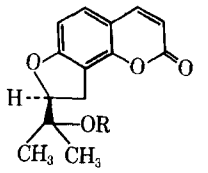
Coumarins VI

A Revised Structure of Columbianin and Pertinent Mass Spectral Studies

By M. SHIPCHANDLER and T. O. SOINE

The previous erroneous assignment of the structure of columbianin as a D-glucoside of columbianetin has been revised to indicate that it is a β-D-gentiobioside. The mass spectral characteristics of columbianin, its acetate, the true β-D-glucopyranoside of columbianetin, and its acetate have been examined and probable fragmentation pathways proposed.

THE ISOLATION of a new coumarin from the umbelliferous plant, *Lomatium columbianum* Mathias and Const., was reported in 1964 (1). The name assigned to this coumarin, a glycoside, was *columbianin* and it subsequently has been shown to be present in *L. dissectum* var. *multifidum* (Nutt.) Mathias and Const. (2) as well as in *L. nuttallii* (A. Gray) Macbr. (3). Upon acid hydrolysis, columbianin yielded D-glucose and a tertiary alcohol, columbianetin (I). Various chemical and spectral studies (1) indicated the validity of the structure assigned to I which has since been confirmed by Nielsen and Lemmich (4) who extended their studies (5) to show that an 8(S) configuration could be assigned as well. The erroneous assignment of a D-glucoside structure (II) for columbianin was based on the fact that only D-glucose and I were obtained from acid hydrolysis and that the elemental analyses obtained for both the glycoside and its acetate could reasonably have been interpreted as dihydrates, the originally proposed formulase. More recently, an examination of the nuclear magnetic



R	
I	—H
II	—D-Glucosyl
III	—β-D-Gentiobiosyl
IV	—β-D-Glucopyranosyl

resonance (NMR) spectrum of the acetate of columbianin showed that it was inconsistent with the proposed structure and, therefore, rendered untenable the structure of the parent glycoside.[1]

Specifically, the spectrum of the glycosidic acetate showed the *O*-acetyl methyls appearing as four peaks at 7.92, 8.00, 8.04, and 8.07 τ and the *gem*-dimethyls of the columbianetin moiety at 8.69 τ. The denouement, however, was the surprising peak ratio of 3.52:1 between the *O*-acetyl methyls and the *gem*-dimethyls since a ratio of 2:1 was expected. Additional analytical and degradative studies were indicated by this finding.

An ebullioscopic determination on the acetate, while not necessarily definitive, suggested the molecular weight to be about 800 rather than the lower value required by the original structure. Supporting this value was the observance of a rather weak molecular ion peak at *m/e* 864 (±4) in the mass spectrum. More definitive was an *O*-acetyl value that was about 7% higher than the expected value for the dihydrate of a tetraacetate. Finally, elemental analysis of a carefully purified sample of the acetate showed close correspondence to the required $C_{40}H_{48}O_{21}$ from the mass spectral study.

These data, of course, suggested that a disaccharide moiety was present in columbianin since the acetate was obviously a heptaacetate rather than the earlier suggested tetraacetate. To identify this disaccharide moiety, columbianin was subjected to a partial hydrolysis under mild conditions using a cation-exchange resin in the acid cycle (6). The hydrolysate was examined by paper chromatography using the silver nitrate technique for carbohydrate spot detection (7). Two spots appeared, one of which was identical in mobility with D-glucose and the other having a mobility consistent with a 1 → 6 linked disaccharide. The two possibilities, isomaltose (1 → 6,α) and gentiobiose (1 → 6,β) have nearly the same R_f values. Preparative paper chromatography made possible the isolation of this disaccharide ($[\alpha]_D^{20}$ −6° [c 2.0, H_2O]). The specific rotations (8) of the two possibilities are rather far apart—*isomaltose* $[\alpha]_D^{24}$ +120° (H_2O); *gentiobiose* $[\alpha]_D^{20}$ +8.7° (H_2O)—and, therefore, it ap-

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[1] A preliminary communication covering the revised structure appeared in *J. Pharm. Sci.*, **56**, 661(1967).

pears that gentiobiose must be the disaccharide under consideration with the small differences being attributed to impurities extracted from the paper during the workup. All doubt on this score was eliminated by showing that acetylation of the disaccharide provided β-D-gentiobiose octaacetate which was identical in all respects to an authentic sample. These data prove that columbianin is a gentiobioside of I but do not clarify the configurational α- or β-linkage of the disaccharide to the aglycone.

