TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASES FROM PIGEON LIVER AND *E. coli* B

No.	Reversible Inhibition, K_i (M)[a] Pigeon Liver	Reversible Inhibition, K_i (M)[a] *E. coli* B	Irreversible Inhibition[b] Pigeon Liver μM Concn. Inhib.	Pigeon Liver μM Concn. TPNH	Pigeon Liver % $E\cdots I$	Pigeon Liver Time, min.	Pigeon Liver % Inactivation	*E. coli* B μM Concn. Inhib.	*E. coli* B μM Concn. TPNH	*E. coli* B % $E\cdots I$	*E. coli* B Time, min.	*E. coli* B % Inactivation
I[c]	3×10^{-5}[d]	1×10^{-3}	40	0	57	12	50[d]	100	0	10	120	59
II[c]	4×10^{-5}[d]	5×10^{-4}	40	0	50	12	50[d]	50	0	10	120	22
III	7×10^{-9}[e]	1×10^{-7}	0.01	12[f]	59	60	0[e]	0.10	30[f]	50	120	0
IV	1×10^{-7}[e]	1×10^{-7}	0.25	12[f]	72	60	0[e]	0.10	30[f]	50	120	33

The technical assistance of Barbara Baine with these assays is acknowledged. [a] K_i was estimated (18) from the concentration of inhibitor necessary to give 50% inhibition in the presence of 6 μM dihydrofolate as previously described (10); K_m = 2.5×10^{-5} M was used for *E. coli* B (20) and 1×10^{-6} M for pigeon liver (18). [b] Inactivation at 37° and pH 7.4 was measured as previously described (21); for the assay of remaining *E. coli* enzyme, 60 μM each of dihydrofolate and TPNH were used to make the rate of reaction dependent on enzyme concentration. [c] All assays contained 10% *N,N*-dimethylformamide to aid solubility. [d] Data from *Reference 19*. [e] Data from *Reference 22*. [f] TPNH is required for optimum reversible complexing of 2,4-diaminoheterocycles, but not for 2-amino-4-hydroxy heterocycles; see Perkins, J. P., and Bertino, J. R., *Biochemistry*, **5**, 1005(1966).

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Received January 9, 1967.
Accepted for publication March 1, 1967.

This work was supported in part by grant CA-08695 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md.
J. H. Jordaan wishes to thank the Atomic Energy Board, Republic of South Africa, for a fellowship.
Previous paper: Baker, B. R., and Lourens, G. J., *J. Pharm. Sci.*, to be published.

Revised Structure of Columbianin

Sir:

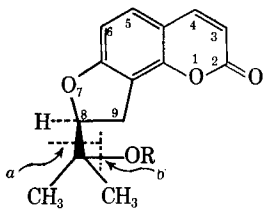
Columbianin is a naturally-occurring glycosidic coumarin isolated from *Lomatium columbianum* Math. and Const. (1) and from *L. dissectum* var. *multifidum* (Nutt.) Math. and Const. (2). Willette and Soine (1) suggested structure I for columbianin on the basis that, on acid hydrolysis, it yielded D-glucose and a tertiary alcohol (*i.e.*, columbianetin). The structure of the aglycone (II) was firmly established by spectral studies as well as by chemical degradative evidence and has since then been confirmed (3) and extended (4) by Nielsen and Lemmich to show that the absolute configuration is 8(*S*). Since no quantitative estimation of the D-glucose content was made in the earlier study and because the analytical data (*i.e.*, assuming water of crystallization) corresponded within limits for I and its tetraacetate, the structure assignment was considered adequate.

Recently, in connection with another study, we had occasion to examine the nuclear magnetic resonance (NMR) spectrum of acetylated columbianin and noted that the *O*-acetyl methyls were to be observed as four peaks at τ 7.92, 8.00, 8.04, and 8.07; whereas the *gem*-dimethyls of columbianetin were found as a singlet at τ 8.69. To our surprise, the peak ratio between the *O*-acetyl methyls and the *gem*-dimethyls was found to be 3.52:1 instead of the expected 2:1. This led us to re-examine the structural assignment for columbianin by means of additional analytical data as well as by further degradative studies.

