CONTROLLING ACCESSIBILITY OF TASTANT TO RECEPTORS VIA BINDING TO A CARRIER PROTEIN: MODERATING THE TASTE OF REBAUDIOSIDE A

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

By

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May 2015

ABSTRACT

Intake of added sugars exceeds discretionary dietary allowances, regardless of energy needs of the US population and often leads to adverse health conditions. Half of the added sugars consumed in the United States are in the form of high fructose corn syrup (HFCS), a major sweetener in beverages. Therefore, the food industry has been constantly evaluating and using several alternate high intensity sweeteners (HIS) to duplicate the taste of sugar, usually with no calories. Last century led to the inventions of several artificial sweeteners which are often questioned for their safety and role in controlling energy intake and weight management. Growing demand for natural ingredients has led to a rise in the popularity of steviol glycosides, natural sweeteners extracted from the leaves of Stevia rebaudiana that have been in use for centuries in Paraguay. High purity rebaudioside A (Reb A), one of the steviol glycosides is GRAS for use as a table top sweetener. Though regarded as a promising alternate, Reb A is often associated with bitter after-taste, limiting its applications.

In this study, a new approach that uses bovine serum albumin (BSA) to control the receptor-accessible part of Reb A was developed. The critical micelle concentration (CMC) of Reb A was established to be 4.5 mM and 5 mM at pH 3 and 6.7 respectively. All experiments were conducted at concentrations of Reb A below its CMC in order to have Reb A available as a monomer for interaction with BSA. The first part consisted of evaluating the stability of bitter inhibitor developed by Kurihara et al. 1998. Particle size analysis data and spectrofluorometric measurements of aqueous solutions of varying phosphatidic acid (PA) and β-lactoglobulin

(BLG) concentrations confirm that PA existed as a separate layer of vesicles at neutral pH and two different temperatures viz. 30°C and 50°C. The light scattering experiments further confirmed that it is almost impossible to have PA bind to BLG in aqueous environment leading to a conclusion that lipoprotein made up of PA and BLG is unstable under conditions pertinent to food systems.

Second part of the study consisted of characterizing the binding between Reb A (1 mM) and BSA (20 µM) via saturation transfer difference (STD) NMR. A binding epitope was established via slopes of saturation transfer to define the receptor accessible and non-accessible part of Reb A. STD NMR experiments confirm that the RPC has fast exchange of the bitterness-instigating hydrophobic diterpene of Reb A into the binding sites of BSA. This study shows that below its CMC Reb A binds weakly to BSA to generate a Reb A-protein complex ("RPC"). NMR titration studies were conducted to evaluate the stability of RPC, which shows that RPC is only modestly stable under varying conditions of pH (3.0-6.7) and temperature (4°C-40°C). The binding affinities of the Reb A-BSA complex were determined to be in the range of 5-280 mM. These results indicate that present approach can be used for Reb A and other amphiphilic tastants to alter (1) the strength of their interaction with receptors to varying degrees and (2) their accessibility to taste receptors as a result of their binding to a carrier protein.

BIOGRAPHICAL SKETCH

Samriddh Mudgal was born in Delhi, India, on January 12, 1990. He lived there for 18 years until he graduated from high school. In 2008 he joined the National Institute of Technology Warangal for a bachelor's program in Biotechnology until its completion in 2012. He spent summer of 2011 as a research intern at Central Food Technological Research Institute, Mysore, India, where he worked on a green tea solids extraction project. Upon graduation he joined Cornell University as a non-degree graduate student during the summer of 2012 where he worked on developing a protocol to enrich branched chain fatty acids from milk fat. In January 2013 he enrolled in the Master of Science program at Cornell University with a major in Food Science & Technology and a minor in Biophysics.

This thesis is dedicated to my grandfather, श्री राम चन्द्र मुद्गल, my mother, श्रीमती ऊमा मुद्गल, my father, श्री आनन्द मुद्गल and my brother सात्विक मुद्गल

ACKNOWLEDGEMENTS

I am grateful to Prof. Syed S.H. Rizvi for his guidance and encouragement during my research work in the food engineering lab. I am equally grateful to my minor committee member, Prof. Gerald Feigenson, who has shaped my research with his expertise. I would like to extend my gratitude to Dr. Ivan Keresztes (Director, Cornell Chemistry NMR Facility), who has contributed immensely to the NMR part of my research.

I would like to thank Dr. Robin Dando (Assistant Professor, Dept. of Food Science, Cornell University) who provided me with insightful comments and helped me understand the sensory aspect of my research. I owe special thanks to Dr. Teresa Porri & Ms. Penny Burke (Nano-Biotechnology Center, Cornell University) for their help in particle size analysis. I would like to thank Ms. Carol Bayles, who provided technical support for conducting spectrofluorometric measurements. I owe special thanks to my lab mates Mike Wagner, Adi Sikin, Jacky Tsai for their support and advice during my stay here in Ithaca.

I would like to express my gratitude to my family and friends especially, Ms. Anchal Sareen, Mr. Manish Kumar and Mr. Maneesh Pillai. Finally, I am grateful to the Republic of India and its people for providing the necessary resources essential for growth and development during my formative years.

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Chapter 1

High Intensity Sweeteners (HIS)

1. Introduction

Since ancient time humans have been looking for various sweet tasting substances, with honey and fruits being the earliest discoveries (Inglett, 1976). During the modern era, however, sucrose gained huge popularity among humans as a primary source of sweetener for coffee, tea, chocolate, rum and many other food applications. Americans started making corn sugar by hydrolyzing corn starch in mid-18th century. The process evolved and in the 1960s, the sweetener industry replaced acid with an enzyme, glucoamylase, for making corn sugar (Kooi, Harjes, & Gilkison, 1962). Further advancement led to production of high-fructose corn syrups (HFCS) by enzymatic isomerization of glucose to fructose (Inglett, 1976). Ever since then HFCS has been used widely as a sweetener in many food applications especially in soft drinks (Brooks, Warnecke, & Long, 1973). In the United States, the largest single source of fructose in the diet is from added sugars, accounting for approximately two thirds of total fructose intake, according to the National Health and Nutrition Examination Survey (NHANES). Added sugars are sugars and syrups that are not naturally present in foods, including sugar, corn syrup, and HFCS, which are added to foods during processing or preparation. Half of added sugars consumed in the United States are in the form of HFCS (Gao et al., 2007). Interestingly a non-caloric and highly intense sweetener saccharin was accidentally discovered in 1879. However, repeated questions on its safety as a food additive led to inventions of other artificial HIS such as Cyclamates (1940s), later banned by FDA for use in the U.S. in 1969, Aspartame (1969) and Sucralose (1976). Apart from the artificially developed high intensity sweeteners, many naturally occurring sweeteners

are consumed by people around the world. Among these, steviol glycosides extracted from the leaves of *Stevia rebaudiana* and glycyrrhizin from licorice root Glycyrrhiza glabra are widely popular and used in commercial applications. Table 1.1 compares the relative sweetness of HIS to sucrose, their composition and acceptable daily intake (ADI). Presence of these HIS, also called as non-nutritive sweeteners, low-calorie sweeteners, intense sweeteners and non-sucrose sweeteners (Fitch & Keim, 2012), in the market developed a niche for low-calorie foods due to increased evidence of health problems associated with increased sugar intake in late 20^{th} century. Studies have suggested that controlled energy intake among individuals increased their lifespan and reduced the occurrence of serious illnesses (Levin, Zehner, Saunders, & Beadle, 1995), therefore, replacement of sugar across the spectrum of foods such as baked goods, beverages, ice cream, frozen desserts etc. with HIS may lead to a greater control on energy intake. Therefore, HIS are considered to be a promising sugar alternate. However, widespread use of these HIS amid growing concerns of their safety has stimulated research on their sweetness profile, taste-structure relationships and ADI. The advances in the body of knowledge regarding HIS have led to their appreciation as well as criticism.

2. Current Status

In the modern and rapidly urbanized world a diet with low food energy intake is highly desirable. Several studies have shown relationship between excessive consumption of fat and several health diseases such as obesity, cancer and cardiovascular diseases (Sandrou & Arvanitoyannis, 2000). A study by Kranz, Smiciklas-Wright, Siega-Riz, & Mitchell, 2005, concluded that increasing the added sugar consumption among preschoolers led to a decrease in nutrient and food intake.

Table 1. 1. Composition, relative sweetness and acceptable daily intake (ADI) of high intensity sweeteners (HIS)

(Fitch & Keim, 2012).

Sweetener	Composition[a]	Relative Sweetness to Sucrose	Acceptable Daily Intake (ADI) (mg/kg-body weight/day)
Acesulfame-k	Derivative of acetoacetic acid	200	15
Aspartame	Dipeptide of aspartic acid and phenylalanine	160-220	50
Cyclamate	Cyclamic acid, calcium cyclamate, or sodium cyclamate	30	11
Saccharin	Cyclized derivative of orthosulfamoylbenzoic acid	300-500[a]	5[b]
Sucralose	1,6-dichloro-1,6-dideoxy-D-fructofuranosyl- 4-chloro-4-deoxy-α-D-galactopyranoside	600	5
Rebaudioside A	Steviol glycoside	242[c]	6[d]

‘a’: Sandrou & Arvanitoyannis, 2000; ‘b’: Leclercq, 1999; ‘c’: Ohtani & Yamasaki, 2002; ‘d’: Carakostas, Curry, Boileau, & Brusick, 2008

As a result, increasing proportions of children could not meet the DRI (dietary reference intake) recommended by National Academy of Sciences (NAS). The study further explains that main food sources of added sugar intake are food products with low nutrient profile such as soda drinks and juices. Excessive added sugar intake is linked to several metabolic abnormalities and adverse health conditions, as well as to shortfalls of essential nutrients. Cross-sectional studies in humans link soft drink consumption to higher energy intake, greater body weight, and poor nutrition, suggesting that excessive consumption plays a role in the epidemics of insulin resistance, obesity, hypertension, dyslipidemia, and type 2 diabetes mellitus in humans (Malik, Popkin, Bray, Després, & Hu, 2006). In order to limit the intake of added sugars, the United States Department of Agriculture (USDA), the World Health Organization, and the National Academy of Sciences have all issued dietary guidelines that encourage limiting consumption of added sugars to less than 10% of total energy intake (Guthrie & Morton, 2000). However, in spite of such dietary recommendations, added sugar intake in the US population far exceeds the allowance for discretionary calories, regardless of energy needs.

Studies have shown that foods that are combinations of sugar and fat are universally preferred, whereas there is widespread dislike of tastes that are bitter. Children especially love sweet and dislike bitter tastes and also eat more of the foods they like best (Drewnowski, 1997). To cater to the innate preference for sweet taste, the food industry has been constantly evaluating and using various HIS in attempts to duplicate the taste of sugar, preferably with no calories. Over the past few decades the consumer preference for healthier foods has steadily increased, for instance, in between 1991 and 2001, the proportion of U.S. adults consuming low-calorie food and beverages grew from 48% to 60% of the population (a 2.3% annual compound rate), and the proportion of

U.S. consumers trying to eat a healthy diet grew at a 6% annual rate (Chandon & Wansink, 2007). Consumption of HIS has increased substantially since 1999–2000 in both children and adults. While reasonably successful in beverages, their utility in other foods is still limited (Fig. 1.1). Safety standards for consumption of commercially available HIS are already in place. The US FDA, the Joint Commission of Experts on Food Additives (JECFA) of the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the European Food Safety Agency (EFSA) have established Acceptable Daily Intakes (ADIs) for all HIS that are GRAS.

3. Influence on Energy balance & Weight Control

HIS are also associated with criticism that question their credibility as a sugar alternate, especially for their long term viability to control energy balance. The influence of HIS on appetite, energy intake, and body weight has been the topic of a number of scholarly reviews (Levin et al., 1995). A study by Stellmann & Garfinkel, 1986, does not support the hypothesis that long term consumption of HIS prevents weight gain or helps weight loss. However, it must be noted that the study was conducted using self-reported weight data of the subjects. The study also clearly states that their results mostly but not exclusively pertain to saccharin as at that time other HIS were not widely available. Recent studies have reduced most of these concerns and suggest that recommended total energy intake can indeed be moderated by HIS. A review by Rolls, 1991, explains elaborately that there is no evidence that consumption of foods and beverages made up of HIS

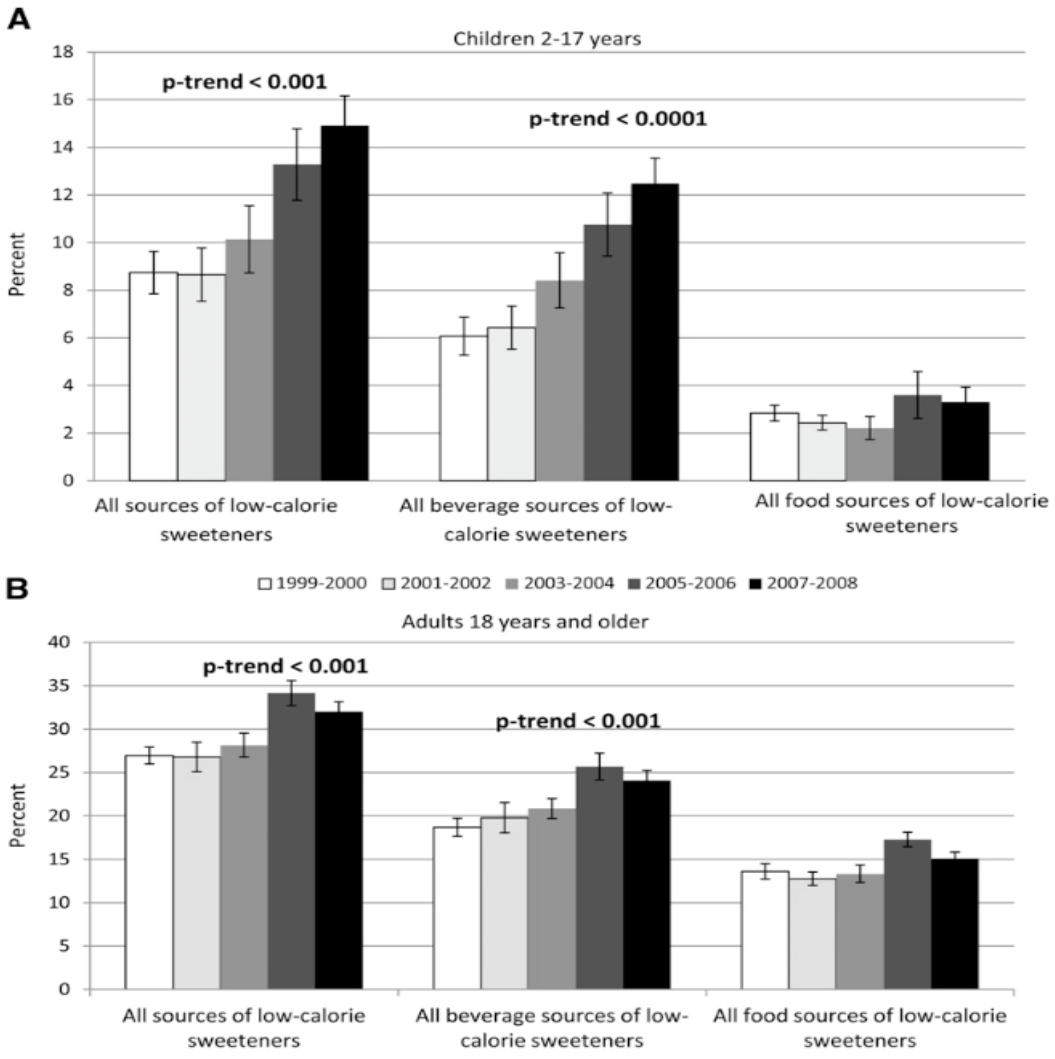


Fig. 1. 1. Mean (±SE) percentage of children (n = 16,716) and adults (n = 26,737) who reported consuming food or beverage sources of HIS in each NHANES cycle from 1999–2000 to 2007–2008 (Sylvetsky, Welsh, Brown, & Vos, 2012).