Gentiobiose is known to occur in nature in the glycosidic form (8). A well-known example is the cyanogenetic glycoside, amygdalin, which was studied by Fischer (9). He showed that amygdalin liberates a molecule of glucose and a new glucoside when treated with an enzyme isolated from yeast extract. Very recently, the hydrolysis of amygdalin, catalyzed by β-glucosidase from almonds, has been studied by Haisman and Knight (10). The enzyme has been shown to cleave β-linked glucose units in a stepwise manner from the nonreducing end of the sugar chain (10, 11). Amygdalin is hydrolyzed in three steps, the last being the hydrolysis of the cyanohydrin to benzaldehyde and HCN. In a similar manner, if columbianin is a β-gentiobioside, it should be hydrolyzed in two steps and no gentiobiose should be observed in the hydrolysate as opposed to acid hydrolysis where the cleavage is random and gentiobiose is observed (10). In considering whether the disaccharide unit is bonded to I by an α- or β-linkage it would be expected that enzymatic hydrolysis with β-glucosidase would stop after the first step if columbianin is an α-gentiobioside. On the other hand, if columbianin is a β-gentiobioside, it would proceed to I, albeit slowly, since tertiary alkyl β-glucosides are known to hydrolyze slowly (12). Experiment showed that columbianin liberated the aglycone in good yield when

$$\text{columbianin} \xrightarrow{\text{fast}} \text{glucose} + \text{monosaccharidic coumarin} \xrightarrow{\text{slow}} \text{glucose} + \text{aglycone (I)}$$

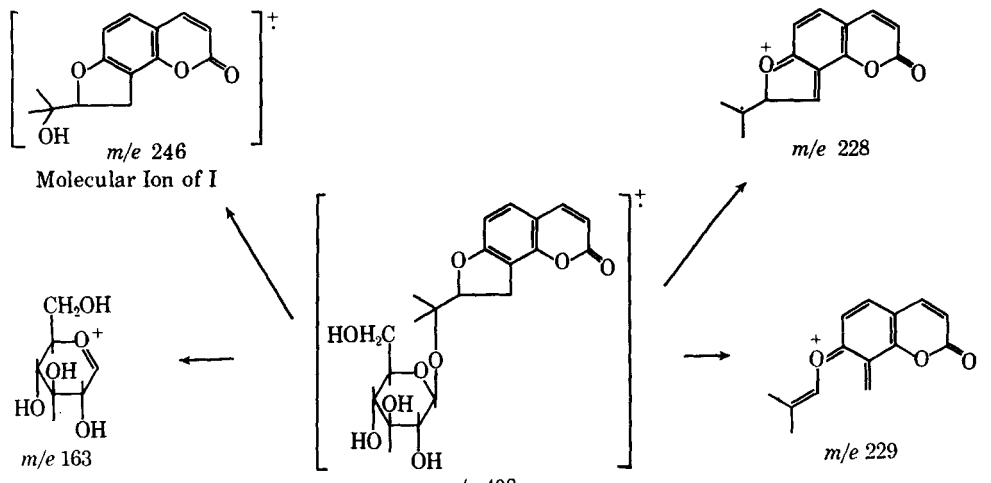
hydrolyzed with β-glucosidase in a prolonged experiment, paper chromatograms showing no gentiobiose but an abundance of glucose. However, paper and thin-layer silica gel chromatograms showed a fluorescent spot having a lesser polarity than the original glycoside but having a greater polarity than I. This material has been isolated in fairly good yield by the use of silica gel and preparative paper and thin-layer chromatography on an enzymatic hydrolysate that had been allowed to run for only a limited amount of time. The isolated compound did not exhibit a definite melting point but did give a small molecular ion peak in the mass spectrum corresponding to *m/e* 408 (*i.e.*, $C_{20}H_{24}O_9$). It provided a tetraacetate, m.p. 163.5–164.5°, in which the ratio of *O*-acetyl methyl peaks and *gem*-dimethyl peaks in the NMR spectrum was 2:1 as expected. The acetyl value as well as the elemental analysis was consistent with the $C_{28}H_{32}O_{13}$ formula of a tetraacetate. The acetate showed a fairly strong molecular ion peak at *m/e* 570 which was in accord with the assigned formula. The enzymatic study of columbianin proves conclusively that columbianin is the β-D-gentiobioside (III) of I. The name, columbianetin β-D-glucopyranoside, is assigned to the monosaccharidic coumarin since all of the analytical and spectral data are consistent with the structure (IV) assigned to it.

