reaction was repeated; the chemical as well as spectroscopic data on the resulting products provided some interesting information on their structures and the mode of their formation.

When helenalin (I) was dissolved in anhydrous chloroform saturated with hydrogen chloride at room temperature and the resulting products were chromatographed on deactivated neutral alumina according to Herz *et al.* (1), the expected mexicanin A (II) was obtained in 25% yield as reported (1); but instead of neohelenalin (III)[1] accompanying II, traces of 1-epiallohelenalin (IV) and a new compound, an oxide (V), isolated in 5% yield, were obtained. However, these compounds (II, IV, and V) were not obtained before the treatment of the reaction products with neutral alumina. This finding will be covered later.

DISCUSSION

Compound V, m.p. 188–190°, had the composition $C_{15}H_{18}O_4$ and showed a molecular ion peak at *m/e* 262 in the mass spectrum. Compound V revealed IR bands at 1760 and 1655 cm.$^{-1}$ and a pair of low-field doublets in the NMR spectrum at 6.29 (1H, J = 3), and 5.61 (1H, J = 3). This is characteristic of a γ-lactone conjugated with an exocyclic methylene grouping, a feature common to helenalin (I) and mexicanin A (II). The NMR spectrum of V showed the lactonic proton at C-8 as a multiplet at 4.60. Two methyl groups at C-10 and C-5 in V were seen as a doublet at 1.18 (3H, J = 6) and a sharp singlet at 1.07 (3H), respectively. The presence of a cyclopentanone ring system in V was first suggested by the appearance of a strong IR band at 1740 cm.$^{-1}$ and substantiated by the absence of the characteristic conjugated cyclopentenone olefinic protons.

The IR spectrum of Compound V taken in mineral oil showed the absence of a hydroxyl group. This was further confirmed by the fact that V could not be acetylated with acetic anhydride in pyridine. This evidence, coupled with the fact that the empirical formula of Compound V ($C_{15}H_{18}O_4$) was identical with those of helenalin and mexicanin A, would indicate that the remaining fourth oxygen must form an oxide ring. Models show that one of the two possible structures, V or VI, can be assigned to this compound[2]. The former is regarded as the most likely because the signal for the proton at H-2 in Structure V appeared as a multiplet at 4.76, and it would be expected to move further downfield if Structure VI is assigned since H-3 in VI is adjacent to the C-4 carbonyl group. The upfield shift of the oxide proton at C-6 (3.79, 1H, d, J = 5.3) in V, compared with that in the NMR spectrum of helenalin (I) in which the H-6 signal appeared at 4.45 (1H, d, J = 4.5), might be due to the anisotropic effect of the α-methylene grouping of the γ-lactone.

The foregoing evidence suggests the assignment of Structure V with complete stereochemistry for the oxide.