promotes food intake and weight gain in dieters. Moreover, it has been proved with certain HIS such as aspartame that its consumption lead to either no change or reduced food intake. Furthermore, a review on HIS and effect of their consumption on appetite and food intake by Mattes & Popkin, 2009, suggests that incorporation of HIS in energy yielding products is not associated with hunger heightening. The study clearly states that consumption of non-nutritive sweeteners as a substitute for higher energy yielding sweeteners have the potential to aid in weight management. Brown, Banate, & Rother, 2010, made similar observations. The study concludes that it is difficult to establish the causality between HIS consumption and weight gain in children. The study further establishes the general trend that use of HIS leads to reduction in calories intake when consumed between meals however when consumed with the meals, the loss in calorie intake is compensated due to increase in meal associated calories among children. Bellisle & Drewnowski, 2007 have shown that diet beverages made up of HIS have the advantage of reducing energy density of the product down to zero, something that is not easily achieved with solid or semi-solid foods. The study explains that diet beverages might represent the optimal use of high intensity sweeteners in the context of a weight control strategy.

4. Potential Health Risks & Persistent Problems

Apart from the effect on weight control and energy intake, it is important to explore and define health hazards, if any, associated with the consumption of HIS in humans. There have been a number of studies showing ill effects of excessive aspartame consumption using animal models (Soffritti, Belpoggi, Esposti, Lambertini, Tibaldi, & Rigano, 2006, and Magnuson et al., 2007). However, most of these claims have been disapproved by the national experts of advisory forum of EFSA (European Food Safety Authority). The national experts reviewed papers relating to

consumption of aspartame with brain function, satiation and appetite, allergenicity and immunotoxicity, metabolic effects and diabetes, carcinogenicity and genotoxicity and noted that there is no substantial evidence that aspartame can induce such effects (http://www.efsa.europa.eu/en/supporting/doc/1641.pdf; 9 April, 2015). Saccharin being the first artificial sweetener that has been in use as a sweetener for over a century and faced a ban in Canada in 1977 and a potential ban in US by FDA since a study conducted in 1960 showed that high levels of saccharin may cause bladder cancer in laboratory rats (Tandel, 2011). However, congress imposed a moratorium on such ban. Further investigation revealed that saccharin causes cancer by a non-DNA-reactive mechanism that is not relevant to humans (Tandel, 2011). Later in 2001, US revoked the warning label requirement on products using saccharin as an ingredient. Sucralose, discovered in 1976, is the only zero-calorie sweetener made from sugar and is considered safe for all section of population including those with health problems such as diabetes (Grotz et al., 2003). Acesulfame K was approved for use in liquid non-alcoholic beverages by the US FDA in 1998 and has been stated as safe for use by The Joint Expert Committee on Food Additives (Tandel, 2011). Steviol glycosides, the sweetener principles principal of Stevia rebaudiana are popular natural sweeteners that meet the purity criteria of JECFA. The US FDA has granted GRAS status to high purity (>97%) rebaudioside A (steviol glycoside) for use as a tabletop sweetener.

Consumption below ADI for all HIS is considered to be safe for people of all ages and therefore the sole factor that diminishes the potential for extended use of HIS is their ubiquitous taste issues. None of the available HIS consistently provides a sugar-like taste profile. A study by Schiffman, Booth, Losee, Pecore, & Warwick, 1995, showed that the bitterness ratings for HIS

increase with increasing concentration in aqeous solutions, whereas for sweeteners having similar sweetness potency to sucrose (glucose, fructose), the biterness ratings decrease with increase in concentration (Fig. 1.2). Further, Wiet & Beyts, 1992, showed that the non-sweet aftertaste (any off-flavor or taste other than sweetness) for most HIS is significantly higher when compared to sugars. Sweet and bitter taste properties are not interdependent but instead are independent of one another (Cardello, 1981). Many food applications require a certain minimum concentration of HIS in order to attain required sweetness intensity but at that concentration the bitterness intensity of HIS can be well above the acceptable standards, limiting their applications. Considering the huge potential of HIS, often limited by their taste issues, our studies were focused on mitigating them and simultaneously expanding the knowledge base to improve their utilization.

5. Research Needs

The global stevia market is expected to account for 15% of overall sweetener market by 2020 as per the Global Stevia Market Analysis & Opportunity Assessment, 2014 – 2020 (http://www.foodmag.com.au/news/global-stevia-market-registers-a-robust-growth; 9 April, 2015). The species *Stevia rebaudiana*, commonly known as sweet leaf, sugar leaf, or simply stevia, is widely grown for food uses. Steviol (Fig. 1.3) exists in the form of glycosides having up to 300 times the sweetness of sugar and has garnered attention with the rise in demand for low-carbohydrate, low-calorie food sweetener (Ohtani & Yamasaki, 2002). Because stevia

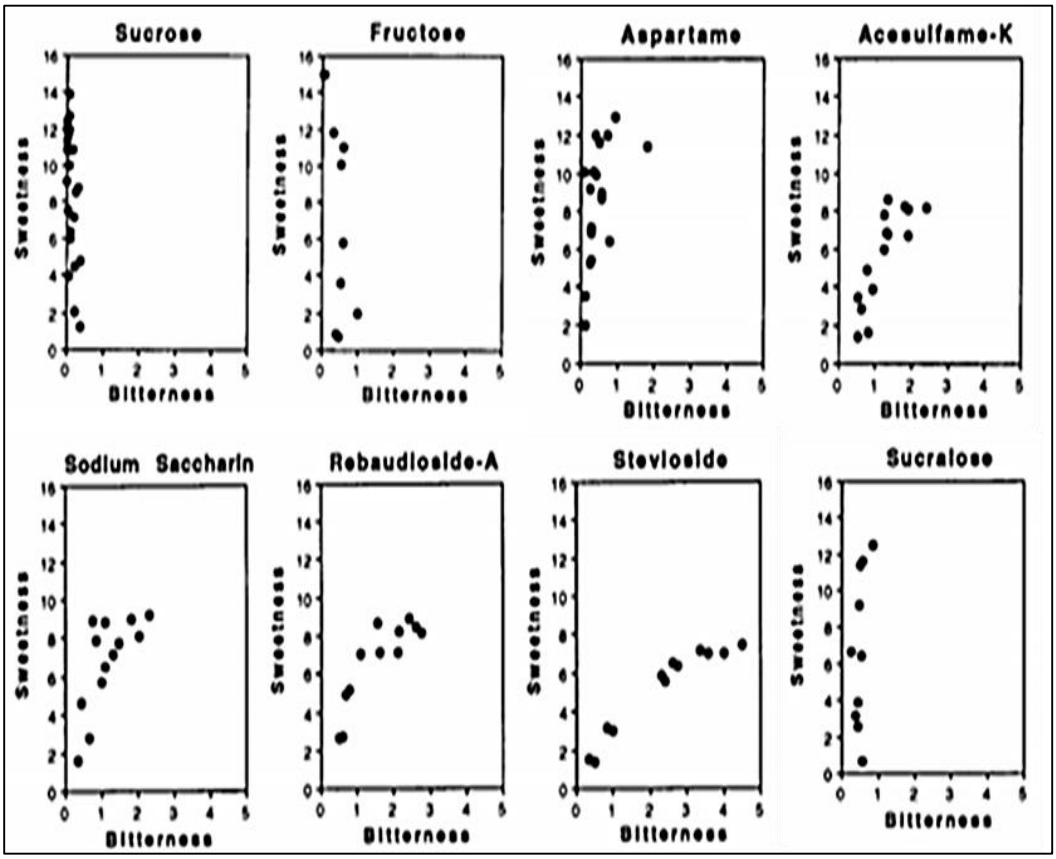
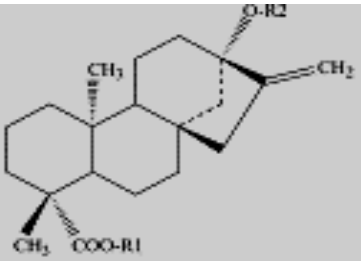


Fig. 1. 2. Relationships between mean sweetness and bitterness ratings for sugars and HIS with increasing concentrations (Schiffman et al., 1995).



	Compound name	R1	R2
1	steviol	H	H
2	steviolbioside	H	β-Glc-β-Glc(2→1)
3	stevioside	β-Glc	β-Glc-β-Glc(2→1)
4	rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
5	rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
6	rebaudioside C (dulcoside B)	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
7	rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
8	rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
9	rebaudioside F	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)
10	dulcoside A	β-Glc	β-Glc-α-Rha(2→1)

Fig. 1. 3. Structures of stevioside and related compounds (Geuns, 2003)

has a negligible effect on blood glucose, it is attractive as a natural sweetener to people on carbohydrate-controlled diets. Glycosides are molecules that contain glucose and other non-sugar substances called aglycones. Some of the tongue's bitter receptors react to the aglycones. The structure of glycoside molecules plays a key role in determining sweetness or bitterness in *Stevia.* For instance, rebaudioside D comprises five glucose molecules (Fig. 1.3) and is around five times sweeter and two-thirds less bitter than dulcoside A, which has just two glucose molecule (Ohtani & Yamasaki, 2002). Stevia is sometimes associated with metallic and bitter aftertaste, including liquorice and astringent notes. The sweetness of stevia has a slower onset and longer duration than sugar; both of these attributes are undesirable. Reb A activates two bitter receptors on the human tongue, namely, hTAS2R4 and hTAS2R14 with threshold concentrations of 200 μM and 600 μM respectively (Hellfritsch, Brockhoff, Stähler, Meyerhof, & Hofmann, 2012).

The present investigation on steviol glycosides, especially rebaudioside A (Reb A), was undertaken to undersand the underlying mechanisms for their aftetaste and way to ameliorate it.

6. Rationale & Objectives

Studies in the past have characterized bitter inhibition of foods and drugs using sodium (Breslin & Beauchamp, 1995), lipoprotein (Katsuragi, Sugiura, Lee, Otsuji, Kurihara, 1995), and flavanones from Herba Santa (Ley, Krammer, Reinders, Gatfield, and Bertram, 2005). Lipoprotein (Katsuragi et al., 1995) made up of phosphatidic acid (PA) and β-lactoglobulin (BLG) was a potential candidate for reducing bitterness of Reb A owing to its ability to 1) inhibit extremely bitter and hydrophobic molecules such as quinine hydrochloride and propranolol hydrochloride, and 2) get adsorbed on frog tongue surface and bind to the receptor sites for bitter substances on the taste receptor membranes (Katsuragi, Yasumasu, & Kurihara, 1996).

Therefore, our studies started with making lipoprotein as per the method outlined by Katsuragi et al., 1995. It must be noted that Katsuragi et al., 1995 did not study the stability of lipoprotein under aqueous conditions pertinent to food systems and therefore several experiments such as particle size analysis, light scattering experiments were conducted to quantify the binding constant of PA to BLG. Katsuragi et al., 1995, suggested that complex formation between protein (BLG) and phospholipid (PA) was necessary for bitter inhibition as BLG alone didn't suppress bitterness. However, the study did not elaborate on the mechanisms of bitterness inhibition of lipoprotein and possible role of protein alone in controlling the accessibility of tastants to bitter receptors. More importantly, the study tested many extremely bitter drug molecules but did not include any HIS as a tastant and, therefore, the ability of PA-BLG to inhibit bitter after-taste of HIS, specifically Reb A, was of deep interest.

Another study on evaluating the bitterness suppression of bitter molecules such as quinine-HCl, naringin, caffeine and others by riboflavin binding protein (RBP) concluded that protein alone can be used as a means to suppress bitterness (Maehashi, Matano, Nonaka, Udaka, & Yamamoto, 2008). The study compares the bitterness inhibition of RBP with bovine serum albumin (BSA), ovalbumin (OVA) and BLG and states that BSA, OVA and BLG failed to inhibit bitterness of compounds tested (didn't include any HIS) in contrast to RBP. The study shows that both OVA and RBP binds to quinine-HCL, while, RBP is able to inhibit bitterness of quinine-HCl, OVA didn't show any change in its bitterness perception. Therefore, study concludes that bitterness inhibition is not a common attribute of all proteins. However, it must be noted that the study didn't characterize the binding of RBP to receptor sites or any of the tastants. Moreover, the study found that increasing the RBP concentration from 0.1 and 0.25 mM for

fixed 0.25 mM quinine, the bound quinine increased from 46% to 85% and the resulting bitterness intensities decreased from 98% to 66% of the original 0.25 mM quinine, acknowledging direct interaction between quinine and RBP as a possible mechanism of its bitterness suppression along with the masking of bitter receptor sites. In case of the other molecules, the primary reason for bitterness inhibition of RBP was suggested to be the masking of bitter receptor sites. Although it is true that masking of receptor site could lead to suppression of bitterness, it can't be denied that binding of protein to tastant could control its accessibility to receptor site and therefore modulate bitterness levels.

