[CONTRIBUTION FROM THE BUREAU OF ENTOMOLOGY AND PLANT QUARANTINE, AGRICULTURAL RESEARCH ADMINISTRATION, U. S. DEPARTMENT OF AGRICULTURE]

Wilfordine, an Insecticidal Alkaloid from *Tripterygium wilfordii* Hook*

BY FRED ACREE, JR., AND H. L. HALLER

Swingle, Haller, Siegler and Swingle[1] have reported on the history, the introduction into this country, and the preliminary entomological tests of *Tripterygium wilfordii* Hook (family *Celastraceae*). This plant is a perennial twining vine commonly called lei kung teng or thunder god vine in China, where the root bark has been used extensively to kill garden insects.

From extracts of the root bark Chou and Mei[2] isolated dulcitol and the insecticidally inert red pigment, tripterine, which Schechter and Haller[3] found to be identical with the red pigment, celastrol, that Gisvold[4] isolated from *Celastrus scandens*, the common American bittersweet. Chou, Hsu and Hwang[5] described detailed entomological tests and the isolation of glucose, fructose, dulcitol, and the toxic alkaloid fraction which they named "tripterygine." Hwang[6] further described this product as a colorless, amorphous powder precipitated in 0.08% yield (root bark) when petroleum ether was added to a benzene solution of the acid-soluble products separated from the ether extractive of the root bark. Tripterygine precipitated the usual alkaloid reagents, melted at 160–165°, and was assigned the formula $C_{32}H_{38}O_{11}N$.

The early efforts in this laboratory to isolate the toxic principle from the ground whole roots of *Tripterygium wilfordii*[7] resulted in the isolation of dulcitol from both the aqueous and the ethanolic extractive. The ether extractive yielded a very small quantity of amorphous alkaloid mixture when subjected to ether–alkali–acid partitions performed in one day's time. Subsequently, the alkaloid was demonstrated to be labile upon remaining overnight in contact with the cold alkaline mother liquor. On the other hand, the material appeared to be stable in contact with ammonium hydroxide even at room temperature. In addition an aqueous suspension of the ground whole roots was found to give an acid reaction.

When the drug (by then approximately one year old) was treated with dilute ammonium hydroxide prior to extraction with ether, the total crude-alkaloid fraction (approximately 0.48% of the roots) was isolated readily from the acid-soluble portion of the ether extractive by precipitation with ammonium hydroxide. Thus obtained, the crude-alkaloid fraction was a colorless amorphous powder, which melted at 163–170° and gave a positive test for nitrogen and negative tests for sulfur and halogen. Efforts to obtain crystalline derivatives of this material with the general alkaloid reagents were unsuccessful.

The crude-alkaloid fraction was separated into fractions readily soluble and insoluble in dilute acetic acid. The readily soluble fraction, after having been treated first with lead acetate and then with silicotungstic acid, yielded a silicotungstate, from which the alkaloid mixture was liberated with ammonium hydroxide. This alkaloid mixture crystallized in part from solution in a mixture of dilute ethylene glycol–methanol as thin, birefractive, equilateral triangular plates that melted at 187–188°. On recrystallization from methanol alone this product crystallized in the same form, but it melted to a resin at 173.5–174° and flowed at 188–189°. The melting point was not changed after several recrystallizations from methanol and from methanol–acetone mixture. The product is therefore considered to be a pure compound, for which the name "wilfordine" is suggested.[8] The acetic acid-insoluble fraction, by way of the silicotungstate, yielded an alkaloid mixture from which, by crystallization from methanol alone, an additional quantity of wilfordine was obtained. The non-crystalline alkaloid mixture was recovered from the mother liquors, but it failed to produce additional crystals on further treatment with solvents. This mixture still contains insecticidally active material,[8] but it has not been examined further.

Reference has been made[1] to the loss in toxicity of the root bark during storage for periods of over one year. This loss may be due to decomposition of the alkaloid, as indicated by the fact that fresh roots yield approximately five times the quantity of crystalline material isolated from roots one year old. Furthermore, the crystalline alkaloid can be obtained from fresh roots simply by allowing the precipitated crude-alkaloid fraction to crystallize from methanol without prior purification by lead acetate and silicotungstic acid as described above.