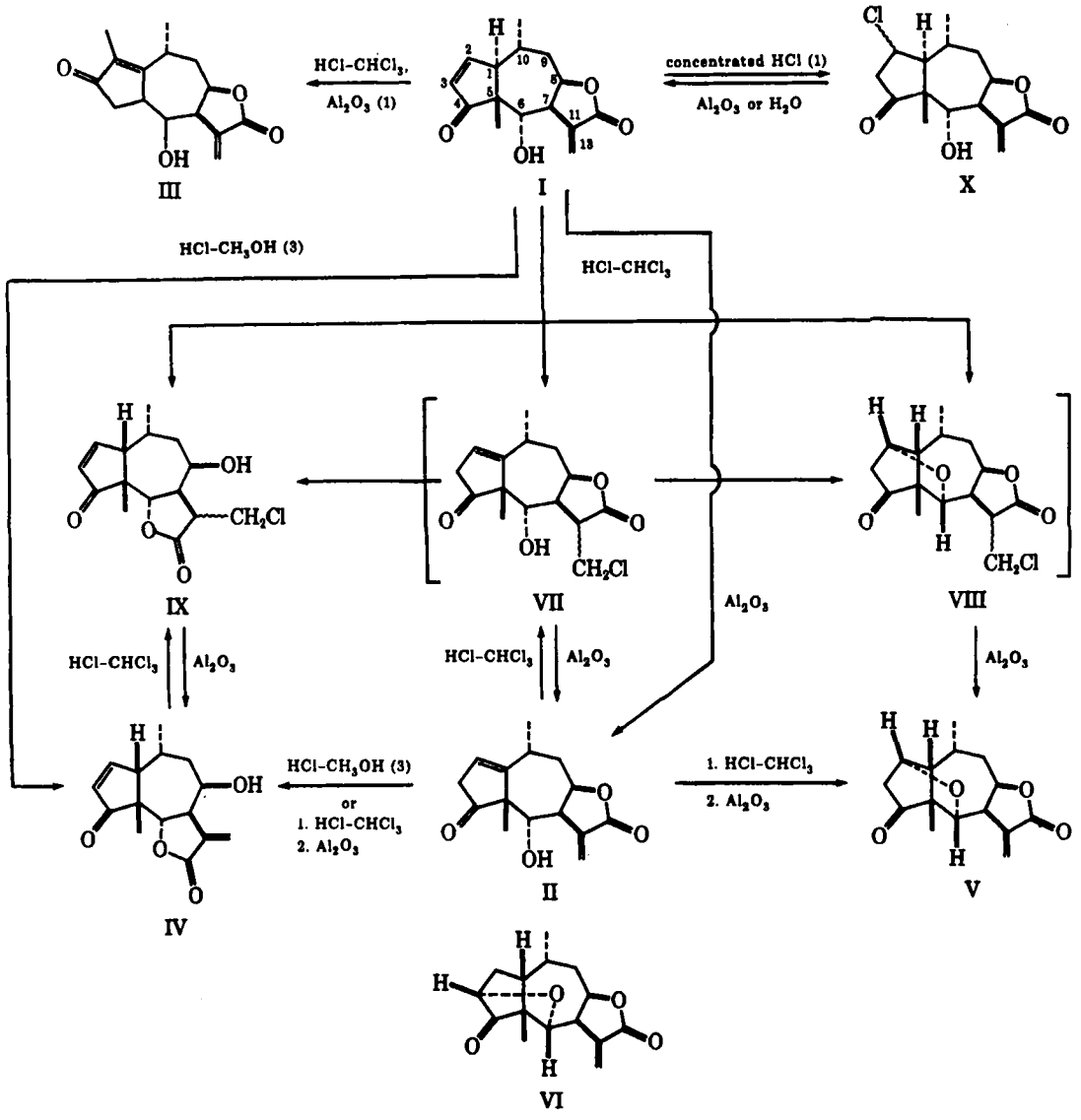
The other product besides II and V in this reaction of I with hydrogen chloride–chloroform and neutral alumina was the 1-epiallohelenalin (IV). This compound was obtained in only about 2% yield and was shown to be identical with the major product obtained by the treatment of I with hydrogen chloride–methanol as reported previously (3).

As already mentioned, during the action of hydrogen chloride–chloroform on helenalin, it was found that the initial reaction products prior to chromatography on neutral alumina were not mexicanin A (II), 1-epiallohelenalin (IV), and the oxide (V). They were mainly the new intermediates that resulted from the addition of hydrogen chloride to the exocyclic methylene grouping of the γ-lactone. These observations were verified by the fact that the intermediates were obtained in almost quantitative yield and showed the characteristic isotopic molecular ions at *m/e* 298 and 300 ($C_{15}H_{19}{}^{35}ClO_4$ and $C_{15}H_{19}{}^{37}ClO_4$) in the ratio of approximately 3:1, indicating the incorporation of 1 mole of hydrogen chloride into the molecule. These intermediates have proven to be elusive as far as separation is concerned[3], but their isolation as a mixture (likely Compounds VII, VIII, and IX) was easily achieved by silica gel chromatography. The NMR spectrum of this mixture further confirmed the absence of the characteristic pair of low-field doublets which corresponds to the α-methylene-γ-lactone grouping. Treatment of mexicanin A (II) with hydrogen chloride–chloroform in a manner similar for helenalin (I) gave reaction products whose NMR spectrum was completely identical with the one obtained from the

[1] Analysis of the crude reaction mixture in high concentration by NMR spectroscopy revealed the absence of any detectable vinyl methyl protons in the high field region (1.60 ~ 2.10). Neohelenalin was reported to show a vinyl methyl group at C-2 as a doublet at 1.68 (J = 2) (1).