The suppression of bitterness could possibly be achieved by two means:

1. Introducing a molecule with a higher affinity towards taste receptors than bitter molecules. This mechanism can be considered as masking of bitter receptors and it is in a way more effective for extremely bitter drug molecules that have affinity towards multiple protein-coupled receptors (T2Rs).
2. Introducing a molecule with a higher affinity towards bitter molecule itself than affinities of either of these molecules towards receptor. This method can be described as controlling the accessibility of tastant to receptor and is expected to be more efficient towards molecules with affinity towards limited T2Rs.

Studies explaining masking of bitter receptors have been done in the past. However limited work has been done on controlling the accessibility of tastant towards receptor molecules. Therefore, it is important to elucidate the mechanisms and characterize the binding

Table 1. 2. Contact angles of 1% (w/v) of HIS, 10% (w/v) of polyols and sugars on a hydrophobic polyethylene surface (Hutteau & Mathlouthi, 1998)

Compound	Contact Angle (°)	Quality of Taste
Xylene	0	—
Sucralose	82.59±2.10	Instant onset of clean sweet taste
Aspartame	88.98±2.30	Lingering sweet-bitter aftertaste
Acesulfame K	92.10±0.52	Lingering bitter and chemical synthetic aftertaste
Sodium Saccharin	94.74±1.69	Bitter, metallic and astringent aftertaste
Sucrose	96.30±1.44	—
Water	100	—

affinity between tastant and protein molecules and its impact on bitterness of tastant. Protein, specifically BSA was chosen, as it is known to bind specifically and non-specifically to several hydrophobic molecules. Moreover, HIS are sparingly hydrophobic (Table 1.2) and sparingly bitter in contrast to the compounds tested in the above studies (Katsuragi et al., 1995 & Maehashi et al., 2008). As opposed to the techniques followed by these studies, this study was undertaken to characterize the binding of BSA to Reb A using NMR spectroscopy which has been used extensively to study ligand-protein binding mechanisms. Study was done under a range of temperatures and pH taking into account the production and storage conditions of food products, especially beverages, as well as the stability of BSA and Reb A interactions.

The specific objectives of the study were as follows:

1. To quantify the critical micelle concentration (CMC) of Reb A via spectrofluorometric assay.
2. To evaluate the stability of bitter inhibitor (lipoprotein made up of phosphatidic acid and bovine serum albumin: PA-BSA) developed by Katsuragi et al., 1995, under aqueous conditions.
3. To identify and characterize the binding of bovine serum albumin (BSA) and Reb A via saturation transfer difference (STD) NMR technique under acidic conditions.
4. To estimate the range of binding affinities of Reb A–BSA interaction under temperature ranging from 4 – 40°C and pH range of 3.0 – 6.7.

Chapter 2 discusses the method that was developed to control the accessibility of the Reb A to the bitter receptors. The underlying principle was to provide a non-polar surrounding in the form of BSA as a means to compensate for any drop in system entropy due to the presence of hydrophobic groups of Reb A in saliva, which is mostly water. Considering that all HIS are relatively hydrophobic in comparison to sucrose, the principle can also be applied to other HIS.

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Chapter 2

Controlling Accessibility of Tastant to Receptors via Binding to a Carrier Protein: Moderating the Taste of Rebaudioside A

1. Abstract

We illustrate a method that uses bovine serum albumin (BSA) to control the receptor-accessible part of rebaudioside A (Reb A). The critical micelle concentration (CMC) of Reb A was found to be 4.5 mM and 5 mM at pH 3 and 6.7 respectively. NMR studies show that below its CMC, Reb A binds weakly to BSA to generate a *Reb A-protein complex* ("RPC"), which is only modestly stable under varying conditions of pH (3.0-6.7) and temperature (4°C-40°C) with its binding affinities determined to be in the range of 5–280 mM. Furthermore, saturation transfer difference (STD) NMR experiments confirm that the RPC has fast exchange of the bitterness-instigating diterpene of Reb A into the binding sites of BSA. Our method can be used to alter the strength of tastant-receptor interaction, as a result of binding of tastant to a carrier protein, ultimately leading to moderation of its taste.

2. Introduction

Scientists have long searched for means to alter the taste perception of tastant molecules to attain a desired flavor profile for food applications. Growing global awareness and concern related to excessive consumption of added sugars have led to an increase in demand for low-calorie foods and beverages (Sandrou & Arvanitoyannis, 2000) that often use high intensity sweeteners (HIS),

both artificial such as saccharin, acesulfame-K, aspartame, and sucralose, and natural, primarily steviol glycosides, to replace sugar. Although used all over the world, to many consumers the foods and beverages prepared with HIS "just don't taste right". Reported off-notes include a delay in the onset of the perceived sweetness, a lingering sweetness, and a bitter or metallic aftertaste. Studies have also shown that most of these HIS have a distinctive sweetener concentration-to-sweetness equivalency ratio that deviates significantly from that of sugar beyond a certain threshold concentration, which limits their application in many foods (Schiffman, Booth, Losee, Pecore, & Warwick, 1995 and Wiet & Beyts, 1992). Thus, altering the taste profile of HIS has been a subject of keen interest to researchers.

Existing approaches to improve the taste profile of HIS are mainly limited to chemical structural modifications, and the use of flavor enhancers and taste modifiers. Flavor enhancers are used to round out the sweetness profile or to mask off-flavors. In contrast, taste modifiers use mechanisms of adaptation, cross-adaptation, taste blocking, and taste modification. The food and beverage industry frequently uses sweetener blends (a form of cross adaptation) to overcome the sensory limitations of individual artificial HIS (Zhao & Tepper, 2007). Blending tends to enhance the sweetening power of HIS, resulting in improved flavor profiles. Past attempts to improve the taste profile of stevia-based steviol glycosides have involved, (1) selective β-glucosylation of 'high yield-less sweet' congeners such as 'stevioside' to produce more sweet tasting compounds such as 'rebaudioside A' (Reb A), extracted otherwise in poor yield, by addition of sugar moieties; and (2) isolation of other related diterpene glycosides such as 'suaviosides' and 'rubusosides' (Ohtani & Yamasaki, 2002). These attempts to improve the taste quality of steviol glycosides met with only partial success owing to poor yield of end products (modified structures) that were typically formed by use of chemical modification (benzylidene

derivatives, BaO, MeOH etc.) or rare enzyme-catalyzed reactions (CGTase, Pullulanase etc.), also raising food safety concerns. Similarly, artificial HIS modifications have also not been completely successful owing to exclusive reliance on flavor enhancers/blends, neglecting the understanding of taste modification at a molecular level. Thus, In order to bring the taste impression of amphiphilic tastant molecules such as Reb A much closer to sugar, a molecular level understanding of the mechanism of taste perception is helpful, which in turn can lead to their increased utilization.

A small structural modification in a tastant molecule can lead to a change in taste perception (Schiffman et al., 1995 and Bartoshuk, 1993). In the present study we have developed a method to physically modify Reb A through controlled interactions with a binding protein, bovine serum albumin (BSA) by applying the principle of the hydrophobic effect, in order to generate a modestly stable *Reb A-protein complex (RPC)*. We show that RPC in an aqueous solution spatially orients such that (i) the hydrophilic sugar moieties of Reb A are exposed to water and available to interact with receptor membranes and (ii) the bitterness-causing hydrophobic diterpene of Reb A is less available for interaction with receptor membranes because of binding to hydrophobic cavities of the protein. Development of an effective RPC involves thermodynamic effects of binding affinity between Reb A and protein molecules, and possibly kinetic effects that lead to a dynamic equilibrium. Here, we explain the binding of Reb A with BSA qualitatively as well as semi-quantitatively.

3. Materials and methods

3.1. Materials

98% pure Reb A (PureCircle Ltd) was used for the study. Fatty acid and protease free, low endotoxin, reagent grade lyophilized powder of BSA was procured from Proliant health and Biologicals. Potassium dideuterium phosphate (98 atom% D), 98% pure diphenylhexatriene (DPH) and lyophilized β-Lactoglobulin (≥ 90% pure by PAGE) were purchased from Sigma-Aldrich. Deuterium Oxide (D, 99.9%) was procured from Cambridge Isotope Laboratories, Inc. 100% orange juice (Florida's Natural; no pulp and no added sugars) was purchased from a local market. L-α-phosphatidic acid (Soy, sodium salt) was from Avanti Polar Lipids Inc.

3.2. Methods

3.2.1. CMC measurements

CMC was measured by the protocol developed by others (London & Feigenson, 1974). Samples were prepared by adding 2 µl of 3 mM DPH dissolved in tetrahydrofuran (THF, Mallinckrodt AR) to 3 ml of Reb A aqueous solutions of varying concentrations of Reb A from 0.5 to 10.0 mM. Samples were incubated at 40°C for 1 hour prior to fluorescence measurements on a Photon Technology International (PTI) spectrofluorometer also maintained at 40°C. Care was taken to avoid exposure of samples to room light once DPH was added.

3.2.2. Properties of lipoprotein

Particle Size analysis: Stock solutions of L-α-phosphatidic acid (PA) and β-Lactoglobulin (BLG) each 0.5 mM were prepared and stored under controlled temperature. Mixtures of PA and BLG were prepared by sonicating PA and BLG at controlled temperature. Particle size was measured using the Malvern Nano ZS (Zetasizer) at 40°C

Intensity of scattered light: A sample of 3 ml aqueous suspension of 0.5 mM PA was prepared and stored at room temperature. The intensity of 400nm light scattered by the sample was

detected using a Photon Technology International (PTI) spectrofluorometer. Excitation and emission slit widths were 2 nm, and emission spectra between 390 and 410 nm were recorded. For every successive addition of 50 µl of BLG aqueous solution the intensity of scattered light was recorded.

3.2.3. NMR Experiments

Sample Preparation: Stock solutions of Reb A and BSA were prepared by dissolving weighed solids in D_2O buffered with 10 µM KD_2PO_4 at room temperature. Sample series were prepared by serial dilution with final sample volumes of 600 µl. Samples were transferred to 5 mm NMR tubes (Wilmad-LabGlass, 535-PP-7) and incubated at 40°C for 12 hours prior to NMR analysis. All spectra were recorded on an Aglient (Varian) INOVA-600 spectrometer at 4 and 40°C to mimic the temperatures of both the refrigerated product and the human taste receptors, respectively. Chemical shifts were referenced to a residual HOD signal at 5.01 and 4.61 ppm at 4°C and 40°C, respectively (Gottlieb, Kotlyar, & Nudelman, 1997). NMR spectra were processed and analyzed using the MNova 9.0.1 software package (Mestrelab Research, S.L.)

Titration experiments were conducted at pH 3.0 or 6.7 to approximate the pH in carbonated beverages and in the vicinity of taste receptors (6.2 - 7.2), respectively. Reb A at 0.5 mM was titrated with BSA that ranged from 0.05 to 1.26 mM, at 4°C and 40°C. Then BSA at 0.88 mM was titrated with Reb A that ranged from 0.09 to 3.0 mM at 40°C. All titration experiments were recorded within 24 hours of sample preparation.

STD NMR experiments were performed on a sample containing 1 mM Reb A and 20 µM BSA at 40°C and pH 3, using the STD_ES sequence as provided in VnmrJ 3.2A (Agilent Inc.). The sequence incorporates excitation sculpting for water suppression and a T_1rho filter for

reducing protein signals. Aromatic protein resonances at 7.19 ppm were saturated with a train of 50 ms Gaussian pulses at 24 dB nominal transmitter power, and 1 ms interpulse delays. Difference spectra were generated internally by phase cycling. Off-resonance saturation was at 30 ppm. Saturation times were varied from 0.1 to 3.5 s to generate build-up curves.

Although the chemical shift assignments of Reb A in pyridine-d_5 have been published (Steinmetz & Lin, 2009), we are not aware of published assignments in D_2O. In addition, we found the chemical shifts of some Reb A resonances to be temperature and concentration dependent in D_2O. In order to facilitate interpretation of the titration and STD experiments, ^{1}H and ^{13}C NMR chemical shifts of Reb A at 0.5 mM in D_2O were assigned at pH 3 and 40°C, based on 2D gCOSY, TOCSY, HSQCAD and gHMBCAD experiments. An assignment table and annotated 2D spectra are shown in appendix table A.1 and appendix figures A.1-A.19 respectively.

To evaluate the stability of RPC in a complex food matrix we performed STD NMR experiments with Reb A and BSA in filtered orange juice. Raw orange juice was filtered under vacuum using a Buchner funnel with a 4-8 µm filter. D_2O (10% v/v), 2mM Reb A and 50 µM BSA were added to the filtrate which was incubated at 25°C for 12 hours before STD NMR experiment was performed with an on-resonance and off-resonance saturation at 8.56 and 31 ppm at 25°C respectively.

3.2.4. Mathematical Modeling and Binding Parameters

Klotz (1982) summarized mathematical models of multisite binding; the equations are further discussed elsewhere (Fielding, 2007). BSA has 7 binding sites available for interaction with fatty

acids (Bujacz, 2012). We adopted the Klotz model assuming that all 7 binding sites are equivalent and non-interacting and are all involved in binding with Reb A,

$$BSA + 7Reb\ A \rightleftharpoons BSA{-}Reb\ A_7 \quad (2)$$

$$K_d = ([BSA].[Reb\ A]^7)/[BSA - Reb\ A_7] \quad (3)$$

The bound population of ligand is given by,

$$X_{L\ (bound)} = \alpha - (\alpha^2 - \beta)^{\frac{1}{2}}, \quad (4)$$ Where,

$$\alpha = ([Reb\ A]_0 + 7 \times [BSA]_0 + K_d)/2 \times [Reb\ A]_0 \quad (5)$$

And,

$$\beta = (7 \times [BSA]_0)/[Reb\ A]_0 \quad (6)$$

Values of bound NMR parameters were calculated using the following equation:

$$M_{obs} = X_{L\ (free)}M_{L\ (free)} + X_{L\ (bound)}M_{L\ (bound)} \quad (7)$$

where, $\boldsymbol{M}_{obs}$ is any NMR observable characteristic of the equilibrium system, $X_{L(free)}$ and $X_{L(bound)}$ are the mole fractions of free and bound ligand, and $\boldsymbol{M}_{L(free)}$ and $\boldsymbol{M}_{L(bound)}$ are the NMR parameters of the ligand in its free and bound states, respectively.

3.2.5. Statistical Analysis

JMP Pro 10 was used to obtain the 95% confidence intervals for observed chemical shifts.