Wilfordine gave no evidence of containing hydroxy, methoxy or methylenedioxy groups, of being a glycoside, or of containing double bonds. Wilfordine is a dextrorotatory compound containing one tertiary nitrogen, and its formula has been calculated from molecular weight, carbon, hydrogen, and nitrogen determinations

(1) Swingle, Haller, Siegler and Swingle, *Science*, **93**, 60 (1941).

(2) Chou and Mei, *Chinese J. Physiol.*, **10**, 529 (1936).

(3) Schechter and Haller, THIS JOURNAL, **64**, 182 (1942).

(4) Gisvold, *J. Am. Pharm. Assoc.*, **28**, 440 (1939); **29**, 432 (1940).

(5) Chou, Hsu, and Hwang, *Chedah Agr. Quart.*, **1**, 3 (1937).

(6) Hwang, *Kwangsi Agr. Expt. Sta. Bul.*, No. 5 (1939).

(7) Whole roots of the plant were furnished by the U. S. Bureau of Plant Industry, Soils, and Agricultural Engineering.

(8) Unpublished entomological notes of E. H. Siegler, of the Bureau of Entomology and Plant Quarantine. Both this crystalline product and the non-crystalline mixture were found to be extremely toxic to codling moth larvae.

to be $C_{42}H_{47}O_{19}N \pm CH_2$ or $\pm$ O. The compound is a very weak base, since its crystalline picrate after having been recrystallized from methanol several times yielded the free alkaloid.

Wilfordine is an ester alkaloid, it yielded eight equivalents of acid upon saponification with ethanolic potassium hydroxide. The acid components were shown to consist of six steam-volatile equivalents composed of five moles of acetic acid together with one mole of benzoic acid, and two non-volatile equivalents. No evidence could be obtained that any other volatile products had been formed.

The acidified aqueous residue remaining after removal of the volatile acids yielded an ether-soluble acid product that melted at 193–194° with darkening and evolution of gas. It contained nitrogen and gave a negative carbylamine test, and its neutral equivalent was found to be 121. From determinations for carbon, hydrogen and nitrogen, this acid product was calculated to be a dicarboxylic acid of formula $C_{11}H_{13}O_4N$. The quantity of acid obtained was approximately one-half of that calculated for an acid of molecular weight 223. Although attempts to separate the polyhydroxy nucleus from the ether-extracted aqueous residue were unsuccessful, it was possible to obtain a colorless, amorphous, very hygroscopic precipitate that contained nitrogen. It was presumed to be the salt of the half-ester of the dicarboxylic acid.

From another experiment, in which wilfordine was saponified with methanolic barium hydroxide, in addition to the products mentioned above, a small quantity of the polyhydroxy compound was isolated from the ether-insoluble portion of the acidified hydrolysate and it contained neither nitrogen nor sulfur. The formula for this substance, which melted at 309–310° with decomposition, was calculated from values for carbon and hydrogen determinations to be $C_{15}H_{26}O_{10}$.

Although these results show that wilfordine is an ester-alkaloid, a discrepancy exists between the sum of the isolated component parts, $C_{43}H_{49}O_{18}N$, and the formula $C_{42}H_{47}O_{19}N$, calculated for wilfordine. It is not known whether this discrepancy is due to the failure to detect a CH_2 or an oxygen. This difference might conceivably be eliminated through a study of the products of saponification by means of chromatography, particularly by the application of partition chromatography to the separation of the acid products. More recent evidence obtained in the course of another problem has demonstrated that the steam-distillation procedure is inadequate for the separation of volatile fatty acids from mixtures with volatile aromatic acids.

Experimental[9]

Isolation of Crude Alkaloid Fraction.—Approximately 3 kg. of finely ground whole roots was moistened with 1200 ml. of 10% ammonium hydroxide, thoroughly mixed, and allowed to stand in a closed vessel for two to three hours. The plant material was then completely extracted by percolation with ether. The ether extract was concentrated to about 500 ml., cooled, and then completely extracted with several portions of dilute hydrochloric acid. The combined acid extracts were warmed slightly, with stirring to expel the dissolved ether, and then cooled. After the addition of an excess of ammonium hydroxide, the colorless, amorphous precipitate of crude-alkaloid fraction that formed was separated, washed with water, and dried in vacuum. It weighed 14.5 g. and melted at 163–170°.