[2] Dreiding models indicate that the formation of such an oxide ring between C-6 and C-2 or C-3, as shown in V or VI, requires the inversion of the asymmetric center at C-1 of helenalin. No other oxygen bridge could be constructed when H-1 is in an α disposition. As shown in Structure V, the oxide bridge involved C-6 α, C-2 α bonds.

[3] These mixtures were not very stable and tended to generate mexicanin A gradually upon prolonged sitting at room temperature.



Scheme I

reaction of I with hydrogen chloride–chloroform and yielded products II, IV, and V upon chromatography on neutral alumina.

In conclusion, the results obtained indicate that mexicanin A (II) is the common intermediate for the 1-epiallohelenalin (IV) and the oxide (V) in the reaction of helenalin with hydrogen chloride–chloroform and neutral alumina (Scheme I).

Similar treatment of 1-epiallohelenalin (IV) with hydrogen chloride–chloroform led to the formation of the corresponding hydrogen chloride adduct of the α-methylene-γ-lactone grouping (IX) in quantitative yield. Compound IX, m.p. 234–236°, analyzed for $C_{15}H_{19}ClO_4$, showed a molecular ion peak at *m/e* 298 along with an isotopic ion at *m/e* 300. The NMR spectrum of IX showed the disappearance of the characteristic exocyclic methylene protons and the generation of a new two-proton multiplet at 3.80, attributable to the protons attached to chlorine atom. Compound IX regenerated IV upon treatment with neutral alumina.

Reaction of helenalin (I) with concentrated hydrochloric acid elaborated 2,3-dihydro-2-chlorohelenalin (X) in quantitative yield. The following evidence supported Structure X for this reaction product: the presence of the isotopic molecular ions at *m/e* 298 and 300 in the mass spectrum, the absence of signals corresponding to the olefinic protons at C-2 and C-3 as found in I, and the presence of the well-defined pair of low-field doublets ascribed to the γ-lactone-α-methylene protons in the NMR spectrum. Further treatment of X with neutral alumina or water led to the complete recovery of I. Further reaction of I with deactivated neutral alumina in chloroform led to the direct formation of II in 15% yield.

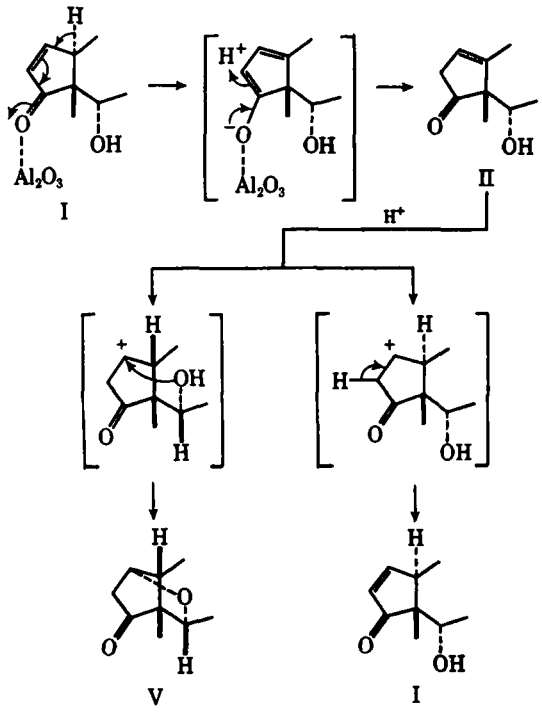
The formation of II and V can be accounted for by the mechanism postulated in Scheme II in which alumina is considered to act as a Lewis acid.

EXPERIMENTAL[4]

Helenalin (I) was obtained from extracts of either *Helenium microcephalum* or *Balduina angustifolia* as previously reported (4, 7).