4. Results

4.1. Critical Micelle Concentration of Reb A

Studies have indicated that steviol, a diterpene present in all steviol glycosides is hydrophobic (Srimaroeng, Chatsudthipong, Aslamkhan, & Pritchard, 2005). Thus all steviol glycosides molecules are likely to form micelles. Wan, Wang, Wang, Yang, and Yuan (2013) quantified the

CMC of stevioside at 4.94 mg/ml (6.1 mM). However, the CMC of Reb A has not been quantified so far in the published literature and therefore, we examined the CMC of Reb A. Increase in the fluorescence of DPH above the CMC of a molecule is because of its incorporation in the hydrophobic interior of a micelle (Chattopadhyay & London, 1984). Above 4.5 mM and 5 mM of Reb A at pH 3 and pH 6.7 respectively the fluorescence of DPH rises rapidly indicating micelle formation (Fig. 2.1). Beyond the CMC, more Reb A micelles are formed resulting in further rise in fluorescence of DPH. Beyond 8 mM of Reb A the fluorescence levels off as all DPH molecules are bound to Reb A micelles. The ability of Reb A molecules to form micelles confirms the existing knowledge that the molecule is hydrophobic.

4.2. Why use of lipoprotein is not appropriate for Reb A

The bitter inhibitor developed by Kurihara, Kashiwagi, Yasumasu, Mitsui, Inaoka, and Katsuragi, 1998 was a lipoprotein of phosphatidic acid (PA) and BLG. Kurihara et al. 1998, advocate for a lipoprotein made by sonicating and lyophilizing aqueous mixtures of PA and BLG (2:5:50 mass ratio = PA:BLG:water) to be used as bitter inhibitors for a range of food products and drugs. Our preliminary studies started by forming that lipoprotein using PA and BLG. We used this lipoprotein in varying concentrations in an aqueous Reb A (500 ppm) solution at acidic pH. The recommended amount of lipoprotein to modify the taste of food was 0.1 to 3.0% by weight. For any molecule/complex to be used as a bitter inhibitor, it is important for it to be stable under conditions pertinent to food systems. However, the lipoprotein is not stable at higher concentrations under acidic conditions, with PA releasing from binding sites on BLG and precipitating after 10 days of storage. Ragona et al. 2000, have made similar observations about palmitic acid binding to BLG. Incubation of a complex made with palmitic acid and BLG at pH

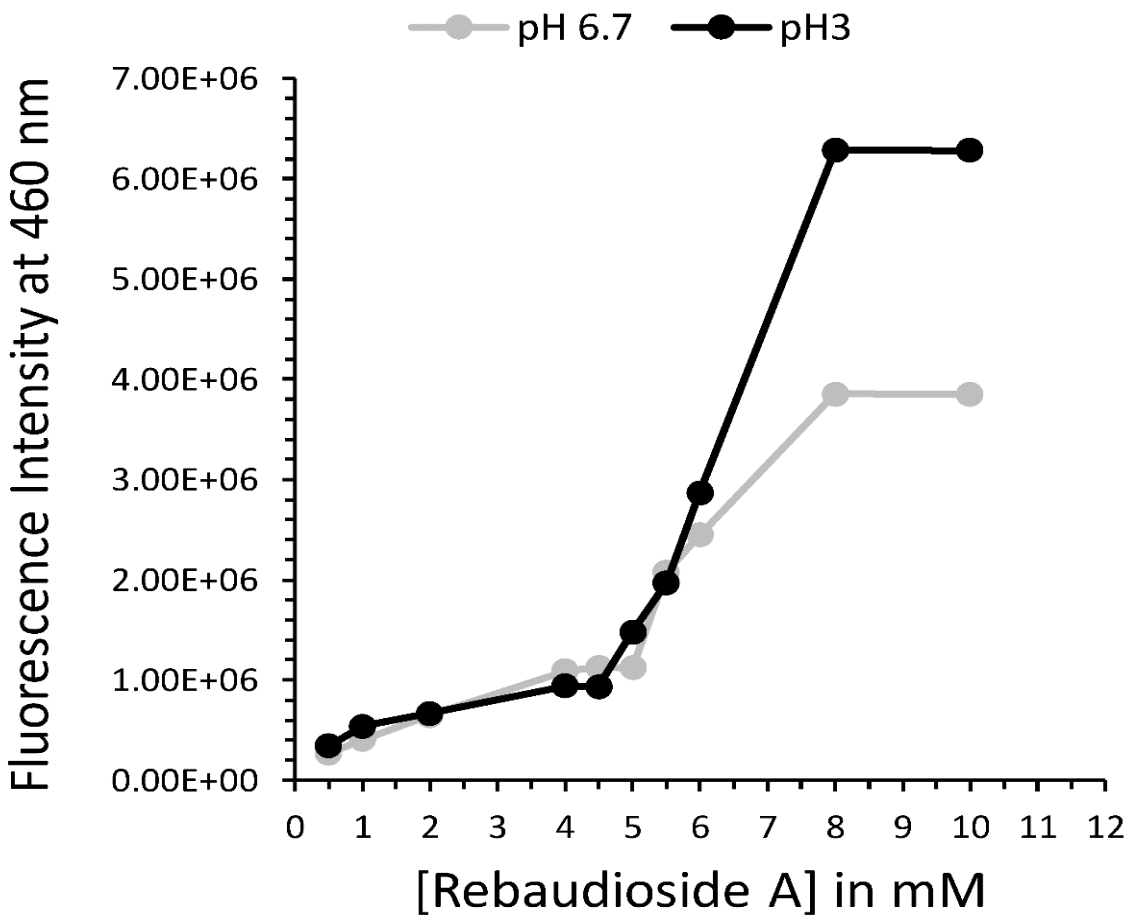

Fig. 2. 1. Plot of fluorescence intensity (measured at 460 nm) plotted against Reb A concentration. Upon micelle formation a sudden jump in fluorescence intensity is observed due to presence of hydrophobic environment for the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH).

2.1 and 37°C released 78% of bound palmitic acid after 2 hours of storage. Instability of the above lipoprotein bitter inhibitor can be attributed in part to a neglect of the molecular binding mechanism. PA:BLG in the recommended weight ratio is equivalent to a molar ratio of 10:1. However, BLG has only one primary fatty acid binding site (Frapin et al. 1993), thus each BLG cannot bind 10 molecules of PA. In preliminary studies, we formed lipoprotein from PA and BSA in the same recommended mass ratio, since BSA has ~7 primary binding sites available for interaction (Bujacz 2012). However, this lipoprotein complex of PA-BSA is also unstable, with PA precipitation observed after 10 days of storage. Phosphatidic acid, being extremely hydrophobic, is insoluble in water, existing as a separate phase of suspended vesicles in contrast to a macromolecule existing as a monomer of unit of size ~ 2nm in the aqueous solution (Fig. 2.2). Adding more BLG to a 0.5 mM aqueous solution of PA decreases the intensity of scattered light initially, but for BLG concentration > 0.23mM, further addition increases the intensity of scattered light, indicating that addition of BLG does not help to solubilize PA (Fig. 2.3). The extreme hydrophobicity of PA might therefore provide powerful competition for modestly hydrophobic steviol of Reb A, and it is quite possible that PA binding reduces the interactions of the binding sites of protein to Reb A, thus leaving steviol exposed to receptors. In short, the desired molecular interaction between BLG and PA seems impossible to achieve and does not serve any purpose in the case of modestly hydrophobic amphiphilic molecules such as Reb A.

4.3. Identification and characterization of Reb A-BSA binding

An important physiological function of serum albumins is to facilitate the transport in blood of hydrophobic molecules, including long-chain fatty acids. (Spector, John, & Fletcher, 1969). Given BSA's ability to bind a wide range of hydrophobes, we anticipated that it may bind amphiphilic Reb A. NMR spectroscopy has been widely used for the qualitative and quantitative

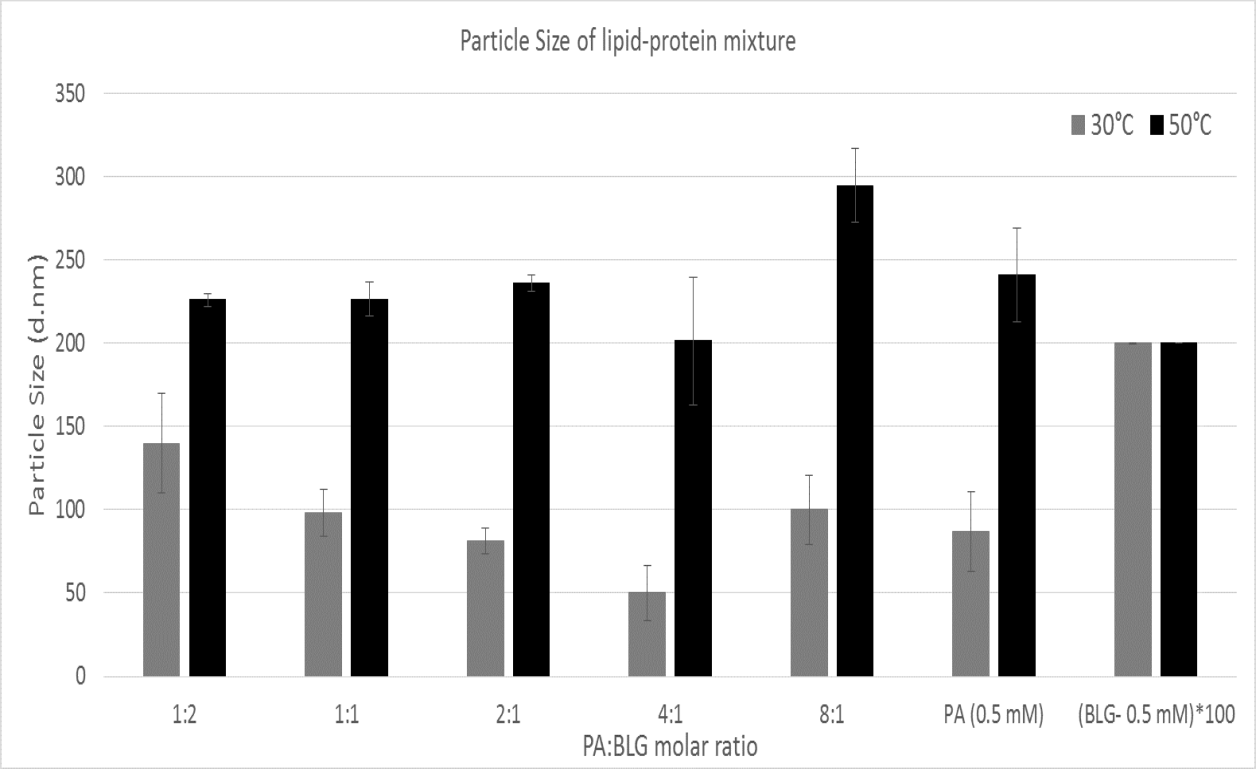

Fig. 2. 2. Particle size analysis of phosphatidic acid (PA) and β-lactoglobulin (BLG) mixtures recorded using Malvern Nano ZS (zetasizer) at 30°C and 50°C.

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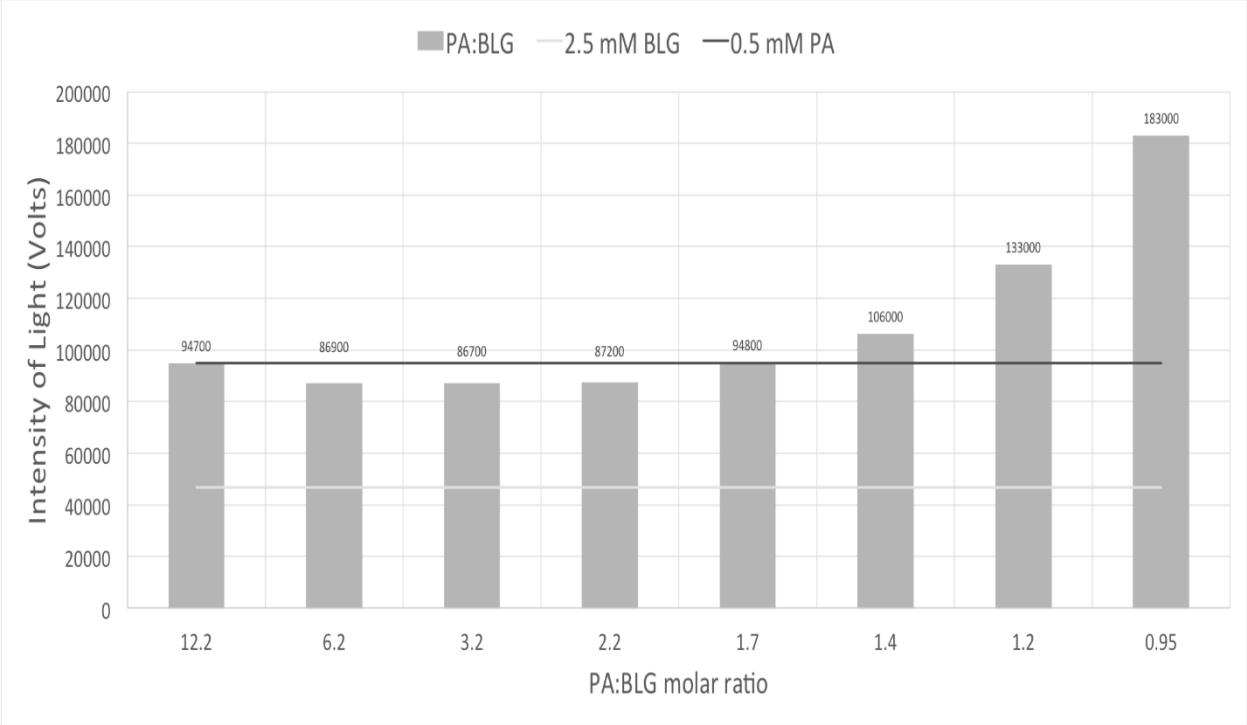

Fig. 2. 3. Variation in Intensity of scattered light with increasing β-lactoglobulin (BLG) concentration for an aqueous solution of 0.5 mM phosphatidic acid (PA) measured using pti spectrofluorometer.

assay of protein-ligand interactions (Fielding, 2007). A first step is to assess binding of the protein to ligand under investigation. In principle, almost any NMR spectroscopic parameter can serve as a gauge for the binding affinity of a ligand to a protein. In practice however, only parameters that can be obtained easily and with high sensitivity are useful (Fielding, 2007). Klein, Meinecke, Mayer, and Meyer (1999), established a new and fast method referred to as saturation transfer difference (STD) NMR spectroscopy to screen ligands for binding to proteins. The method is based on the transfer of saturation from the protein to the bound ligands, which exchange into solution where they are detected as a reduction in the intensity of the free ligand signal. Subtracting a spectrum in which the protein is saturated from one without protein saturation produces a difference spectrum in which only the signals of the ligand(s) remain (Meyer & Peters, 2003). The method has been effectively used to identify binding epitopes, because the ligand residues in direct contact with the protein show faster build-up of STD signals (Angulo & Nieto, 2011). The method involves a several-fold excess of ligand concentration over protein, allowing low mM protein concentrations to be used.