Mass Spectral Considerations—The mass spectrum of IV retains almost all of the features characterizing the fragmentation of I (13). The principal fragmentations of the molecular ion have been described in Scheme I[2] whereas the abundance ratios of the ions have been shown in Fig. 1. A peak at *m/e* 246 is probably the molecular ion of I arising from rearrangement of the molecular ion of IV, the probable mechanism being shown in Scheme II. Hence, it is expected that all of the peaks found in the mass spectrum of I will also be found in the present case, assuming that the ion arising by direct electron impact on I is similar to the one arising by the fragmentation shown in Scheme II. The fragments shown in Scheme I would follow the same fragmentation pathways as described previously (13). Many of these pathways have been supported in our earlier paper (13) by the appearance of the corresponding metastable ion peaks.

The peak at *m/e* 229 is very conspicuous as compared to the corresponding peak in the mass spectrum of I. This is probably due to the increased stability of the glucosyl radical as against the hydroxyl radical. Furthermore, the peak at *m/e* 229 is quite conspicuous in the spectra of the esters of I (13) and may, probably, be ascribed to being due to a similar reason.

The ion of *m/e* 188 could either be generated from the ion of *m/e* 246 (*i.e.*, the molecular ion of I) or could arise directly from the molecular ion of IV or both. This ion could then fragment by two routes as in the case of I, *i.e.*, 188 → 187 → 159 → 131 → 103 → 77 and 188 → 160 → 132 with the latter route being of relatively minor consequence.

[2] The ion structures given are not necessarily accurate representations, being used primarily as a convenient means for showing the fragmentation pathways. Further fragmentation pathways for the coumarinic ions have been discussed in the previous paper.