Treatment of Helenalin (I) with Hydrogen Chloride–Chloroform: Compounds VII, VIII, and IX—This reaction was carried out in the same manner as that reported in the literature (1); *i.e.*, a solution of helenalin (1 g.) in anhydrous chloroform saturated with hydrogen chloride (35 ml.) was allowed to stand at room temperature overnight. The mixture was further refluxed for 30 min., and the product

[4] Melting points were determined on a Thomas-Hoover melting-point apparatus and are corrected. Unless otherwise specified, IR spectra were determined in mineral oil mulls with a Perkin-Elmer 257 grating IR spectrophotometer. NMR spectra were measured in $CDCl_3$ with a Jeolco C 60 HL NMR spectrometer, using tetramethylsilane as an internal standard. All chemical shifts were reported in δ (p.p.m.) values and the coupling constants were reported in hertz values. Signals are characterized in the usual way: s, singlet; d, doublet; t, triplet; q, quartet, and m, multiplet. Mass spectra were determined on an A.E.I. MS-902 instrument at 70 ev. using a direct inlet system. Silica gel for column chromatography refers to Baker A.R. No. 3405, and silica gel for TLC refers to Merck silica gel G developed with chloroform–acetone (3:1) and visualized by spraying with concentrated sulfuric acid and heating. Deactivated neutral alumina for column chromatography refers to neutral alumina AG-7 (100–200 mesh), Bio-Rad, activity grade III. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga.



Scheme II

was washed with 5% sodium hydroxide, washed with water, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to yield a brown syrup. The syrup showed the characteristic isotopic molecular ions at *m/e* 298 and 300 as described in the text. It was chromatographed on silica gel (1.3 × 30 cm.) activated at 120° and impregnated with 10% of water using *n*-hexane–benzene (1:1), benzene, and ethyl acetate as the developing solvents, successively. Thirty-one 25-ml. fractions were collected, and the composition of the fractions was determined by examining thin-layer chromatograms. The first hexane–benzene (1:1) eluate (fractions 1–19) yielded, after evaporation of the solvent, a colorless oil (380 mg.), which appeared as a single fast moving spot. The NMR spectrum of this substance indicated that this was a mixture of the hydrogen chloride adducts, *i.e.*, Compounds VII, VIII, and IX. The subsequent benzene (fractions 20–26) (180 mg.) and ethyl acetate (fractions 27–31) (400 mg.) eluates all contained a mixture of mainly the above-mentioned, fast moving spot and traces of others, and they were not investigated further.

Treatment of Helenalin (I) with Hydrogen Chloride–Chloroform and Deactivated Neutral Alumina—The brown syrup obtained from the repetition of the above experiment with I (1 g.) and hydrogen chloride–chloroform (35 ml.) was chromatographed on deactivated neutral alumina (1.5 × 25 cm.). Elution was effected in 2-ml. fractions with benzene, benzene–chloroform, chloroform, and acetone.

The Oxide (V)—The first benzene eluates (fractions 1–10) afforded, after evaporation of the solvent, a crystalline residue. This residue was recrystallized from dichloromethane–absolute ethanol to give V as colorless needles (50 mg., 5%), m.p. 188–190°. The relevant (IR, NMR, and mass) characteristics have been described in the text.

Anal.—Calc. for $C_{15}H_{18}O_4$: C, 68.68; H, 6.92. Found: C, 68.46; H, 7.01.

Mexicanin A (II)—The subsequent benzene–chloroform (1:1) and chloroform eluates (fractions 11–25) provided a crystalline material, which was recrystallized from acetone–ether to furnish II as colorless prisms (250 mg., 25%), m.p. 140–142° [lit. (1) m.p. 138–140° (acetone–ether)]. The IR spectrum of II was identical with that of mexicanin A as described in the literature (1). The NMR spectrum exhibited signals at 1.21 (3H, s, C-5 CH_3), 1.32 (3H, d, J = 6, C-10 CH_3), 3.59 (1H, d, J = 7.5, H-6), 4.67 (1H, dt, J = 3, 8.3, H-8), 5.89 (1H, q, J = 3.8, 1.5, H-2), 6.02 (1H, d, J = 3, H-13), and 6.35 (1H, d, J = 3, H-13) and was in complete agreement with the indicated structure (II), *i.e.*, mexicanin A.

