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Communication

An Assessment of the Rubisco Inhibitor

2-Carboxyarabinitol-1-Phosphate and D-Hamamelonic Acid 2¹-Phosphate Are Identical Compounds

Erwin Beck*, Renate Scheibe, and Josef Reiner

Lehrstuhl Pflanzenphysiologie (E.B., R.S.) *and Lehrstuhl Organische Chemie I* (J.R.), *Universität Bayreuth, D 8580 Bayreuth, Federal Republic of Germany*

ABSTRACT

2-Carboxyarabinitol-1-phosphate, the nocturnal inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase is identical with D-hamamelonic acid-2¹-phosphate. Reasoning is based on theoretical considerations as well as on mass spectra and ¹H- and ¹³C-NMR spectra of the phosphate-free compounds. D-Hamamelonic acid-2¹-phosphate is interpreted as a metabolic derivative of D-hamamelose-2¹,5-bisphosphate which originates in the chloroplast from fructose-1,6-bisphosphate. A simple method for the synthesis of the inhibitor is suggested.

PROOF OF IDENTITY OF 2-CARBOXYARABINITOL AND D-HAMAMELONIC ACID

A phosphorylated low mol wt carbohydrate has recently been purified from leaves of potato (7, 8), bean (4), and tobacco (14) which proved to be a potent inhibitor of Rubisco[1] in the dark or under low light intensities (9). The compound isolated from potato and bean leaves was identified as 2CA1P (4, 7) which could be addressed as derivative of a branched-chain monosaccharide. Unfortunately, the structure of branched-chain monosaccharides is but partly covered by the rules of carbohydrate nomenclature (11) and hence misunderstanding of a compound's structure cannot completely be ruled out. Following the general principle of organic nomenclature to number the carbons of an aliphatic compound starting with the most oxidized group, and observing rules 6 and 9 of carbohydrate nomenclature, 2CA1P must be addressed as 2-C-(hydroxymethyl)-D-ribonic acid 2¹-P. A similar term, namely (2C-phosphohydroxymethyl)-D-ribonic acid, has been mentioned once (4) but has been then replaced by the commonly designation 2CA1P. According to Shafizadeh's proposal (15), the carbon of the side-chain is identified by the number of the branching carbon in the straight chain plus a superscript (which gives the number of the carbon[s] of the side chain). Admittedly, the correct term is more circumstantial than 2CA1P, but since this compound is a member of a naturally occurring family of branched-chain monosaccharides, a simple common name, *viz.* HA2¹P, should be used (for a review of those compounds, see Beck [1]).

The identity of 2CA1P and HA2¹P is not quite obvious and therefore requires substantiation. In Figure 1 both carbohydrates are shown according to their designation. In 2CA1P the straight carbon chain starts with the (phosphate carrying) hydroxymethyl group; the same configuration of the straight chain is obtained by turning the bond between C-2 and C-3 by 90° which brings the carboxyl-group into position 1, whereupon the straight chain clearly represents D-ribonic acid, the backbone of D-hamamelonic acid.

Identity of 2CA1P and HA2¹P was also experimentally established by comparison of the mass spectrum of the TMS-derivative of the latter with that of $(TMS)_6$-2CA (from Berry *et al.* [4]) and of the ¹H-NMR spectrum of D-hamamelonic acid with that reported by Gutteridge *et al.* (7) for 2CA1P and for the dephosphorylated inhibitor, respectively. Figure 2 shows both mass spectra and in addition that of the lactone which easily forms upon oxidation of D-hamamelose 2¹-P to the corresponding acid. The fragment patterns of both acids as well as the relative intensities (peak size) are perfectly identical.