This product gave a positive test for nitrogen and negative tests for sulfur and halogens. Solutions of the product gave amorphous precipitates when treated with silicotungstic acid, picric acid, and chloroplatinic acid, and formed indefinite products when treated with picrolonic acid, methyl iodide, and semicarbazide hydrochloride. It was not possible to obtain the base hydrochloride or sulfate in solid form from aqueous media.

Purification of Crude-Alkaloid Fraction.—The crude alkaloid fraction was suspended in water to which acetic acid was added with vigorous stirring as long as any base appeared to dissolve. The undissolved material (see below) was separated from the acid solution, to which an excess of lead acetate was then added and the precipitated lead salts were removed. The crude alkaloids were then precipitated from the filtrate by addition of an excess of silicotungstic acid. After having been separated and washed with water, the silicotungstate precipitate was suspended in water and vigorously stirred during the addition of an excess of ammonium hydroxide. The liberated alkaloid fraction was separated by extraction with ether, washed with water, and then dried. After removal of the solvent, the ether solution yielded an amorphous residue, which was dissolved in dilute hydrochloric acid, filtered through a bed of charcoal, and then precipitated by the addition of an excess of ammonium hydroxide. The separated purified alkaloid fraction weighed 2.55 g. after having been washed with water and dried. Another extraction of 9 kg. of root yielded an additional 4.1 g. of this purified alkaloid fraction.

The acetic acid-insoluble portion of the crude alkaloid fraction described above together with the comparable material obtained from the 9 kg. batch of root was dissolved in dilute hydrochloric acid and precipitated with an excess of silicotungstic acid. The silicotungstate yielded 3.8 g. of purified alkaloid fraction after having been separated and treated as described above.

Isolation of Crystalline Wilfordine.—A portion of the purified-alkaloid fraction from the acetic acid-soluble portion described above was dissolved in hot ethylene glycol, from which it precipitated gummy when the solution was diluted to turbidity with water. An equal volume of methanol was added and the precipitate was dissolved by heating. The hot solution was allowed to cool slowly, and the crystals of wilfordine that separated melted at 175–180°. The product was recrystallized from the same solvent mixture and once from methanol and then melted at 187–188°. Wilfordine occurs in the form of very thin, birefractive, equilateral triangular plates. An additional quantity of wilfordine was obtained when the remaining portion of this purified alkaloid fraction was dissolved in hot methanol and seeded while still warm. In this case the crystals that separated were recrystallized twice from methanol and occurred in the same form, but they first melted to a resin at 173.5–174° and then flowed at 188–189°. The 6.1 g. of this purified alkaloid fraction yielded 135 mg. of pure wilfordine.

The 3.8 g. of purified-alkaloid fraction obtained from the acetic acid-insoluble material described above was partly dissolved in methanol and crystallized spontaneously before sufficient methanol had been added to effect solution. Additional methanol was added to dissolve the amorphous material, and the crystals were separated after the solution had been cooled. The crystalline product was recrystallized from methanol and furnished 620 mg. of wilfordine, which melted to a resin at 173.5–174°

(9) All melting points are corrected and all analyses were made by micro methods.

and flowed at 188–189°. The three portions of crystalline wilfordine were combined and recrystallized from methanol and then from equal volumes of methanol–acetone, and the separated product melted to a resin at 173.5–174° and flowed at 188–189°; $[\alpha]^{20}D$ + 15.4° (*c*, 1.266 in HCl); + 11.9° (*c*, 3.974 in acetone). The product lost no solvent when it was dried over boiling methanol, *p* = 0.1 mm.

Anal. Calcd. for $C_{42}H_{47}O_{13}N$: C, 58.04; H, 5.45; N, 1.61; mol. wt., 869.8. Found: C (av. 8 anal.), 57.98; H, 5.52; N (av. 6 anal.), 1.62; mol. wt., 853, 862 [isothermal distillation].[10]

Wilfordine gave negative tests for methoxy and methylenedioxy groups, and it did not reduce Fehling solution either before or after hydrolysis with acid. The alkaloid was recovered unchanged from the reaction mixture after attempts were made to acetylate and to hydrogenate it. When wilfordine was allowed to react with cyanogen bromide, it gave a weak but positive test for tertiary nitrogen and it also failed to react with nitrous acid.