1-Epiallohelenalin (IV)—The final acetone eluates (fractions 26–39) gave an oily residue, which was seeded with one crystal of 1-epiallohelenalin obtained from the treatment of helenalin with hydrogen chloride–methanol (q.v.). The colorless crystals which formed were collected and recrystallized from dichloromethane–ether to yield 20 mg. of IV, m.p. 179–180°. The identity of IV was established by direct comparison with an authentic sample of 1-epiallohelenalin by mixed melting point, TLC, and superimposable IR and NMR spectra.

Attempted Acetylation of Oxide (V)—A solution of V (35 mg.) in acetic anhydride–dry pyridine (2:1) (0.5 ml.) was kept at room temperature overnight. The product, recovered by working up the reaction mixture in the usual way, was the unchanged starting material (33 mg.).

Treatment of Mexicanin A (II) with Hydrogen Chloride–Chloroform and Deactivated Neutral Alumina: Compounds VII, VIII, and IX and Compounds II, V, and IV—Compound II (300 mg.) was treated with anhydrous chloroform saturated with hydrogen chloride (15 ml.) in an analogous manner as that for helenalin, furnishing a brown oily residue. The residue was identified as the mixture of Compounds VII, VIII, and IX by their superimposable NMR spectra in comparison with the reaction products of helenalin and hydrogen chloride–chloroform already described. The oily residue was chromatographed on deactivated neutral alumina (1.5 × 12 cm.). Elution with benzene (20 ml.) afforded V (30 mg., 10%). Elution with benzene and chloroform–ether (1:1) (25 ml.) yielded II (180 mg., 60%). Elution with chloroform–ether (1:2) (20 ml.) gave IV (18 mg., 6%).

Treatment of 1-Epiallohelenalin (IV) with Hydrogen Chloride–Chloroform: 11,13-Dihydro-15-chloro-1-epiallohelenalin (IX)—Compound IV (100 mg.) was treated with anhydrous chloroform–hydrogen chloride (5 ml.) in an analogous manner as described for helenalin. The oily residue resulting from the removal of solvent crystallized upon trituration with a small amount of anhydrous ether. Recrystallization from dichloromethane–ether gave IX as white crystals in quantitative yield, m.p. 234–236° dec. The IR bands were at 3420 (OH), 1775 (saturated γ-lactone), 1690, and 1590 (cyclopentenone) cm.$^{-1}$. The NMR spectrum exhibited signals at 1.22 (3H, d, J = 6.8, C-10 CH_3), 1.38 (3H, s, C-5 CH_3), 3.80 (2H, m, CH_2Cl), 4.05 (1H, m, H-8), 4.77 (1H, d, J = 8, H-6), 6.33 (1H, dd, J = 6, 2.3, H-3), and 7.82 (1H, dd, J = 6, 2.3, H-2).

Anal.—Calc. for $C_{15}H_{19}ClO_4$: C, 60.29; H, 6.41. Found: C, 60.25; H, 6.51.

1-Epiallohelenalin (IV) from 11,13-Dihydro-13-chloro-1-epiallohelenalin (IX) and Neutral Alumina—Chromatography of IX (20 mg.) on deactivated neutral alumina (1.2 × 7 cm.) and elution with chloroform afforded IV as colorless needles in quantitative yield. Compound IV showed undepressed melting point on admixture of an authentic sample, and their IR and NMR spectra were identical.

Treatment of Helenalin (I) with Concentrated Hydrochloric Acid: 2,3-Dihydro-2-chlorohelenalin (X)—A solution of I (100 mg.) in concentrated hydrochloric acid (1 ml.) was heated at 40° for 5 min. The colorless crystalline product that deposited was filtered and dried to give quantitative yield of X, m.p. 134–135° dec. [lit. (3) m.p. 143–145° dec.]. Compound X showed IR bands at 3480 (OH), 1765 (sh), 1752, 1650 (α-methylene-γ-lactone), and 1735 (cyclopentanone) cm.$^{-1}$. Compound X also displayed NMR (dimethyl sulfoxide-d_6) signals at 0.87 (3H, s, C-5 CH_3), 1.15 (3H, d, J = 6, C-10 CH_3), 5.86 (1H, d, J = 3, H-13), and 6.19 (1H, d, J = 3, H-13).