Figure 3 presents the ¹H-NMR spectrum of D-hamamelonic acid and Table I shows the chemical shifts of the protons of

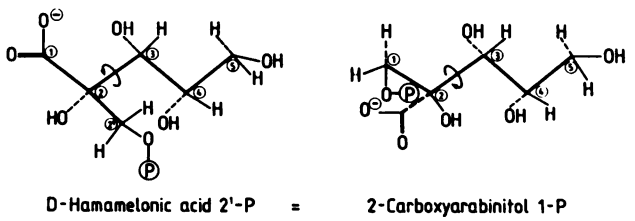

Figure 1. Structural formulas showing the interconvertibility of D-hamamelonic acid-2¹-P and 2-carboxyarabinitol-1-P. For reasons of clearness the main carbon chains are drawn linearly instead of the natural annular-like configuration. Dashed bonds lead to a plane behind that of the paper.

[1] Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; 2CA1P, 2-carboxyarabinitol-1-phosphate; FBP, fructose-1,6-bisphosphate; HA2¹P, D-hamamelonic acid-2¹-phosphate; HBP, D-hamamelose-2¹,5-bisphosphate; MSTFA, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide; TMS, trimethylsilyl

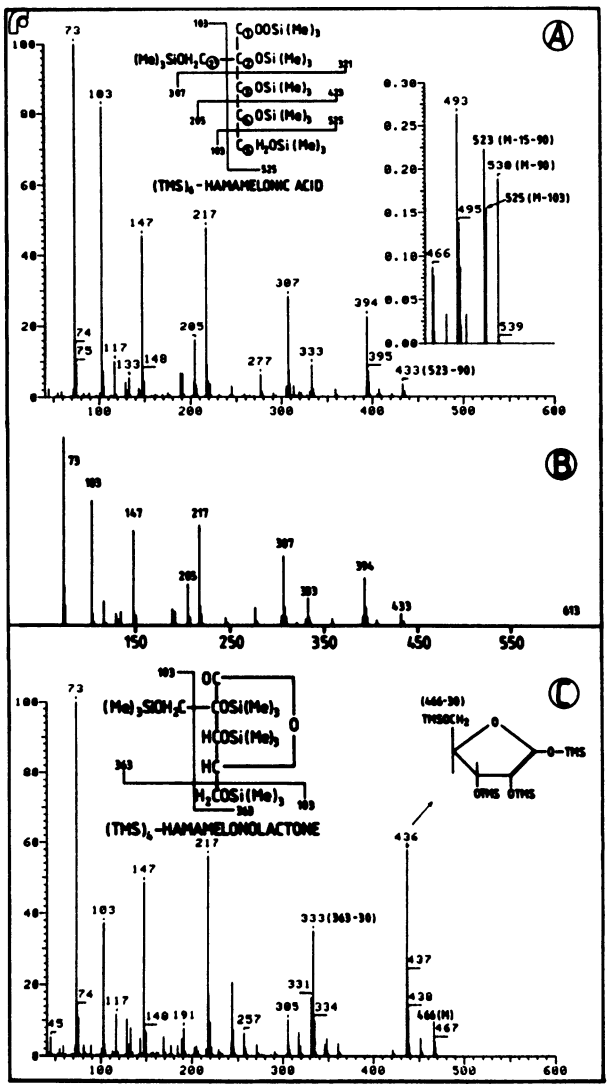

Figure 2. Mass spectrum and interpretation of the fragments of the TMS-derivatives of D-hamamelonic acid (A) and of the dephosphorylated Rubisco-inhibitor (B, from Berry *et al.* [4]), respectively. C, Mass spectrum of the corresponding TMS-derivative of D-hamamelonolactone. Hamamelonic acid was prepared by hypoiodite oxidation of crystalline D-hamamelose (6). A mixture of D-hamamelonic acid and its lactone was silylated with MSTFA in THF and separated by GC on a 30-m glass capillary column (OV 101). The mass spectra were produced with a Finnigan MAT 312 MS system.

D-hamamelonic acid (and its lactone) in comparison to those published by Gutteridge *et al.* (7) for 2-carboxyarabinitol and its diastereoisomer 2-carboxyribitol. Again, the agreement of the data of 2-carboxyarabinitol and D-hamamelonic acid is excellent. The patterns of the coupling constant corroborate the structure of a hydroxymethyl-branched pentonic acid. The ^{1}H-NMR-spectra of the inhibitor, as published by Gutteridge *et al.* (7, 8), are not directly comparable to those of the dephosphorylated compound because the phosphate group strongly interferes with the protons, especially with those at C-2^1.