A STD spectrum of 1 mM Reb A and 20 μM BSA at pH 3.0 and 40°C with saturation of the aromatic residues of BSA at 7.19 ppm showed all expected Reb A resonances (Fig. 2.4) indicating that Reb A binds to BSA. In order to characterize the Reb A-BSA binding interaction, we investigated the build-up of saturation transfer by recording STD NMR spectra for various time periods (Appendix figure A.20).

$$y = C + A(1 - e^{-Rt}) \qquad (1)$$

The peak areas were fit to a first-order equilibrium equation (1) and rates of saturation build-up (R) were evaluated (Appendix figures A.21-A.23 and Appendix table A.2) to determine the binding epitope of Reb A (Fig. 2.5). Steviol protons acquired saturation at a faster rate than

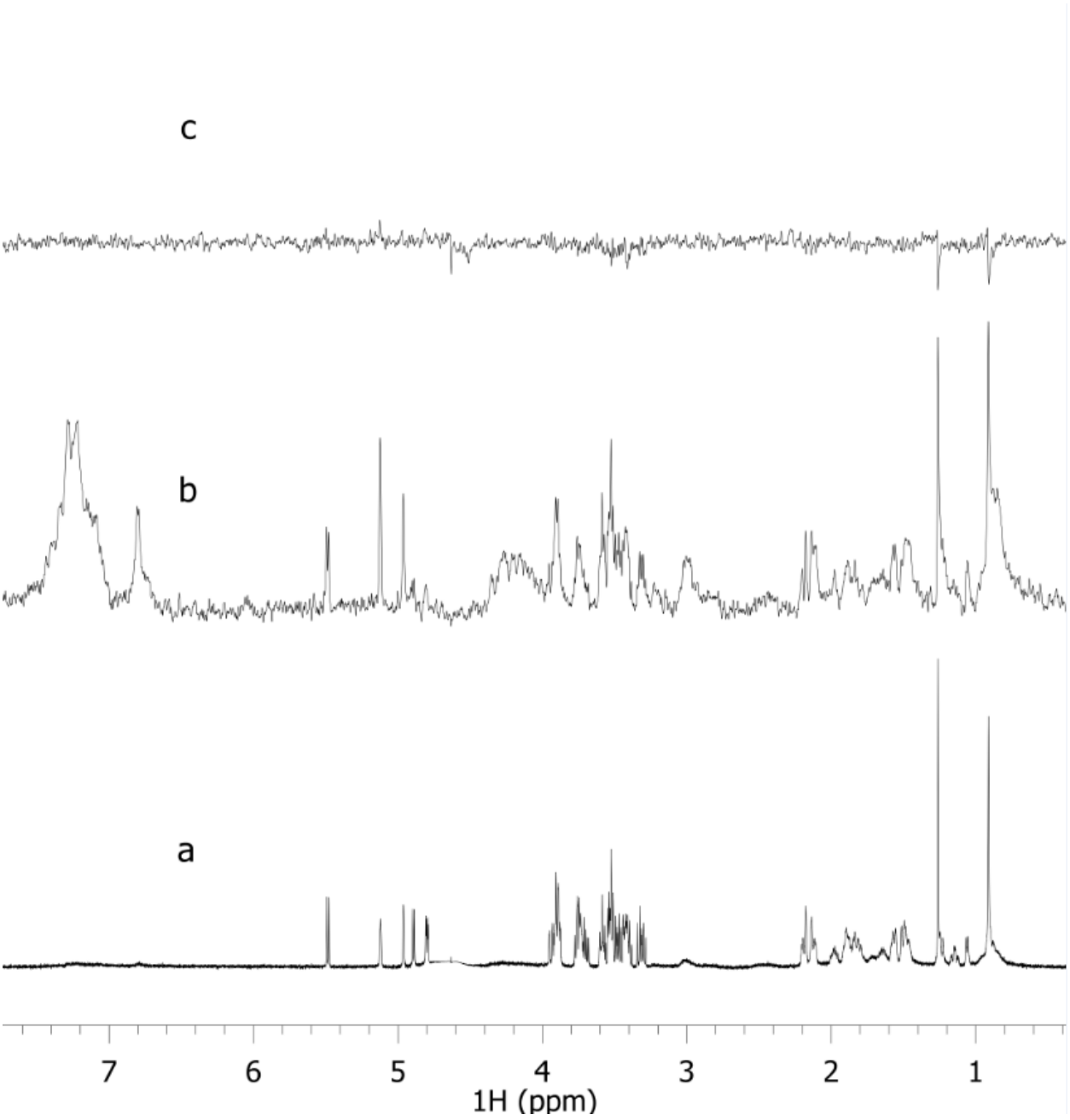

Fig. 2. 4. (a) Pre-saturated spectrum of a sample containing 1 mM Reb A and 20 µM BSA at 40°C and pH 3. (b) STD spectrum of the same sample. Aromatic protein resonances at 7.19 ppm were used for saturation and difference spectra were generated internally with off-resonance saturation at 30 ppm. (c) STD spectrum of control containing 1 mM Reb A at 40°C and pH 3 acquired under identical conditions as spectrum (b).

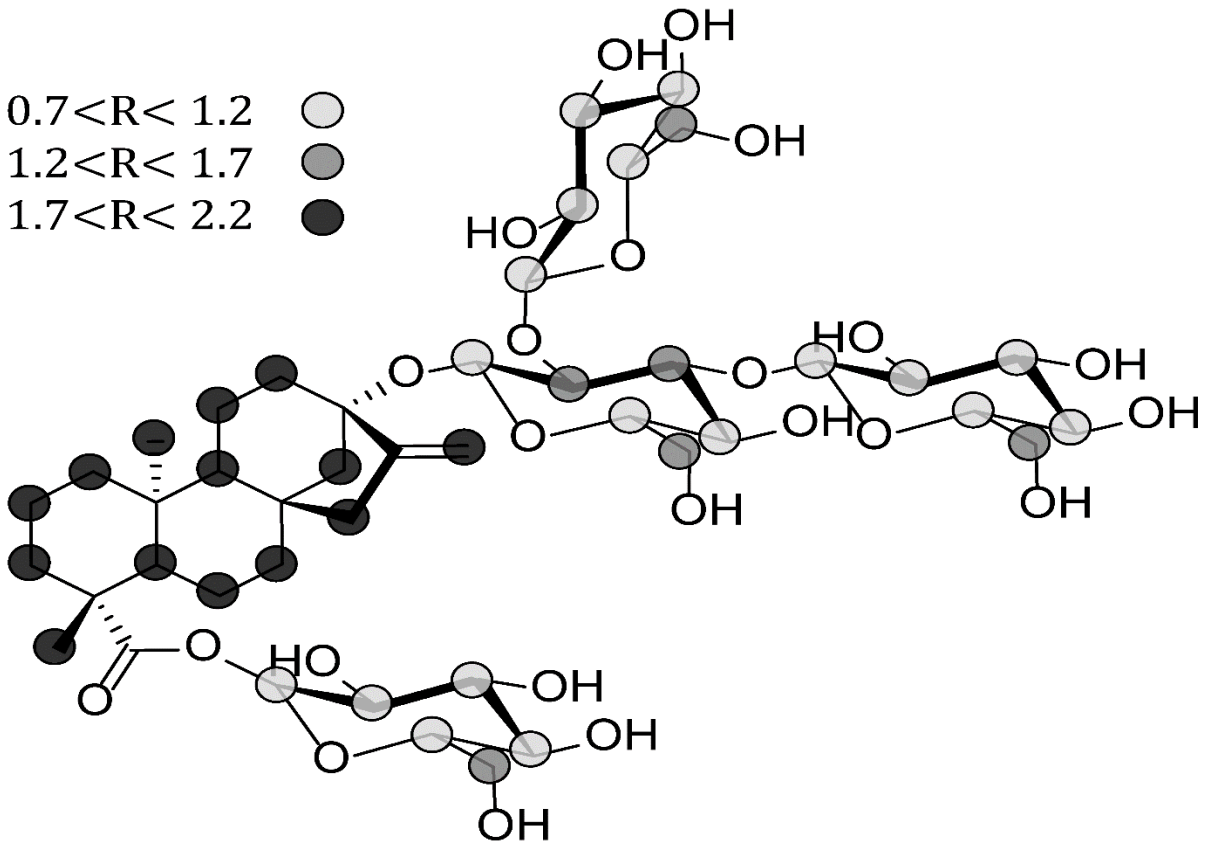

Fig. 2. 5. Reb A (epitope) mapping based on slopes of magnetization (R) acquired from saturation transfer difference (STD) experiment conducted on a sample containing 1 mM Reb A and 20 µM BSA at 40°C and pH 3. The peak intensities were fit into the first order-kinetics equation$y = C + A(1 - e^{-Rx})$. For a given Reb A proton, higher values of R represent a faster rate of magnetization transfer from protein protons, indicating intimate contact with protein in the RPC.

glucosidic protons. Based on these STD experiments we conclude that (1) Reb A binds to BSA; and (2) the binding interaction involves primarily the diterpene (steviol) rings of Reb A, indicating that the binding of Reb A and BSA is, as expected, hydrophobic in nature.

A STD spectrum of 2 mM Reb A and 50 μM BSA in filtered orange juice was recorded to validate the stability of RPC in a natural and complex food matrix. To establish the specificity of BSA binding to Reb A, a control STD experiment was performed without BSA. The sample with BSA showed strong saturation transfer to Reb A and citric acid (2.83 and 2.71 ppm) resonances (Figure 2.6). The control sample without BSA showed similar levels of saturation transfer for citric acid resonances, but strongly reduced transfer to Reb A signals. Raw orange juice contains about 0.7 wt% protein and 10 wt% carbohydrates including 0.2 wt% dietary fiber (http://www.ars.usda.gov/News/docs.htm?docid=18880). The main fiber component is pectin (Grigelmo-Miguel & Martín-Belloso, 1998), some of which is methylated at the carboxylic acid. At least some pectin is known to exist as protein-pectin complex (Klavons, Bennett, & Vannier, 1991). Since on-resonance saturation was performed at 8.56 ppm—well outside the chemical shift range for carbohydrates—all observed STDs are likely to be mediated by proteins. Therefore, we attribute the observed saturation transfer to citric acid, both in the presence and absence of BSA, to its binding to pectin-protein complexes in solution. The weak saturation transfer for Reb A indicates that it also binds to soluble proteins or pectin protein complexes, either via hydrophobic interactions with the steviol or hydrogen bonding between the glucose residues and pectin. The large enhancement of Reb A STD signals upon the addition of BSA indicates that specific binding between Reb A and BSA occurs even in a complex food matrix such as orange juice.

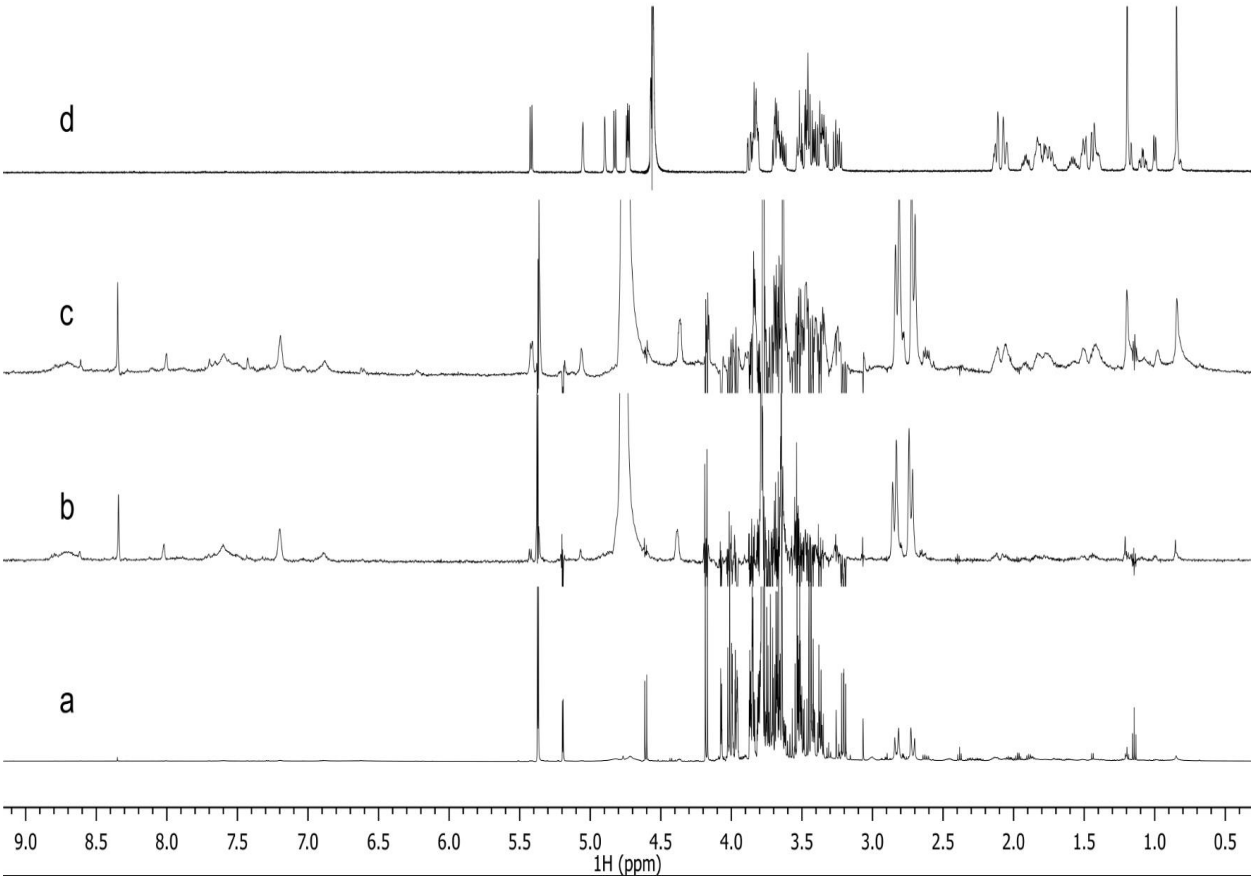

Fig. 2. 6. (a) Pre-saturated ^{1}H NMR spectrum of 2 mM Reb A and 50 µM BSA in filtered orange juice with 10% D_2O at 25°C. (b) STD spectrum of 2 mM Reb A in filtered orange juice with 10% D_2O at 25°C. On- and off-resonance saturation was at 8.56 and 31 ppm, respectively. Sub-spectra were acquired independently and subtracted during processing. (c) STD spectrum of 2 mM Reb A and 50 µM BSA in filtered orange juice with 10% D_2O at 25°C acquired under similar conditions as spectrum (b). (d) Pre-saturated ^{1}H NMR spectrum of a sample containing 0.5 mM Reb A at 40°C and pH 3.