Helenalin (I) from 2,3-Dihydro-2-chlorohelenalin (X)—Chromatography of X (50 mg.) on deactivated neutral alumina (1.7 × 7 cm.) with elution with acetone (50 ml.) recovered I quantitatively. Compound I was also obtained by extraction of X with water and chloroform. The chloroform extract was dried and evaporated to yield a crystalline residue, which was recrystallized from benzene–ether to give I as colorless needles, m.p. 170–172°. The IR spectrum of this compound was identical with that of helenalin.

Treatment of Helenalin (I) with Deactivated Neutral Alumina: Mexicanin A (II)—A solution of I (70 mg.) in chloroform (5 ml.) was added to deactivated neutral alumina (1 g.). The reaction mixture, after standing at room temperature for 3 days with occasional shaking, was filtered and evaporated to yield an oil[5]. Preparative TLC on silica gel[6] furnished mexicanin A (II) (10.5 mg., 15%), m.p. 140–142°, identical (IR and mixed melting point) with an authentic sample, and unreacted I (43 mg.).

[5] Examination of this oil by TLC revealed the absence of Compounds IV and V.

[6] Merck silica gel GF-254 with chloroform–acetone (3:1).

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DRUG STANDARDS

Quantitative Fluorometric Determination of Pancreatic Lipase in Pharmaceuticals

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Abstract □ A rapid and reliable assay for pancreatic lipase is described. The method utilizes microquantities of enzyme and a fluorescent substrate. Among the many fluorescent substrates available, 4-methylumbelliferone laurate was chosen as the most suitable for this method. The rate of hydrolysis at different enzyme levels was determined at 25°. Interference due to nonspecific esterases and the effect of some pharmaceutical agents were evaluated. The fluorometric assay procedure was compared to the NF XIII potentiometric method. Pancreatic lipase in concentrations as low as 0.15 NF unit of activity could be detected. Various complex pharmaceutical preparations were compared using the fluorometric and the potentiometric methods. The fluorometric procedure was found to be reproducible and useful in determining content uniformity.

Keyphrases □ Pancreatic lipase—quantitative fluorometric determination in pharmaceuticals □ Fluorometry—quantitative determination of pancreatic lipase in pharmaceuticals □ Enzymes, pancreatic lipase—quantitative fluorometric determination in pharmaceuticals

For many years, pancreatic enzymes have been used as digestive aids. A number of pharmaceutical preparations contain pancreatic enzymes alone or in combination with other therapeutic agents. Many assay procedures have been devised to determine the potencies and activities of the lipolytic enzymes in these preparations. Aldridge (1) first described a manometric method utilizing carbon dioxide liberation from bicarbonate buffer to detect lipases in biological extracts. However, titrimetric measurement of acid liberation from natural fats was found to be more convenient (2, 3). Over the years, this procedure has been greatly modified and refined to improve its reliability and reproducibility (4). The potentiometric titration method, although reliable, is cumbersome and time consuming for routine analysis. This procedure also lacks the versatility needed for testing complex pharmaceutical preparations and ascertaining content uniformity.

Fluorometric procedures have a distinct advantage over other methods in that they are very sensitive. For a number of years, biochemists and clinical chemists detected minute amounts of esterase in microorganisms by utilizing fluorogenic substrates such as 1-naphthyl esters (5). Most fluorometric procedures for hydrolytic enzymes involve hydrolyzing a nonfluorescent substrate to a highly fluorescent product. 1-Naphthyl phosphate, a nonfluorescent substrate, is readily hydrolyzed by the enzymes, acid phosphatase and alkaline phosphatase, producing the free fluorescent product, 1-naphthol, which is then measured quantitatively by the fluorometric method. Similarly, umbelliferone and 4-methylumbelliferone are highly fluorescent compounds while their ester derivatives are nonfluorescent. These derivatives are ideal substrates for many enzymes (6). Recently, a number of investigators (6–8) reported new derivatives of fluorescein and 7-hydroxy-4-methylcoumarin to detect and locate minute quantities of lipase in plants