Scheme I

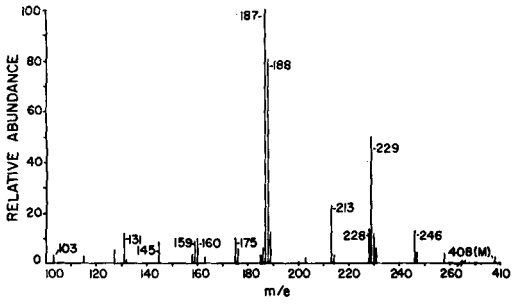


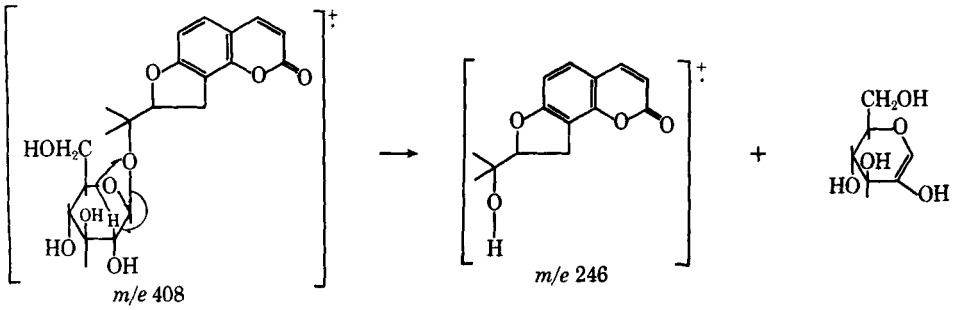
Fig. 1—Mass spectrum of IV. Peaks of abundance ratio less than 3% have been omitted in all spectra unless a reference has been made to them in text. Numbers in all figures correspond to those cited in the text.

The ion of *m/e* 187 forms the base peak which, in addition, could arise by two more routes, *i.e.*, M → 246 → 231 → 187 and M → 229 → 187. The transformation 229 → 187 is supported by the appearance of a metastable peak at *m/e* 152.8. A pathway of minor consequence is 231 → 203 → 175.

The ion of *m/e* 228 arises from the molecular ion by loss of a molecule of glucose. A mechanism similar to that proposed in the case of I for its formation (13) can be proposed in this case also. The ion fragments by the route 228 → 213 → 185.

In addition to the features that can be ascribed to the coumarinic part, there are peaks which arise from the carbohydrate portion although these are of lesser consequence. Reed *et al.* (14) have studied the mass spectrum of methyl β-D-glucopyranoside and suggest that the peak at *m/e* 163 can be ascribed to an ion arising from the cleavage of the bond between the anomeric carbon and its substituent. This ion, in turn, loses H_2O to generate an ion of *m/e* 145. The latter ion has been shown to fragment into ions of *m/e* 85, 74, 71, and 60 (14).

The tetraacetate of IV shows quite a predictable mode of fragmentation (see Fig. 2) characteristic of the parent compound and also of the polyacetate derivatives of glucose. The peaks which can be said to have originated from the sugar part of the molecule are quite prominent with the peak at *m/e* 169 forming the base peak. Pearl and Darling (15) have recently studied a number of natural glucoside acetates and have found that all of these, in general, possessed a similar pattern as far as the sugar portion is con-



Scheme II

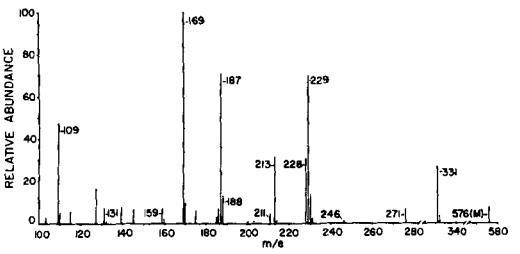


Fig. 2—Mass spectrum of the acetate of IV.

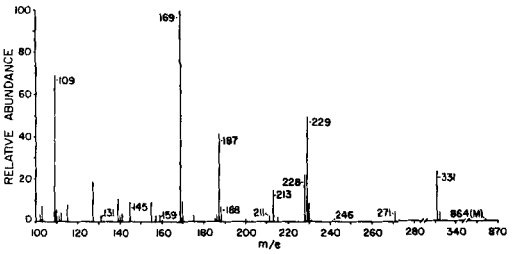
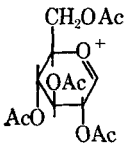


Fig. 4—Mass spectrum of the acetate of III.

cerned. Biemann *et al.* (16) have shown that the peak at *m/e* 331 in the spectra of glucose pentaacetate and the tetraacetates of methyl and phenyl glucosides is due to V, *i.e.*, the ion arising from cleavage between the anomeric carbon and its substituent. Pearl and Darling (15), likewise, found this type of ion in a number of glucoside acetates. This ion fragments by two routes, *i.e.*, 331 → 271 → 229 → 169 → 109 and 331 → 211 → 169 (16,17). Triacetyl and diacetyl oxonium ions at *m/e* 145 and 103 are also observed. One would expect to find almost all of the ion peaks in



m/e 331

V

the spectrum of the tetraacetate as are found in the spectrum of IV as far as fragments arising from the coumarinic portion of the molecule are concerned.