4.4. Reb A-BSA binding affinities

Binding of small-molecules to macromolecular receptors, usually proteins, results from equilibrium of association and dissociation events. The strength of binding of Reb A and BSA is critical in order to know what fraction of Reb A molecules are bound to BSA at any given time and the nature of exchange (slow, intermediate or fast) between the molecules. This information can be useful in understanding the overall transduction mechanism and the binding of the Reb A-BSA complex with taste receptors that are largely hydrophobic membranes. Also, while formulating a product having other hydrophobic components, knowing the binding affinity can be important. NMR spectroscopy has been used widely to investigate the binding affinities of protein and small molecules (Fielding, 2007). One of the classical NMR strategies is to observe change in the NMR parameters of the ligand by titrating either ligand or protein with protein or ligand, respectively. We titrated 0.5 mM Reb A with BSA over a concentration range of 0–1.26 mM, and 0.88 mM BSA with Reb A over a concentration range of 0.09–3.0 mM. Appendix figures A.24-A.29 show complete NMR spectra for titrations done under varying conditions of temperature and pH.

There were many unknowns and in order to attain a range of measurements for binding affinity we used the following assumptions and constraints:

1) All of BSA's estimated 7 known primary binding sites are involved in binding with Reb A;
2) For quantitative analysis of the binding affinity it is important to reach saturation, but our experiments show that it is impossible to achieve saturation of BSA binding sites, given the low Reb A monomer concentrations restricted by its CMC. Even increasing the amount of protein beyond 1.26 mM, the Reb A proton shifts were difficult to identify in

the spectra. Increasing the Reb A concentration for a fixed BSA concentration (up to 3 mM) increased the linewidth but had no effect on chemical shifts. Therefore, unable to achieve saturation of binding, affinities are reported only as ranges.

3) Since it was not possible to get bound chemical shift and linewidth values, the K_d values were estimated by fitting upper and lower 95% confidence bounds based on the observed chemical shifts, with free chemical shift found to be the point with maximum confidence.
4) During fitting, NMR parameters for the bound state were limited to reasonable values, based on experimental data.

NMR spectra of Reb A (0.5 mM) titrated with BSA (Fig. (2.7a) 40°C and pH 3.0, (2.7b) 40°C and pH 6.7 and (2.7c) 4°C and pH 6.7) and BSA (0.88 mM) titrated with Reb A (Fig. 2.7d) were investigated to measure changes in the observed chemical shifts and linewidths. At pH 6.7 and 40°C the titration was characterized by changes in chemical shifts and large increases in linewidths for Reb A proton resonances, whereas at pH 6.7 and 4°C, we did not observe significant changes in chemical shift, but large increases in linewidths were observed. At pH 3 and 40°C the changes in chemical shifts were similar to those at pH 6.7, but only moderate increases in linewidths were observed. In addition, the BSA signals were noticeably sharper at pH 3 than at pH 6.7, whereas temperature had only a minor effect. BSA is known to display a pH dependent tendency to self-assemble into large aggregates (Vetri, Librizzi, Leone, & Militello, 2007 and Militello, Casarino, Emanuele, Giostra, Pullara, & Leone, 2004). The rate of exchange between bound and free ligands can depend on temperature, pH, binding surfaces, and presence of any titratable protons that are involved in binding. It is possible that at pH 6.7 and 4°C, the exchange of bound and free Reb A is slow on the NMR timescale—i.e. the exchange rate is slower than the frequency difference between resonances of the bound and free states—therefore

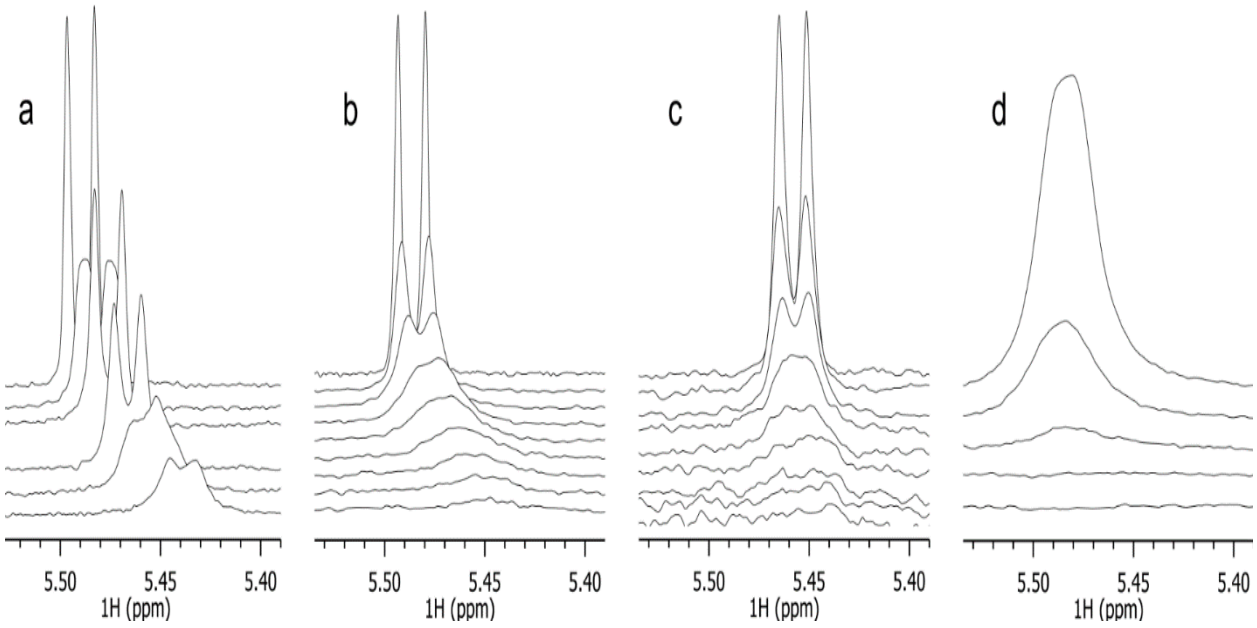

Fig. 2. 7. Changes in observed chemical shifts and line-widths for titration of Reb A (0.5 mM) and varying BSA concentration for samples at (a) pH 3 and 40°C, (b) pH 6.7 and 40°C and (c) pH 6.7 and 4°C. (d) Changes in line-widths for titration of BSA (0.88 mM) and varying Reb A concentration at pH 6.7 and 40°C

the observed free Reb A chemical shifts remain constant. We did not observe the bound Reb A signals in the spectra, most likely due to their large linewidths. At pH 6.7 and 40°C, we observed both chemical shift averaging and exchange broadening of Reb A resonances indicating the exchange is likely in the intermediate regime. At pH 3 and 40°C, fast exchange results in significant chemical-shift averaging of free and bound RebA resonances but only moderate broadening. Titration results shows a K_d range of 5 – 280 mM (Table 1) indicating weak binding between Reb A and BSA. Appendix figure A.30 shows a plot of mole fraction of bound Reb A with concentration fixed at 0.5 mM with increasing BSA concentration. The lower values of K_d in general are indicative of fast exchange of steviol in and out of BSA binding sites, which means that the availability of glucose moieties to interact with receptors remains unaffected in RPC.

5. Discussion

Studies have shown that sensations of bitter and sweet tastes are initiated by the interaction of tastant molecules with G protein-coupled receptors (GPCRs) in the apical membranes of taste receptor cells (Chandrashekar et al., 2000 and Lindemann, 1996). Taste receptor cells (TRCs) are typically clustered in groups within taste buds. The apical surface of TRCs, which makes contact with the oral cavity, is rich in GPCR*s*, ion channels, and other transduction elements (Margolskee, 2002). When a tastant molecule binds to a GPCR, it causes a conformational change in the GPCR that triggers the interaction between the GPCR and a bound G protein. The activation of the G protein leads to generation of signals that are conveyed through TRCs to the brain via taste nerves, resulting in a sensation. Humans can detect thousands of different bitter compounds with a limited set of receptor genes belonging to the TAS2R gene family. The

Table 2. 1. Range of binding affinity estimated based on Klotz model (Klotz et al., 1974) for Reb A (0.5 mM) titrated with BSA under different conditions of temperature and pH

Experimental conditions		Parameter: observed/ fitted (confidence interval)		BSA concentration in mM									Calculated Parameters	
pH	Temp(°C)	Observed	Fitted	0	0.05	0.13	0.25	0.38	0.51	0.63	0.76	0.88	K_d mM	NMR parameter (bound)
3	40	δ_H	NA	5.490	5.482	5.476	5.466	nd	5.452	nd	nd	5.439	NA	5.20[a] – 5.42 ppm
		NA	δ_H (Upper 95%)	5.490	5.486	5.480	5.470	nd	5.450	nd	nd	5.420	39 – 8	
		NA	δ_H (Lower 95%)	5.490	5.487	5.484	5.477	nd	5.465	nd	nd	5.446	24 – 5	
6.7	40	δ_H	NA	5.486	5.484	5.481	5.476	5.471	5.467	5.459	5.452	5.454	NA	4.00[a]–5.40 ppm
		NA	δ_H (Upper 95%)	5.486	5.484	5.481	5.475	5.470	5.464	5.459	5.453	5.448	280 – 15	
		NA	δ_H (Lower 95%)	5.486	5.484	5.481	5.477	5.472	5.467	5.463	5.458	5.453	240 – 13	
6.7	4	LW	NA	8.75	10.2	12.5	15.9	19.7	23.1	26.8	30.6	34.0	NA	200[a]–60 Hz
		NA	LW (Upper 95%)	8.75	10.2	12.4	16.0	19.6	23.3	26.9	30.5	34.2	40 – 12	
		NA	LW (Lower 95%)	8.75	10.2	12.3	15.9	19.5	23.1	26.7	30.3	33.9	40 – 12	

nd - not determined; NA - not applicable; δ_H units are in ppm; LW (line-width) units are in Hz; 'a' represents values that were limited based on rationality of fit or reasonability of parameter

observed mechanism of distinguished taste detection for different tastants is due to exhibition of a broad tuning to a great number of structurally divergent ligands by receptors (Behrens, Brockhoff, Kuhn, Bufe, Winnig, & Meyerhof, 2004). Therefore, it is evident that structural modification in a tastant can lead to a change in taste perception. However, it is critical to understand exactly which part of their molecular structure needs to be modified to achieve reduction in their bitterness while retaining their sweetness.

It is reasonable to expect that the ease of access to the receptor site and the spreading of tastant over the receptor membrane are also of relevance in the mechanism of taste response. Interactions between water molecules present in saliva and tastant molecules have a major effect on water behavior, which activates ion transfer across the receptor membrane and initiates the transduction mechanisms (Mathlouthi & Seuvre, 1988). The presence of hydrophobic and hydrophilic functional groups, configuration and conformation of these groups, degree of their hydrophobicity are some of the key factors that determine the sensory qualities of tastant (Wiet & Miller, 1997). It has also been suggested that disruption of the hydrophobicity of sweet molecules can lead to a disruption of the basic sensory profile of a tastant, which can be explained as follows: the hydrophobic parts of tastant molecules, if any, cannot hydrogen bond to water, so those nearby water molecules must make their hydrogen bonds in a more restricted way with other neighboring waters, lowering the system entropy. The net result, known as "the hydrophobic effect", is to drive the hydrophobic groups out of the water and into nonpolar surroundings, in our case within a receptor membrane. Gardner (1978) made a similar observation about the ability of a tastant to form inter-molecular hydrogen bonds and its correlation to bitterness. The study suggests that with reduction in ability of a molecule to form inter molecular hydrogen bonds to aqueous saliva, the degree to which it penetrates into cell

membrane increases making it more likely to reach the receptor site of action. Price and Desimone (1977) observed that the initial recognition of the sweet compounds occurs at the surface of the cell. However, in case of bitter compounds there is no evidence that their recognition occurs at the surface of a cell (Gardener, 1979). Models have suggested that the bitter tastant must penetrate the cell wall to reach its site of action for the perception of bitterness which goes well with the observation that most of the HIS have several fold higher sweetness and relatively less intense bitterness at low concentrations.

Hutteau and Mathlouthi (1998) showed that all HIS molecules are more hydrophobic than sugar molecules. Like any other HIS, Reb A also includes a hydrophobic diterpene (Srimaroeng et al., 2005). Most of the HIS are capable of crossing the membrane barrier to reach the threshold level of bitterness due to their hydrophobicity (Gardner, 1979). Consequently, as explained above, (i) their interaction with receptors is enhanced, resulting in intensive sweetness and noticeable bitterness; and (ii) their solubility in water is diminished. Both of these attributes lead to undesirable changes in their taste profile, and therefore one of the ways to make the taste profile of HIS more sugar-like is to make their interaction with water more like that of sugar molecules. We achieved this end by modifying the Reb A *accessible* structure via binding its hydrophobic part to nonpolar binding sites on BSA. Binding of Reb A to BSA compensates for the drop in system entropy which otherwise was achieved by driving the hydrophobic groups out of the water into the receptor membrane in the case of pure Reb A. Therefore, the binding of Reb A to a carrier protein helps bring its interaction with surrounding water closer to that of sugar molecules. Our findings from the STD NMR experiments suggest that binding of Reb A to BSA is likely to reduce its bitterness and retain its sweetness.