Non-crystalline Alkaloid Fraction.—The mother liquors obtained during the isolation and purification of wilfordine from the two purified alkaloid fractions were combined and the dissolved material was precipitated by the addition of water. Small portions of the separated amorphous material failed to furnish crystals after standing for several months in solution in different solvents. That this material is for the most part alkaloidal in nature was demonstrated when it precipitated a solution of picric acid and when it showed considerable toxicity to codling moth larvae.[8]

Picrate of Wilfordine.—Twenty milligrams of wilfordine was dissolved in 1 ml. of water containing 2 drops of hydrochloric acid (1:4) and allowed to react with 0.6 ml. of a saturated aqueous solution of picric acid. The crystalline picrate was separated and washed with water. After recrystallization from dilute methanol the product weighed 18 mg. and melted at 158–159° after sintering at 156°. This product was very difficult to burn during analysis.

Anal. Calcd. for $C_{48}H_{50}O_{26}N_4$: C, 52.5; H, 4.55; N, 5.09. Found: C, 51.27, 51.86; H, 4.56, 4.52; N, 5.11, 4.66.

This preparation was repeated, and the separated crystalline picrate was recrystallized several times from dilute methanol in an effort to obtain a constant melting point. The product that separated from the fourth crystallization melted at 173.5–174°, and the melting point was not changed in admixture with wilfordine.

Determination of Acid Equivalents of Wilfordine.—A solution of 1.0011 g. of wilfordine in 15 ml. of ethanolic potassium hydroxide (0.8317 *N*) and 1 ml. of water was refluxed for three hours. The condenser was washed down with 15 ml. of water, and the reaction mixture, back-titrated to phenolphthalein, required 3.15 ml. of 1.043 *N* sulfuric acid. The blank was 0.14 ml. of 1 *N* alkali. Another 5 ml. of ethanolic potassium hydroxide was added to the titrated solution and, after having been refluxed for 2 hours, the reaction mixture was again titrated and required 3.49 ml. of 1.043 *N* sulfuric acid. After correction for the blank (0.28 ml. of 1 *N* alkali), the sample consumed 9.42 ml. of 1 *N* alkali as compared with 9.05 ml. found in the first titration (calcd. for mol. wt. 869.8: 8 eq., 9.20 ml.; 9 eq., 10.35 ml.). These results were essentially duplicated in two check determinations.

Determination of Distillation Curves.—The titrated solution from the first saponification (1.0011 g.) described above was extracted with ether, and the extract was washed with water and dried. On removal of the solvent, the solution yielded only a trace of oily residue.

The wash water was combined with the extracted alkaline solution, which was acidified to congo red with sulfuric acid and then completely steam-distilled. The total distillate of volatile acids required 6.13 ml. of 1 *N* alkali for neutralization (calcd. for 5 eq., 5.74 ml.; 6 eq., 6.89 ml.).

The neutralized volatile acid fraction was acidified with sulfuric acid and steam-distilled at constant volume according to the procedure described[11] for determining distillation constants and distillation curves. When the values obtained in this determination (100 ml., 32.4 per cent. of acid; 200 ml., 54.4; 300 ml., 69.1; 400 ml., 79.1; 500 ml., 86.1) were plotted on log paper a straight line was obtained, which indicated only the presence of acetic acid. However, the slope of the line was steeper than the slope of the line obtained in the same manner with acetic acid and therefore indicated the presence of at least a small quantity of some acid of lower distillation rate. In the meantime, both acetic acid and benzoic acid were isolated as described below. Calculations[12] based on the distillation constants indicated that only these two acids were present in the volatile acid fraction and that they occurred in the ratio 5 to 1, respectively (average values found for 2 detns.: acetic, 4.6 moles; benzoic, 0.93 mole). These results checked those obtained from an experimental mixture containing 5 moles of acetic acid and 1 mole of benzoic acid.

Identification of Acetic Acid.—A portion (250 mg.) of the salt obtained upon evaporation of the volatile acid distillate described above was allowed to react with 700 mg. of *p*-toluidine and 0.3 ml. of concentrated hydrochloric acid in the usual manner. The separated crystals after having been recrystallized from water weighed 100 mg. and melted at 148–149°. The product was identified as *p*-acetotoluidine when, in admixture with an authentic sample (m. p. 149–150°), its melting point was not changed.