Columbianin shows almost all the principal features of IV in the mass spectrum (see Fig. 3), the difference being in the peak ratios. The base peak appears at *m/e* 187 just as in the mass spectrum of IV. We failed, however, to observe a molecular ion peak.

The mass spectrum of the heptaacetate of III (see Fig. 4) resembles that of the tetraacetate of IV, once again the differences being mainly in the abundance ratios. The ion of *m/e* 169 forms the base peak. The molecular ion peak, however, is rather weak.

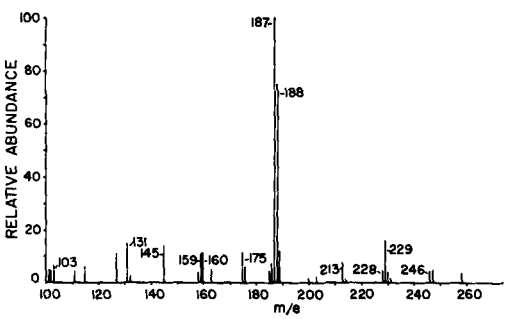


Fig. 3—Mass spectrum of III.

EXPERIMENTAL

Melting points were determined in capillary tubes in a Thomas-Hoover melting point apparatus and are uncorrected.

Infrared spectra were determined on a Perkin-Elmer 237B grating infrared spectrophotometer.

Values of $[\alpha]$ were determined on a Perkin-Elmer 141 polarimeter.

Nuclear magnetic resonance (NMR) spectra were determined on a Varian Associates A-60 instrument in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard.

Mass spectra were obtained by Mr. A. R. Swanson and Mr. R. D. Berg, School of Chemistry, University of Minnesota, employing a Hitachi Perkin-Elmer RMU-6D mass spectrometer. The instrument was operated with a source temperature of 250° and an ionizing voltage of 50 ev. The sample inlet temperature was 300° in the case of IV, 250° for the tetraacetate of IV, 350° in the case of III, and 380° for the heptaacetate of III.

Silica gel for column chromatography refers to Baker Analyst No. 3405 and silica gel for thin-layer chromatography refers to "Chromagram" sheets supplied by Distillation Products Industries, Rochester, N. Y.

Columbianin—The product isolated during the studies of Willette and Soine (1) in these laboratories was employed.

Columbian Acetate—The acetate of columbianin was prepared in the manner previously described (1) and was crystallized from chloroform–cyclohexane, m.p. 218–219°, undepressed on admixture with the authentic acetate.

Anal.[3]—Calcd. for $C_{40}H_{48}O_{21}$: C, 55.55; H, 5.59; mol. wt., 864; *O*-acetyl, 34.84. Found: C, 55.63; H, 5.53; mol. wt. (ebullioscopic), 800; *O*-acetyl, 34.92.

Partial Hydrolysis of Columbianin—Columbianin (20 mg.) was dissolved in 0.5 ml. of water in a small test tube and to this was added 40 mg. of cation-exchange resin in the acid cycle[4] and the mixture placed into a boiling water bath for 1 hr. The

[3] Microanalyses were determined by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis, or by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

[4] Marketed as Amberlite IR-120 by Rohm and Haas Co., Philadelphia, Pa., and converted to the acid cycle with 10% sulfuric acid.

hydrolysate was spotted on standard size Whatman paper No. 1 together with authentic samples of glucose, isomaltose, maltose, and gentiobiose. The paper was developed by the descending chromatographic technique using a pyridine–ethyl acetate–water (2:5:7, upper layer) mobile phase for about 16 hr. The paper was dried and then developed with silver nitrate in acetone in the usual manner (7). Two spots appeared, one of which had moved the same distance as glucose and another which had moved the same distance as isomaltose and gentiobiose, the latter two having traveled almost identically.