A third line of experimental evidence for the identity of 2CA1P and HA2^{1}P was obtained from the ^{13}C-chemical shifts of the carbons (Table II). Since the structure of hamamelonic acid is known, the carbons could be assigned to the resonance signals of the J-modulated ^{13}C-spectrum which allow differentiation between quarternary carbons, CH_2-groups and CH-groups, respectively. Comparison of the resonances of the open chain potassium salt and the lactone showed a pronounced shift of the signal of a CH group (from 71.59–84.13). This shift is conceivable from binding the oxygen to the carbonyl group and since there are only two CH-signals (C3 and C4, respectively) the resonance signal reflecting the greatest variance between acid and lactone must be assigned to C4 and hence evidences the presence of the γ-lactone. The smaller shift of the other CH signal (C3) toward a higher field (from 73.78–67.85) indicates a decrease of the effect of the vicinal hydroxyl at C4 upon lactone formation. The quarternary C2 was identified by the smallness and the direction of the signal. Both primary carbons (C2^1 and C5, respectively) require a substantial higher field for resonance and hence could easily be differentiated from C2. Since C5 appears to be shielded more efficiently than C2^1 the smaller ^{13}C-chemical shift (62.85 ppm, acid, and 60.34, lactone, respectively) must be assigned to it. Comparison of the ^{13}C-chemical shift data of hamamelonic acid with those reported for 2CA1P (4) and 2CABP (10), each in the lactone form, showed good agreement. Smaller deviations must be ascribed to the influence of the phosphate groups on the resonances of the carbons to which they are attached. Table II shows that, on average, a shift of approximately 4 ppm toward lower field results from phosphate ester formation.

NATURAL CHLOROPLAST CONSTITUENTS RELATED TO HAMAMELONIC ACID 2^1-P

The purpose of this assessment, however, is not only to stress and prove the chemically correct designation of the Rubisco-inhibitor but also to comment on its biogenetic provenance. The branched-chain hexose skeleton underlying the structure of the inhibitor arises by an intramolecular rearrangement of FBP yielding HBP (5). This reaction takes place in the chloroplast and an equilibrium between HBP and FBP at a ratio of approximately of 1:10 has been found (1). With leaves of a primrose, (*Primula clusiana* Tausch) metabolic dephosphorylation of HBP has been demonstrated resulting in a mixture of hamamelose-2^1- and -5-P as well as of free hamamelose (5). The formation of hamamelose monoP has not yet been shown to occur in isolated chloroplasts. However, with isolated chloroplasts the fraction of the sugar monoP has never been checked for hamamelose after a substantial dark period.

Conceivably, HA2^{1}P could be produced from HBP by a specific phosphatase and a dehydrogenase. With respect to that dehydrogenation step, the recently reported reductive inactivation of the inhibitor (12) is of particular interest.

PREPARATION OF G-^{14}C-HA2^{1}P

2CA1P has been prepared by cyanohydrin synthesis with ribulose-1,5-bisP, nonselective partial dephosphorylation, separation from the ineffective diastereoisomer (D-epihamamelonic acid-2^1-P) and 5-phosphate by affinity binding to Rubisco and destruction of the enzyme-inhibitor complex (4). A simpler procedure which in particular is recommendable