Saponification of Wilfordine

Five grams of wilfordine was added to 100 ml. of an ethanol solution containing 10 g. of potassium hydroxide and refluxed for five hours in nitrogen which escaped through a scrubber containing dilute hydrochloric acid. The acid solution gave a negative test for volatile amines upon the addition of chloroplatinic acid. Most of the excess alkali in the reaction mixture was neutralized with dilute sulfuric acid, and then the alcohol and some of the water were removed under reduced pressure. The distillate gave a negative test for volatile amines on the addition of chloroplatinic acid. The alkaline residue was extracted with ether, and the extract was washed with water and dried. On removal of the solvent the ether solution yielded only a trace of oily residue, which was discarded. The wash water was combined with the alkaline solution and acidified to congo red with dilute sulfuric acid. The crystals that immediately formed were extracted with three portions of ether, and the extract was washed once with water and dried. The crystalline residue obtained on removal of the solvent weighed 620 mg.

Identification of Benzoic Acid.—The 620 mg. of crystalline residue just described (theory for 1 mole of benzoic acid, 690 mg.) was recrystallized from water, and the separated crystals melted at 120.5–121.5°. The melting point was not depressed in admixture with an authentic sample of benzoic acid. The product (50.8 mg.) was titrated and required 4.02 ml. of 0.1 *N* alkali.

Anal. Calcd. for $C_7H_6O_2$: mol. wt., 122. Found: mol. wt. (by titration), 126.

Isolation of Dicarboxylic Acid.—The wash water from the ether solution of benzoic acid was combined with the extracted acidified aqueous solution described above and continuously extracted with ether. The separated aqueous solution is described below.

The ether extract was titrated with 26.9 ml. of 1 *N* alkali and then the solvent was removed. The residue was dissolved in small volume of water, but when it failed to crystallize after having been acidified with sulfuric acid, it was exhaustively steam-distilled and yielded volatile acid equivalent to 15.96 ml. of 1.055 *N* alkali. The steam-distillation residue was again extracted continuously with ether. The extract on removal of the solvent yielded 670 mg. of crystalline residue which gave a positive

(10) Clark, *Ind. Eng. Chem., Anal. Ed.*, **13**, 820 (1941).

(11) Clark and Hillig, *J. Assoc. Official Agr. Chem.*, **21**, 684 (1938).

(12) Dyer, *J. Biol. Chem.*, **28**, 445 (1917).

test for nitrogen and a negative test for carbylamine. Of this quantity 470 mg. was recrystallized from methanol, and the 171 mg. that separated was recrystallized from water. The 45 mg. thus obtained melted at 191–192° with darkening and evolution of gas (20 mg. required 3.30 ml. of 0.0502 *N* alkali, therefore, neutral equivalent = 121). In attempts to find a better crystallizing solvent, all the acid was lost, except that the aqueous mother liquor yielded about 25 mg., which melted at 193–194° with darkening and evolution of gas.

Anal. Calcd. for $C_{11}H_{13}O_4N$: C, 59.10; H, 5.83; N, 6.28; mol. wt., 223. Found: C, 59.15, 58.72; H, 5.85, 5.75; N, 6.15, 6.01; mol. wt. (above titration), 242.

The isoelectric point of a small quantity of the acid obtained from another experiment was determined by electrometric titration to be *p*H 2.92.

Isolation of Dicarboxylic Acid Half-Ester.—After ether extraction, the acidified hydrolysate described above was neutralized and evaporated but it failed to yield the expected polyhydroxy nucleus. The residue was finally digested with several portions of hot absolute methanol from which, upon the addition of three to four volumes of acetone, a colorless, hygroscopic, amorphous precipitate was separated. It gave a positive test for nitrogen. The product is presumed to be the salt of the half-ester of the dicarboxylic acid described above, but it did not form a definite amide or *p*-phenylphenacyl ester.