Isolation of Gentiobiose—Columbianin (400 mg.) was dissolved in 10 ml. of water and 800 mg. cation-exchange resin in the acid cycle was then added and the reaction mixture heated in a boiling water bath for 5.5 hr. The water lost by evaporation was replaced from time to time. After the end of the heating period the hydrolysate was filtered and the filtrate streaked on standard length Whatman paper No. 3 and developed in the manner described above. After drying, narrow strips were cut from each side of the paper and developed with silver nitrate to locate the zone containing the disaccharide. This zone was cut out, divided into small pieces, and eluted with water. Removal of the water under vacuum followed by drying gave several mg. of the crude disaccharide $\{[\alpha]_D^{20} -6°$ (c 2.0, $H_2O)\}$. The crude product was acetylated with acetic anhydride and sodium acetate in the usual manner to give 30 mg. of β-D-gentiobiose octaacetate, m.p. 191–192°; $[\alpha]_D^{20} -1.86°$ (c 0.64, $CHCl_3$) {lit., m.p. 193°; $[\alpha]_D^{19.5} -5°$ (c 1.8, $CHCl_3$) (8)}. The melting point was not depressed on admixture with an authentic sample of β-D-gentiobiose octaacetate and the infrared spectra were identical.

Isolation of the Aglycone from the Enzymatic Hydrolysis—Columbianin (200 mg.) was dissolved in 20 ml. of water and the aid of heat and to the cooled solution was added 100 mg. of β-glucosidase.[5] The mixture was slowly stirred at room temperature for 56 hr. at which time it was boiled for a few minutes and, after cooling, treated with another 100 mg. of enzyme followed by an additional 36 hr. of stirring. Silica gel thin-layer chromatographic examination in the beginning showed blue fluorescent coumarinic spots when developed with chloroform–ethanol–ethyl acetate (2:3:10) and examined under ultraviolet light. The R_f values of the three principal spots were: 0.07, 0.37, and 0.66 with the first (R_f = 0.07) corresponding to columbianin and the last to columbianetin. Presumably, the spot of intermediate R_f value was an intermediate product of hydrolysis. Paper-chromatographic examination using the pyridine–ethyl acetate–water system previously employed also showed three blue fluorescent spots under ultraviolet light. However, development of the paper chromatogram of the hydrolysate using the silver nitrate technique failed to show the presence of gentiobiose although a spot corresponding to glucose was observed. As the hydrolysis proceeded, the spot corresponding to columbianin slowly lost its fluorescence, whereas the spot corresponding to columbianetin gained in brilliance. When the hydrolysis was terminated the hydrolysate showed only a spot corresponding to columbianetin (R_f = 0.66) and the spot of intermediate polarity (R_f = 0.37). The hydrolysate was extracted with ether until a portion of the ether failed to fluoresce under ultraviolet light when spotted on filter paper. The ether extract was dried with anhydrous sodium sulfate, stripped of solvent, and crystallized to provide 35 mg. (40% of theory) of columbianetin, m.p. 160–161°, which failed to depress the melting point of an authentic sample with which it showed an identical infrared spectrum.

Isolation of Columbianetin β-D-Glucopyranoside—Columbianin (500 mg.) was dissolved in 25 ml. of water with the aid of heat and, after cooling, 100 mg. of β-glucosidase was added. The mixture was stirred for 12 hr. at room temperature at which time thin-layer chromatographic examination showed only traces of unhydrolyzed columbianin, only a small amount of columbianetin, and the major part of the fluorescence in the spot of intermediate polarity. The hydrolysis was then stopped by heating the reaction mixture on a steam bath for several minutes. The isolation, in several runs, was carried out by three different chromatographic techniques. Preparative paper or thin-layer chromatographic procedures employing the previously described solvent systems were employed effectively followed by final elution of the product with methanol. Column chromatography employing silica gel activated at 110° and impregnated with 5% of water appeared to be quite satisfactory also. Elution with ethyl acetate–chloroform (1:1) yields the aglycone and, subsequently, elution with ethyl acetate provides the compound of intermediate polarity (R_f = 0.37) which can be crystallized with difficulty from ethyl acetate–cyclohexane. This compound does not have a definite melting point, softening at *ca* 117–120°, honeycombing at about 130°, and finally becoming transparent at about 180°; $[\alpha]_D^{18.5}$ +206 (c 1.0, CH_3OH). Because of the indefinite melting point and difficulty in crystallization, this compound was analyzed as the acetate derivative.