An ebullioscopic determination on the acetate suggested the molecular weight to be about 800 and an acetyl analysis indicated 34.92%, both values being incompatible with the tetraacetate formulation. On the other hand, the results pointed strongly toward a disaccharide moiety in columbianin and a heptaacetate as the acetylated form. Furthermore, additional elemental analyses on the acetate derivative gave much closer correspondence with the values expected for $C_{40}H_{48}O_{21}$ than for the previously suggested $C_{28}H_{32}O_{13} \cdot 2H_2O$. The question of the presence of sugars other than D-glucose had been ruled out in the earlier work (1).

Partial hydrolysis of the glycoside was carried out using an ion exchange resin[1] in the acid cycle (5). Examination of the hydrolysate by paper chromatography was carried out using an ethyl acetate–pyridine–water mobile phase and developing the chromatogram by using the silver nitrate technique (6) for visualization of the

[1] Marketed as Amberlite IR-120 by Rohm and Haas Co., Philadelphia, Pa.



I, R=—D-Glucosyl
II, R=—H
III, R=—β-D-Gentiobiosyl

carbohydrate spots. Two spots were observed with one of these being identical to D-glucose in its R_f value. The R_f value of the other spot was consistent with that for disaccharides possessing a 1→6 linkage. Thus, the possibilities, virtually indistinguishable by paper chromatography, were isomaltose (α-linkage) and gentiobiose (β-linkage). The same partial hydrolysis carried out on a preparative scale led to the isolation of several milligrams of the crude disaccharide, $[\alpha]_D^{20}$ −6° (c 2.00, H_2O). Since the specific rotations (7) of the two possibilities are rather far apart—isomaltose, $[\alpha]_D^{24}$ +120° (H_2O); gentiobiose $[\alpha]_D^{20}$ +8.7° (H_2O)—the results strongly suggested gentiobiose as the disaccharide, with the small discrepancy probably being accounted for by extraction of impurities from the paper. Acetylation of the disaccharide provided gentiobiose octaacetate, m.p. 191–192°, $[\alpha]_D^{20}$ −1.86° (c 0.64, $CHCl_3$), identical with the authentic material m.p. 193°, $[\alpha]_D^{19.5}$ −5° (c 1.8, $CHCl_3$) (8) as shown by mixed melting point determination and comparison of infrared spectra.[2] These data indicate conclusively that columbianin is a gentiobioside of columbianetin. The question of whether the gentiobiose moiety is attached by an α- or β-linkage to the aglycone was examined by subjecting the glycoside to the action of β-glucosidase.[3] Enzymatic cleavage takes place stepwise from the nonreducing end of the molecule (9) and, in this case, one would expect to find D-glucose, a new monosaccharidic glucoside, and columbianetin as the products of hydrolysis if the linkage were β. On the other hand, if the glycosidic linkage were α, no columbianetin would be observed although the other two products would be expected. Paper and thin-layer chromatographic examination of the hydrolytic products showed no gentiobiose but did show spots corresponding to glucose and columbianetin (fluorescent) as well as a new fluorescent spot with chromatographic behavior indicating a lesser polarity than that of the original glucoside. This material has been isolated in a crystalline state with an indefinite melting point following softening at about 100° with slow effervescence, a behavior not uncommon to glycosides. Examination of this product by mass spectrometry confirmed the molecular weight of 408 for the $C_{20}H_{24}O_9$ monosaccharidic coumarin by virtue of the molecular ion. Furthermore, two major fragmentation peaks at *m/e* 187 and 229 are, very likely, representative of the coumarin portion of the molecule and represent $[C_{11}H_7O_3]^+$ and $[C_{14}H_{13}O_3]^+$ arising from fragmentation at *a* and *b*, respectively, in I. Complete characterization of this compound is being carried out presently and will be reported at a later date. Columbianetin was also obtained in a crystalline form and identified by mixed melting point and spectral comparison with authentic material. The fact that enzymatic cleavage has been accomplished to yield columbianetin as a final product identifies a β-linkage to the aglycone and, therefore, the structure of columbianin may be assigned as the β-gentiobioside of columbianetin (III).

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Received February 3, 1967.
Accepted for publication March 22, 1967.

This investigation was supported by grant No. HE-07101 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

[2] The authors acknowledge with gratitude the assistance of Dr. B. A. Lewis, Biochemistry Department, St. Paul Campus, University of Minnesota, St. Paul, in providing valuable guidance and authentic samples for comparison.
[3] Product of General Biochemicals, Chagrin Falls, Ohio.