Like any model, the RPC model also has limitations. Since the model is based on hydrophobic interactions of Reb A's diterpene with hydrophobic binding sites in BSA, its application is restricted to a food system with a hydrophilic environment. Thus, one of the major applications of RPC is to beverages. Non-polar components present in a food matrix, especially those more hydrophobic than Reb A, may have a preference to bind into the hydrophobic sites of BSA. Our experiments with orange juice confirm that the RPC is stable in a natural and complex food matrix. However, orange juice contains little or no fat therefore it is unknown how the RPC will behave in the presence of food matrices rich in fat. The stability of Reb A-BSA in water depends on the pH, temperature and hydrophobicity of Reb A. The protein's stability primarily depends on the pH and temperature in water. Thermal stability of BSA in aqueous solutions is good in the range 4 - 40°C (Baier & McClements, 2001) so we confined our study to this range. 4°C was chosen to duplicate refrigeration conditions, whereas 40°C was chosen to mimic the temperature of taste receptor cells (~37°C). The entire range of temperature has minimal effect on the protein's native structure, avoiding any denaturation of BSA. A pH range of 3.0-6.7 was chosen, as most of the available beverages fall in this range (Chavalittamrong, Pidatcha, & Thavisri, 1982). Current understanding regarding the stability and functionality of RPC beyond this range of temperature and pH is unknown. The amount of BSA required for binding 0.5 mM of Reb A in a 1l aqueous solution is shown in Appendix table A.3. The other critical parameter associated with functionality and stability of RPC is the hydrophobicity of Reb A. Apart from confirming the hydrophobicity of Reb A, CMC also gives a crucial constraint in the study of the RPC model, since one of the fundamental requirements for realization of RPC is to have Reb A molecules present as monomers. Above the CMC, the micelles present completely different possibilities for interactions, greatly complicating studies, and leading to binding with bitter

receptors in an uncertain manner leading to undesirable notes. Therefore, it is important to have a tastant molecule well below its CMC while forming the RPC. We performed all our studies at 0.5 mM of Reb A, which is sufficient to sweeten most beverages based on its equivalent sweetness to sucrose (Cardello, Da Silva, & Damasio, 1999). To have desired taste moderation, it is important to ensure the availability of hydrophilic functional groups of the tastant to interact with receptors, and binding of hydrophobic groups to a carrier protein for desired spatial orientation resulting in reduction of off-notes. Thus, ultimately the ability of a carrier protein to moderate the taste depends on the strength of its binding to a tastant.

6. Conclusion

Many food applications require a certain minimum concentration of HIS in order to attain a required sweetness intensity. But at that concentration the bitterness intensity of HIS can be well above acceptable standards, limiting their applications. Given the excessive consumption of added sugars together with taste issues associated with HIS, it is imperative to improve their quality by a holistic approach that (1) is commercially feasible, employing relatively inexpensive structural modifications; and (2) expands our knowledge base regarding the principles of tastant-receptor interaction modifications. Here we describe a chemical-free, economical, and purely physical interaction-based approach to control the accessible structure of Reb A, which might prove to be beneficial in improving its taste profile. The estimated K_d range can be useful in determining the amount of protein required to have the desired taste profile, which might reflect the amount of Reb A in the bound state. It is possible that only a small fraction of bound Reb A could result in significant improvement in the taste profile, because the presence of protein can affect the transduction mechanism in several ways, such as changes in diffusion rate and

orientation of tastant. The details of how Reb A binding into BSA cavities improves the taste profile of Reb A cannot be quantified based on current findings, but it can be concluded that the presence of protein will impact the taste profile of Reb A owing to a change in Reb A - taste receptor interactions. This approach is based on a model that reflects an understanding of tastant-receptor interaction and has been translated well theoretically. A solution of the RPC can potentially be more effective by using a carrier protein having a higher or lower binding affinity for Reb A.

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APPENDIX

Table A. 1. NMR chemical shift assignments for 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C[a]

C#[*]	δ ^{13}C (ppm)	δ ^{1}H (ppm)	^{1}H multiplicity	COSY	TOCSY	HMBC[b]
1	39.92	0.88 (ax)	td, J = 13.5, 12.8, 4.3 Hz, 1H	1eq, 2ax, 2eq	1eq, 2ax, 2eq, 3ax, 3eq	20
		1.87 (eq)	dm, J = 13.5 Hz[c]	1ax	1ax, 2ax, 2eq, 3ax, 3eq	
2	18.63	1.79 (ax)	m	1ax, 2eq, 3ax, 3eq	1ax, 1eq, 2eq, 3ax, 3eq	
		1.45 (eq)	dm, J = 15.5 Hz[c]	1ax, 2ax, 3ax, 3eq	1ax, 1eq, 3ax, 3eq, 2ax	
3	37.24	1.12 (ax)	td, J = 13.6, 4.1 Hz, 1H	2ax, 2eq, 3eq	1ax, 1eq, 2ax, 2eq, 3eq	18
		2.11 (eq)	dm, J = 13.8 Hz[c]	2ax, 2eq, 3ax	1ax, 1eq, 2ax, 2eq, 3ax	
4	43.96	–	–	–	–	3ax, 5, 18
5	56.48	1.21	dd, J = 12.4, 1.9 Hz, 1H	6ax, 6eq	6ax, 6eq, 7ax, 7eq	18, 20
6	21.44	1.80 (ax)	app qd, J = 13.5, 4.8 Hz[c]	5, 6eq, 7ax	5, 6eq, 7ax, 7eq	
		1.88 (eq)	dt, J = 13.4, 5 Hz[c]	5, 6ax, 7ax	5, 6ax, 7ax, 7eq	
7	40.70	1.47 (ax)	td, J = 13.4, 3.5 Hz[c]	6ax, 6eq, 7eq	5, 6ax, 6eq, 7eq	8
		1.55 (eq)	dd, J = 13.4, 5.8 Hz[c]	6ax, 6eq, 7ax	5, 6ax, 6eq, 7ax	
8	41.89	–	–	–	-	7ax, 7eq, 9, 11eq
9	52.97	1.036	dm, J = 8.1 Hz, 1H	11ax, 14b, 20	11ax, 11eq, 12ax, 12eq, 20	20
10	39.167	–	–	–	–	20, 9, 11ax, 11eq
11	19.929	1.62 (ax)	ddd, J = 21.4, 14.6, 7.3 Hz, 1H	9, 12eq, 12ax, 11eq	9, 11ax, 12ax, 12eq	
		1.83 (eq)	dd, J = 16.6, 5.5 Hz [c]	11ax, 12ax	9, 11eq, 12ax, 12eq	
12	36.53	1.96 (ax)	ddd, J = 12.8, 11.3, 6.1 Hz, 1H	11ax, 12eq, 11eq	9, 11ax, 11eq, 12eq	9
		1.55 (eq)	tm, J = 10.7 Hz [c]	11ax, 12ax	9, 11ax, 11eq, 12ax	
13	87.65	–	–	–	–	12ax, 17b, 1", 11ax
14	44.2	2.16 (a)	dd, J = 11.7, 3.5 Hz [c]	14b	14a	
		1.48 (b)	dd, J = 11.7, 4.4 Hz [c]	9, 14a	14b	
15	46.86	2.17 (a)	dm, J = 17.0 Hz [c]	15b, 17b, 17a	15b, 17a, 17b	16
		2.09 (b)	dm, J = 17.0 Hz [c]	17b, 17a, 15a	15a, 17a, 17b	
16	153.9	–	–	–	–	12ax, 15a
17	104.2	5.1 (a)	m(br), 1H	15b, 15a	15a, 15b, 17b	
		4.93 (b)	m(br), 1H	15b, 15a	15a, 15b, 17a	
18	27.95	1.239	s, 3H			
19	179.4	–	–	–	–	3ax, 5, 18, 1'
20	15.25	0.889	s, 3H	9	9	5, 9
1'	94.07	5.46	d, J = 8.2 Hz, 1H	2'	2', 3', 4', 5'	2'
2'	72.07	3.50	app. t, J = 9.7Hz [c]	1', 3'	1', 3', 4', 5'	3'

C#[*]	δ ^{13}C (ppm)	δ ^{1}H (ppm)	^{1}H multiplicity	COSY	TOCSY	HMBC[b]
3'	76.12	3.56	app. t, J = 10.3 Hz [c]	2', 4'	1'	2', 4'
4'	69.32	3.44	app. t, J = 10.0 Hz [c]	5', 3'	1'	3'
5'	76.81	3.56	dd, J =10.1, 5.8 Hz [c]	6'a, 6'b, 4'	1'	4'
6'	60.56	3.88 (a)	d, J =12.5 Hz [c]	5'		4'
		3.72 (b)	dd, J = 12.1, 5.9 Hz [c]	5'		
1"	96.03	4.77	d, J = 7.9 Hz, 1H	2"	2", 3", 4", 5"	2"
2"	78.78	3.73	app. t, J = 8.7 Hz [c]	1", 3"	1"	1'", 3"
3"	85.07	3.88	app. t, J = 9.7 Hz [c]	2", 4"	1"	1"", 1", 2"
4"	68.70	3.50	app. t, J = 10.1 Hz [c]	3", 5"	1"	3"
5"	75.29	3.39	dd, J =10.1, 5.8 Hz [c]	4", 6"a, 6"b	1"	4", 6"b
6"	60.98	3.86 (a)	d, J = 12.2 Hz [c]	5", 6"b		4"
		3.70 (b)	dd, J = 12.2, 5.8 Hz [c]	5", 6"a		
1'"	102.1	4.87	d, J = 7.8 Hz, 1H	2'"	2'", 3'", 4'", 5'"	2'", 2"
2'"	74.26	3.37	dd, J = 9.6, 7.9 Hz, 1H	1'", 3'"	1'", 4'", 5'", 3'", 6'"a, 6'"b	3'"
3'"	75.90	3.47	app. t, J = 9.8 Hz [c]	2'", 4'"	1'", 2'", 4'", 5'"	2'", 4'"
4'"	70.28	3.30	dd, J = 9.8, 9.0 Hz, 1H	3'", 5'"	1'", 2'", 3'", 5'", 6'"a, 6'"b	3'"
5'"	76.44	3.39	dd, J =10.5, 6.3 Hz [c]	4'", 6'"a, 6'"b	1'", 2'", 3'", 4'", 6'"a, 6'"b	4'", 6'"a
6'"	61.39	3.87 (a)	d, J = 11.7 Hz [c]	5", 6"b	2'", 4'", 5'", 6'"b	4'"
		3.66 (b)	dd, J = 11.5, 5.6 Hz [c]	5", 6"a	2'", 4'", 5'", 6'"a	
1""	102.3	4.77	d, J = 8.0 Hz, 1H	2""	2"", 3"", 4"", 5""	2"", 3"
2""	73.52	3.37	app. t, J = 8.6 Hz [c]	1"", 3""	1"", 6""a	3""
3""	75.97	3.52	app. t, J = 10.1 Hz [c]	2"", 4""	1"", 6""a	2"", 4""
4""	69.70	3.42	app. t, J = 10.4 Hz [c]	3""	1"", 6""a	3""
5""	76.21	3.499	dd, J =10.5, 6.3 Hz [c]	4"", 6""a, 6""b	1"", 6""a	4"", 6""b
6""	60.78	3.92 (a)	dd, J = 12.4, 2.3 Hz, 1H	5"", 6""b	2"", 3"", 4"", 5"", 6""b	4""
		3.71 (b)	dd, J = 12.4, 6.3 Hz [c]	5"", 6""a	6""a	

[*] Carbon number as in supplementary figure 1

[a] The chemical shifts of Reb A are concentration and temperature dependent

[b] HMBC correlation are to the carbon from the Hs indicated

[c] Multiplicities and coupling constants derived from 2D experiments (HSQC, COSY and/or TOCSY)

Table A. 2. STD build-up rates for Reb A resonances

Integration range	Assignment	STD Build-up rate
0.77–0.84	1ax, BSA methyl	1.9
0.86–0.89	1ax, 20, BSA methyl	2.0
0.92–0.97	BSA methyl	1.1
0.99–1.04	9	1.9
1.08–1.15	3ax	1.8[a]
1.17–1.20	5	2.1
1.20-1.24	18	1.9
1.40-1.49	2eq, 7ax, 14b	2.1
1.51-1.56	7eq, 12eq	2.1
1.57-1.69	11ax	2.0[a]
1.74–1.82	2ax,6ax	2.1[a]
1.82-1.90	1eq, 6eq, 11eq	2.0[a]
2.05-2.19	3eq, 14a, 15a, 15b	2.0
3.24-3.28	2’’’	1.0
3.28–3.32	4’’’	0.9
3.34–3.41	5’’,5’’’,2’’’’,4’’’’	0.9
3.43–3.47	4’	0.9
3.47–3.52	2’, 4’’, 3’’’, 3’’’’, 5’’’’	1.0
3.52–3.58	3’, 5’	1.0
3.63–3.68	6’’’b	1.7
3.68-3.75	6’b, 2’’, 6’’b, 6’’’’b	1.3
3.82-3.89	6’a, 3’’, 6’’a, 6’’’b, 6’’’’a	1.5
3.89–3.93	6’’’’a	1.4
4.75-4.78	1’’, 1’’’’	0.8[a]
4.83-4.88	1’’’	0.8
4.91-4.95	17b	1.8
5.06-5.10	17a	2.1
5.43-5.47	1’	1.1
[a] Peak intensities were very low, but clear exponential build-up was observed. Background areas without Reb A peaks showed only random intensity fluctuations.		