Columbianetin β-D-Glucopyranoside Acetate—Preparation of the acetate in the usual manner employing acetic anhydride and sodium acetate yielded white crystals from a mixture of ethyl acetate and cyclohexane. Since solvation of the product with cyclohexane was evident from the NMR it was dried at 85° for several hours in vacuum to give a product with m.p. 163.5–164.5°; $[\alpha]_D^{19.5}$ +146° (c 1.0, $CHCl_3$).

Anal.—Calcd. for $C_{28}H_{32}O_{13}$: C, 58.33; H, 5.59; *O*-acetyl, 29.86. Found: C, 58.33; H, 5.73; *O*-acetyl, 29.60.

[5] Product of General Biochemicals, Chagrin Falls, Ohio.

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Keyphrases

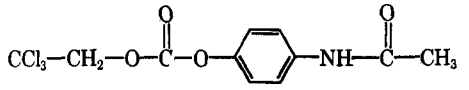
Coumarins
Columbianin structure—revised
Column chromatography—separation
IR spectrophotometry—structure
Mass spectrometry—structure
Paper chromatography—identity
TLC—identity
NMR spectrometry—identity
Polarimetry—identity

4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate Synthesis, Physical Properties, and *In Vitro* Hydrolysis

By J. V. SWINTOSKY*, H. C. CALDWELL, C. W. CHONG, G. M. IRWIN, and L. W. DITTERT*

A trichloroethyl carbonate prodrug ester of acetaminophen has been made by reacting acetaminophen with trichloroethyl chloroformate. It is a virtually tasteless crystalline compound retaining the pharmacologic effects of acetaminophen and trichloroethanol. It is lipophilic; its solubility in water is less than 0.05%. It undergoes base-catalyzed hydrolysis, and at pH 7.4 has a half-life in water solution of about 7 hr. Its stability in the solid state makes this compound amenable to use in dosage forms such as tablets and capsules. Hydrolysis of this compound is not catalyzed by human gastric fluid, but it is catalyzed by human intestinal fluid, rat intestinal mucosa, rat plasma, rat liver, and human plasma. It was surmised, therefore, that following oral administration to animals, a portion of the prodrug may be absorbed intact, but some cleavage may occur before and during absorption from the intestines. Furthermore, since enzymes that hydrolyze it are prevalent in plasma and liver, the prodrug on entering the blood will be hydrolyzed very rapidly.

THIS PAPER deals primarily with the preparation and *in vitro* hydrolysis characteristics of a new prodrug (1, 2) form of acetaminophen and trichloroethanol possessing the following structure:



4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate (ATC)

Albert (3) has used the term "prodrug" to describe compounds which undergo biotransformation prior to exhibiting their pharmacologic effects. Harper (4) has used the term "drug latentiation" to describe chemical modification of a biologically active compound to form a new compound which upon *in vitro* enzymatic attack will liberate the parent compound.

During the past few decades there have been numerous *in vitro* studies describing hydrolysis of esters using serum and other body tissues as enzyme sources. Glick (5) measured the enzymatic hydrolysis of a variety of esters including those of choline. Related studies were undertaken by Adams and Whittaker (6) and Alles and Hawes (7). Levine and Clark (8) studied the relationship between structure and *in vitro* hydrolysis of various esters and amides in human serum. Frazer (9) performed hydrolysis studies on succinylcholine esters using cholinesterases. Other studies of these types have been reported

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