Isolation of Polyhydroxy Nucleus of Wilfordine.—In another experiment 4 g. of wilfordine was saponified with barium hydroxide in 90% methanol, and the reaction mixture was diluted with water. The excess barium was precipitated with carbon dioxide, removed from solution, and washed with water and methanol. The washings were combined with the filtrate and the methanol was removed. The hydrolysate was acidified and continuously extracted with ether. The aqueous residue then was made alkaline and saponified a second time. The reaction mixture was adjusted to *p*H 2.92 and, after the volume had been reduced to about 5 ml., it was diluted with 100 ml. of boiling ethanol. The ethanol solution was filtered hot, and the solvent was removed under reduced pressure. The thick, sirupy residue contained nitrogen. It was dissolved in a little water, and required 53.35 ml. of 0.1059 *N* barium hydroxide (eq. to 631 mg. of dibasic acid) for neutralization to cresol red. Removal of the solvent yielded a sirupy residue containing the barium salt. This residue was digested with four portions of boiling methanol (A), which were filtered from the insoluble part (B). The residue obtained on removal of the solvent from the combined filtrates (A) was dissolved in a few milliliters of water, and just enough sulfuric acid was added to precipitate all the barium. The barium sulfate was separated and the filtrate was evaporated to dryness. The residue thus obtained crystallized when treated with methanol. The crystals were filtered and added to the crystalline fractions obtained as described below, and the methanol filtrate was evaporated. The resulting residue was digested with several portions of boiling acetone, which were filtered. The acetone-insoluble portion (1.2 g.), presumably the half-ester, was set aside. The acetone filtrates were combined, and when the solvent was removed 200 mg. of crystals was obtained.

The methanol-insoluble portion (B) was dissolved in a little water, and the barium was precipitated by the careful addition of dilute sulfuric acid. The barium sulfate was separated and the solvent was removed leaving a residue that was dissolved in a few milliliters of hot methanol and diluted with 3 volumes of acetone. The solution crystallized when cooled and the crystals (35 mg.) were separated. The solvent was removed from the mother liquor and the residue was digested with several portions of hot acetone and then set aside. The acetone solutions were combined, filtered, and reduced to a small volume from which, after cooling, an additional 15 mg. of the crystalline product was obtained.

The four portions of crystalline material were combined and recrystallized four times from equal volumes of methanol–acetone. The separated crystals weighed 170 mg. and the combined mother liquors yielded an additional 35 mg. of the same product which contained neither nitrogen nor sulfur and melted very unsharply, 245–270° (dec.), when heated in the usual manner. However, when plunged into a bath previously heated to 306° and then heated at 4 to 5° per minute, the sample melted at 309–310° with decomposition.

Anal. Calcd. for $C_{15}H_{26}O_{10}$: C, 49.18; H, 7.15. Found: C, 49.03, 49.38, 49.32; H, 7.29, 7.35, 7.05.

Summary

A crystalline, insecticidally active alkaloid has been isolated from the roots of *Tripterygium wilfordii* Hook. This compound has been designated wilfordine and it has been found to be an ester alkaloid consisting of a polyhydroxy nucleus esterified with 5 moles of acetic acid, 1 mole of benzoic acid, and 1 mole of a nitrogen-containing dicarboxylic acid.

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Khellin and Allied Compounds

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Khellin, obtained from the fruits of *Ammi visnaga* (L), has attracted interest, being an antispasmodic and a coronary vasodilator of promise, in the treatment[1] of angina pectoris and bronchial asthma. Comparatively little is known about the chemistry of khellin,[2] its derivatives and analogs. The aim of this paper is to fill this gap; by the preparation of a number of such derivatives, a study of the relationship between chemical constitution and physiological activity was rendered possible.

Khellin (IIIb) being a chromone derivative with a methyl group in position 2, might be expected to condense with aromatic aldehydes as do other 2-methylchromones.[3] This is indeed the case; thus the action of anisaldehyde and sodium alcoholate lead to the formation of the yellow 2-(*p*-methoxybenzylidene)-khellin (IIIf). Khellin gave an orange perchlorate.

(1) Anrep, Barsoum, Kenawy and Misrahy, *British Heart J.*, **8**, 171 (1946); *Lancet*, **I**, 557 (1947).

(2) Mustapha, *Compt. Rend. Acad. Sci., Paris*, **89**, 442 (1879); Fantl and Salem, *Biochem. Z.*, **226**, 166 (1930); Späth and Gruber, *Ber.*, **71**, 106 (1938).

(3) Heilbron, Barnes and Morton, *J. Chem. Soc.*, **123**, 2559 (1923).