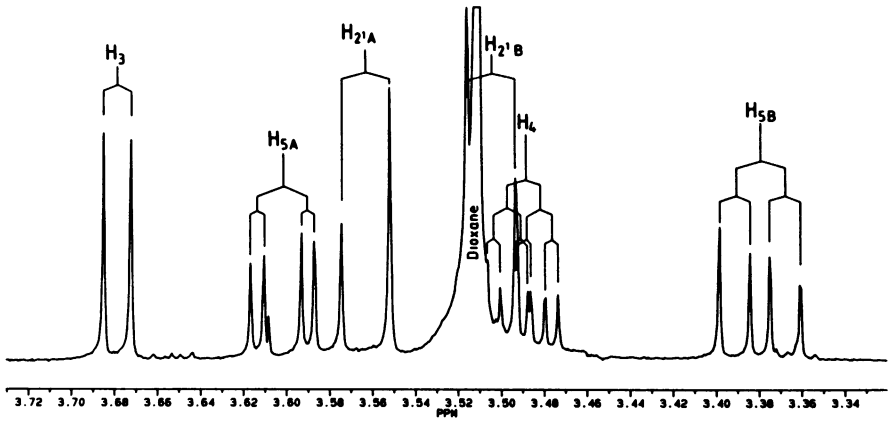

Figure 3. ^{1}H-NMR-spectrum of D-hamamelonic acid and assignment of the resonance peaks to the individual H-atoms. The spectrum was obtained with a Bruker AM 500 instrument (500 MHz).

Table I. *Chemical Shifts of Protons in ppm Relative to Dioxane (3.51 ppm) and ^{1}H-Coupling Constants (J; Hz) of D-hamamelonic Acid and Its Lactone as Compared to ^{1}H-Chemical Shift Data of*

2-carboxyarabinitol and 2-carboxyribitol

Compound	$H2_A{}^1$	$H2_B{}^1$	H3	H4	$H5_A$	$H5_B$
D-Hamamelonic acid	3.56	3.50	3.68	3.47–3.51	3.60	3.40
	d	d	d	m	dd	dd
	$J^1_{2A/2B} = 11.3$		$J3/4 = 6.4$		$J_{5A/5B} = 11.7$	
					$J_{4/5A} = 3.1$	$J_{4/5B} = 7.1$
2-Carboxyarabinitol[a]	3.57	3.53	3.70	3.51	3.63	3.40
2-Carboxyribitol[a]	3.49–3.55		3.49–3.55	3.37	3.49–3.55	
D-Hamamelono-γ-lactone	3.76	3.65	4.31	4.44	3.99	3.77
	d	d	d	m	dd	dd
	$J^1_{2A/2B} = 11.7$		$J_{3/4} = 7.5$		$J_{5A/5B} = 13.2$	
					$J_{4/5A} = 2.6$	$J_{4/5B} = 5.1$

[a] Data from Gutteridge *et al.* (7).

Table II. *^{13}C-NMR Chemical Shifts (ppm) of the Carbons of Hamamelonic Acid and Hamamelono-γ-Lactone (dissolved in D_2O) as Compared to Those of the γ-Lactones of 2CA1P and 2CABP*

The chemical shifts were related to that of dioxane = 67.3 ppm. The spectra were obtained with a Bruker AM 500 spectrometer (125.7 MHz).

Carbon	Compound						
	Hamamelonic Acid, K-salt	Hamamelono-γ-lactone	Hamamelono-γ-lactone-2^1-P[a]	→	Free Acid	Hamamelono-γ-lactone-2^1,5-BP[b]	→ Free Acid
1	178.20	177.19	177			177	
2	80.03	76.01	74.6			76	
2^1	65.85	61.43	64			64	
3	73.78	67.85	67.5		71.9	69	
4	71.59	84.13	83.4		72.0	83.9	72.9
5	62.85	60.45	60			64	

[a] Data from Berry *et al.* (4). [b] Data from Pierce *et al.* (10).

for the preparation of U-^{14}C-labeled HA2^{1}P starts with free ^{14}C-D-hamamelose (from primrose leaves which were allowed to assimilate $^{14}CO_2$ [2]) and uses the hamamelose-kinase of the Enterobacterium *Kluyvera citrophila* 627 (3) which specifically produces hamamelose-2^{1}P. This compound could be easily oxidized to yield HA2^{1}P with hypoiodite according to the procedure of Schaffer and Isbell (13).

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