Table A. 3. Amount of BSA (g) required for binding 0.5 mM Reb A (483 mg/l) in 1 liter aqueous solution. (Calculations based on K_d of 5 mM)		
Bound Reb A (%)	Free Reb A (%)	Mass of BSA Required (g)
0	100	0
25	75	16.6
50	50	49.9
90	10	396

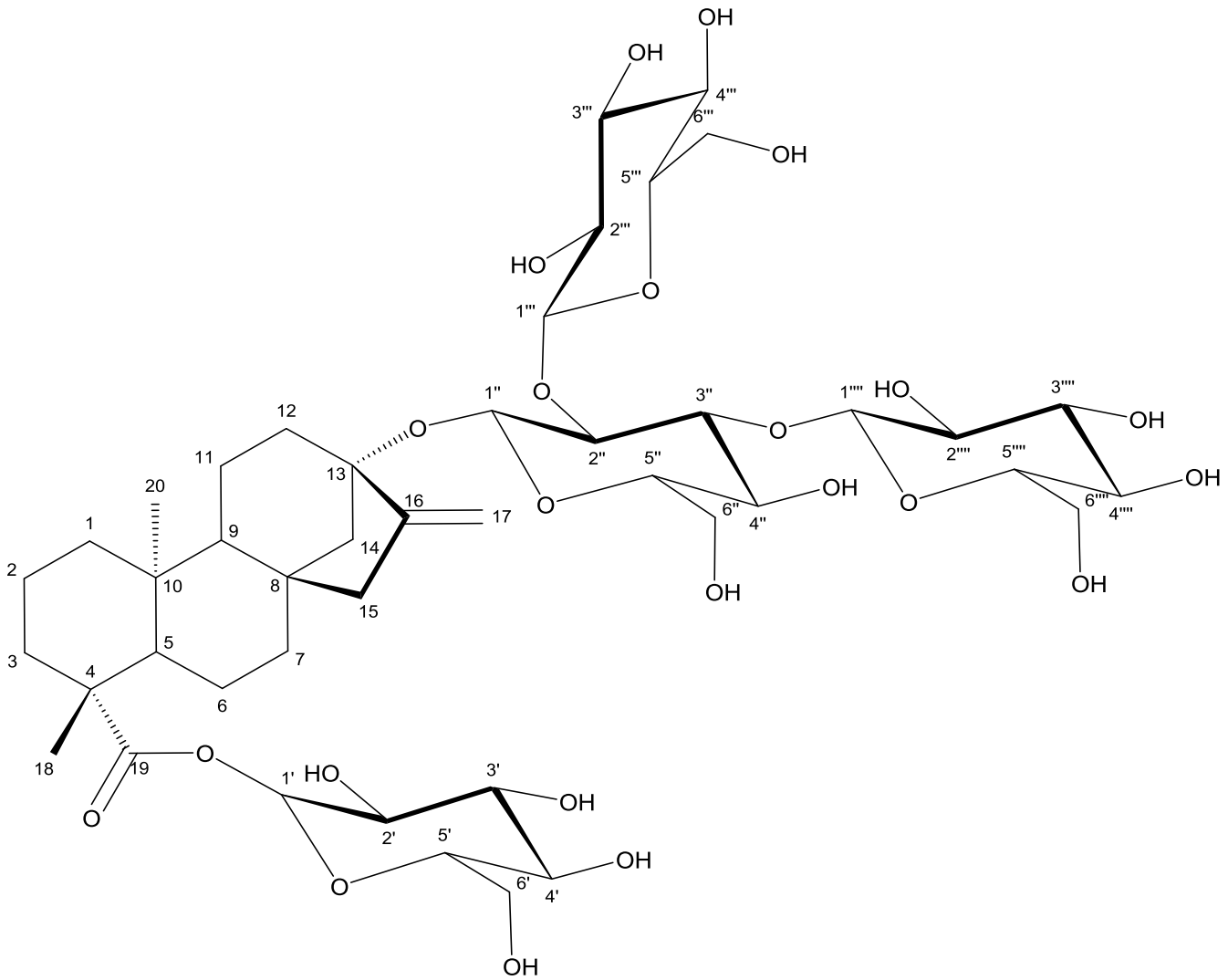

Fig. A. 1. Structure of Rebaudioside A

Parameter	Value
Experiment	1D
Pulse Sequence	PRESAT
Solvent	d2o
Temperature	40.0
Number of Scans	128
Receiver Gain	50
Relaxation Delay	2.0000
Pulse Width	7.2500
Pulse Width 90	7
Nucleus	1H
Spectrometer Frequency	599.50
Spectral Width	9588.9
Lowest Frequency	-1229.9
Acquired Size	19178
Spectral Size	262144

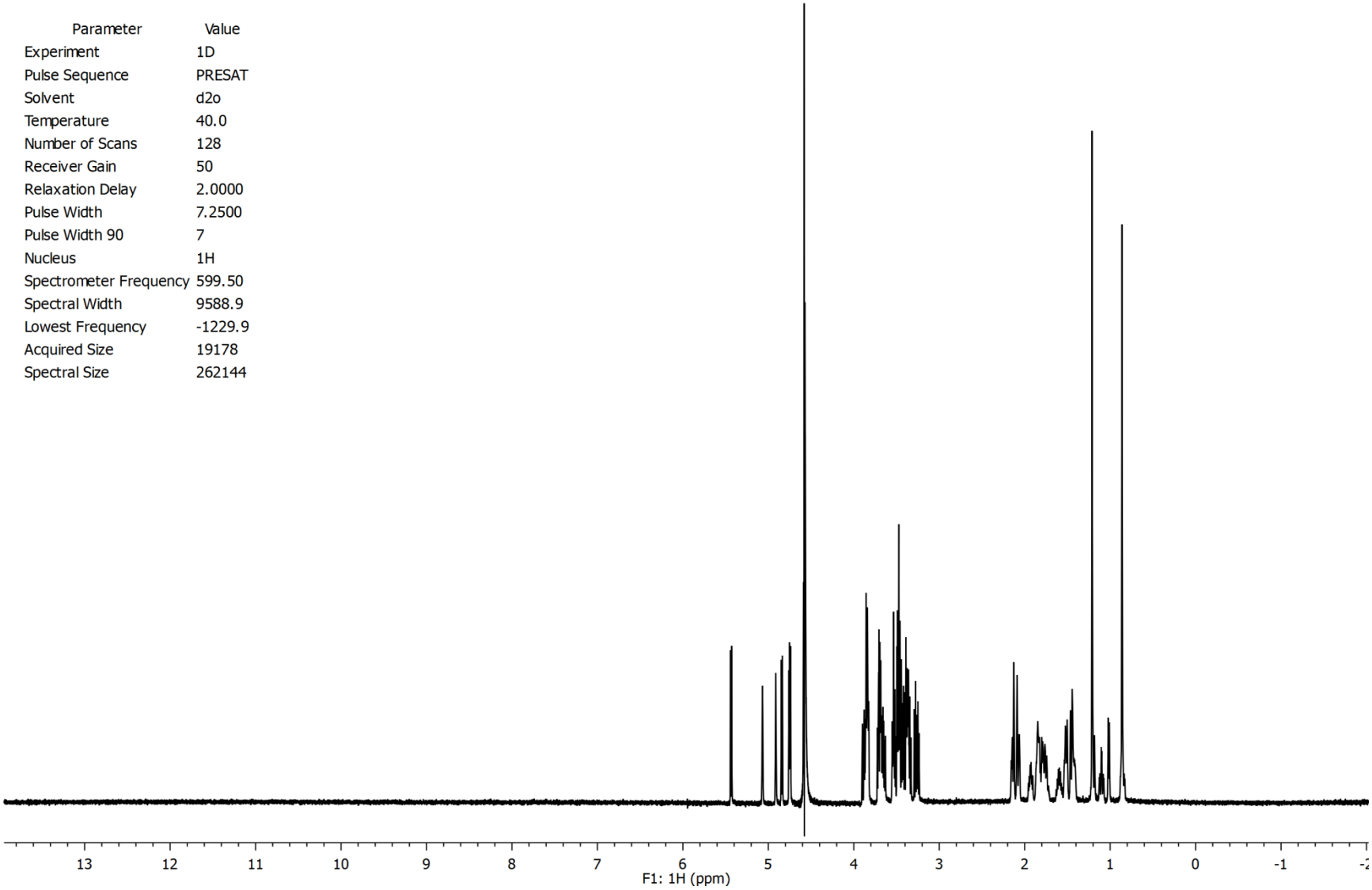

Fig. A. 2. 600 MHz ^{1}H NMR spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. HOD signal was suppressed by presaturation.

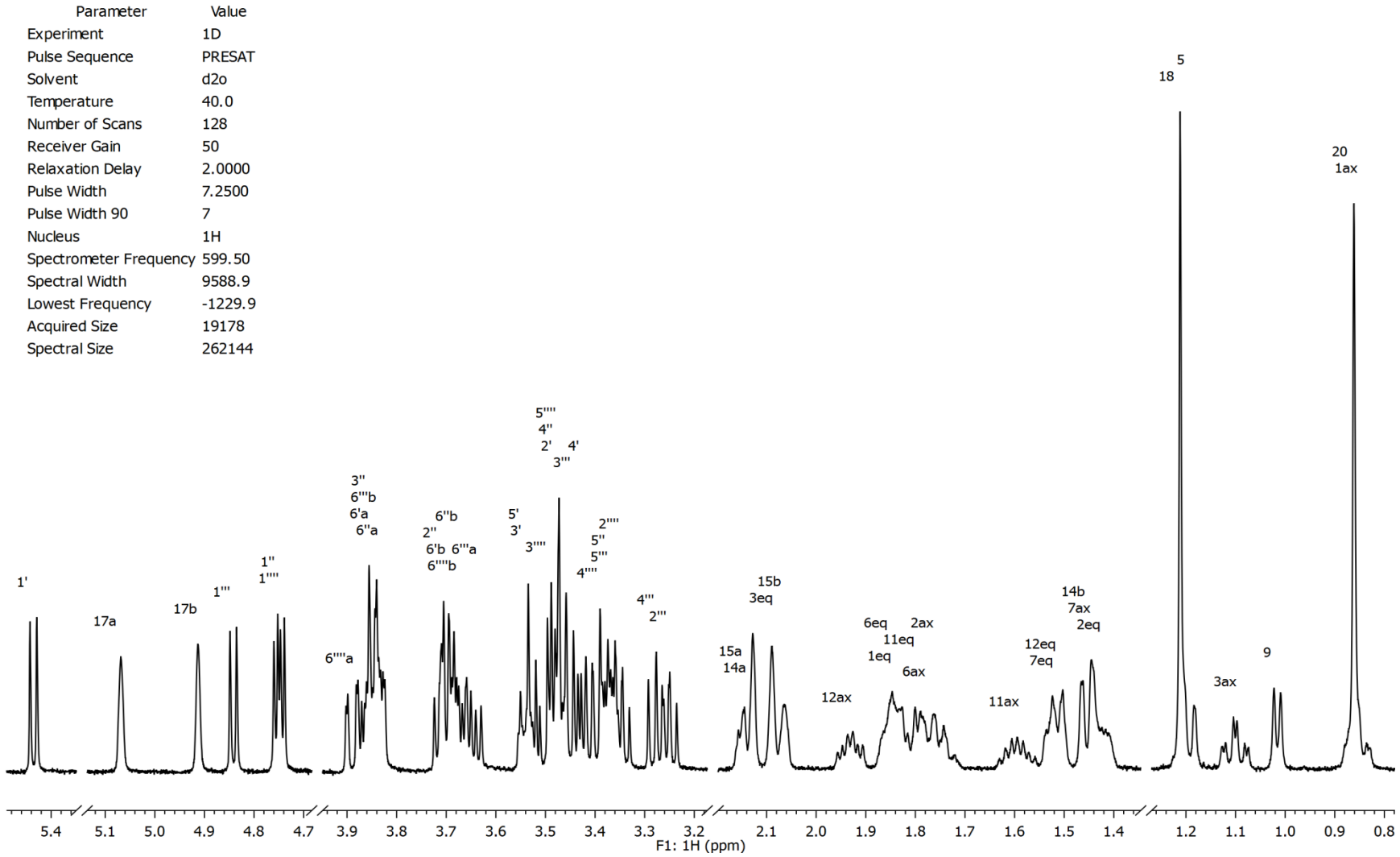

Fig. A. 3. 600 MHz ^{1}H NMR spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Display cut to show only regions of interest. Numbers indicate assignments. HOD signal was suppressed by presaturation.

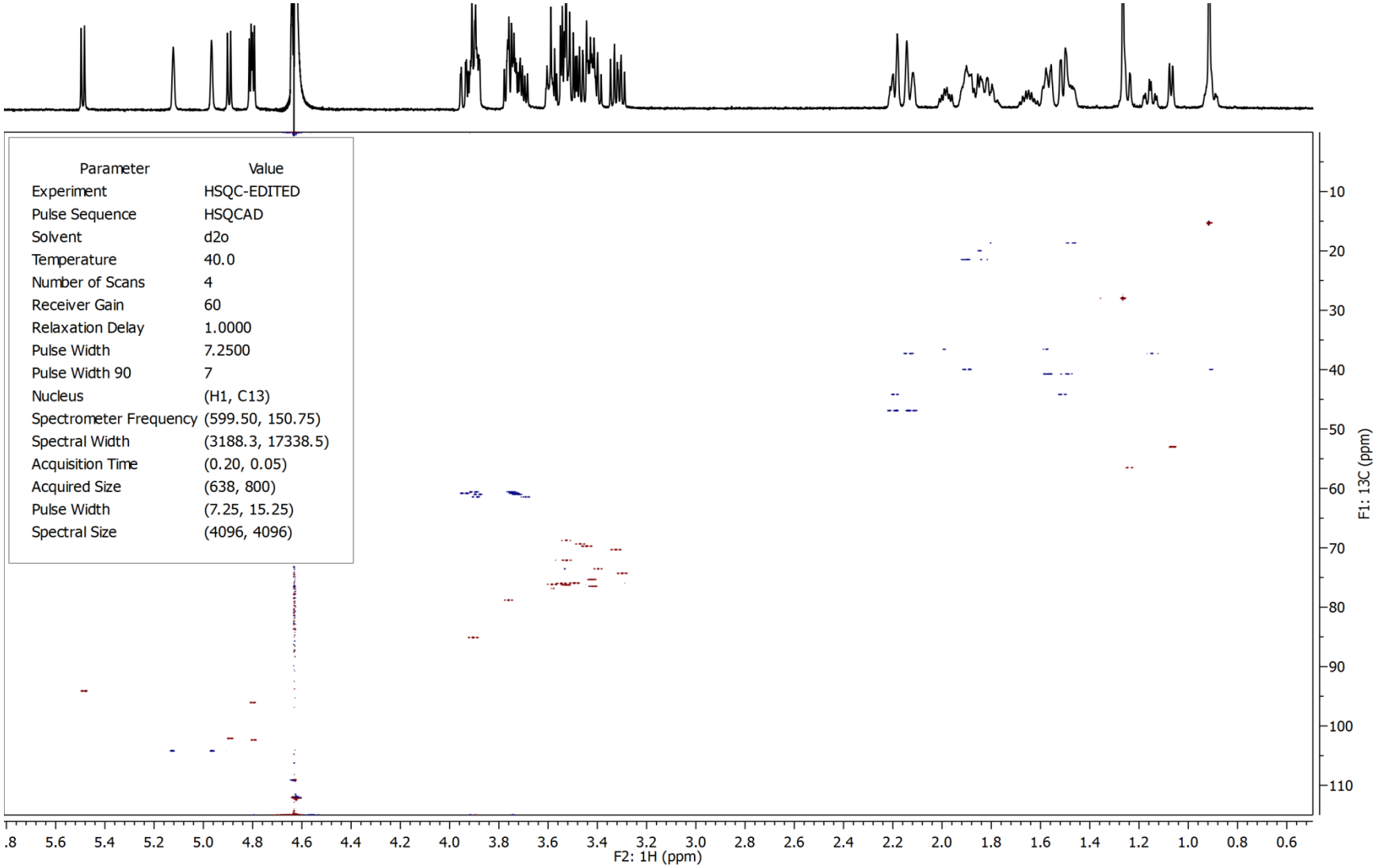

Fig. A. 4. Multiplicity-edited HSQCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C, optimized for $^{1}J_{C,H}$ = 146 Hz. Red contours indicate methines and methyls, blue contours indicate methylenes.

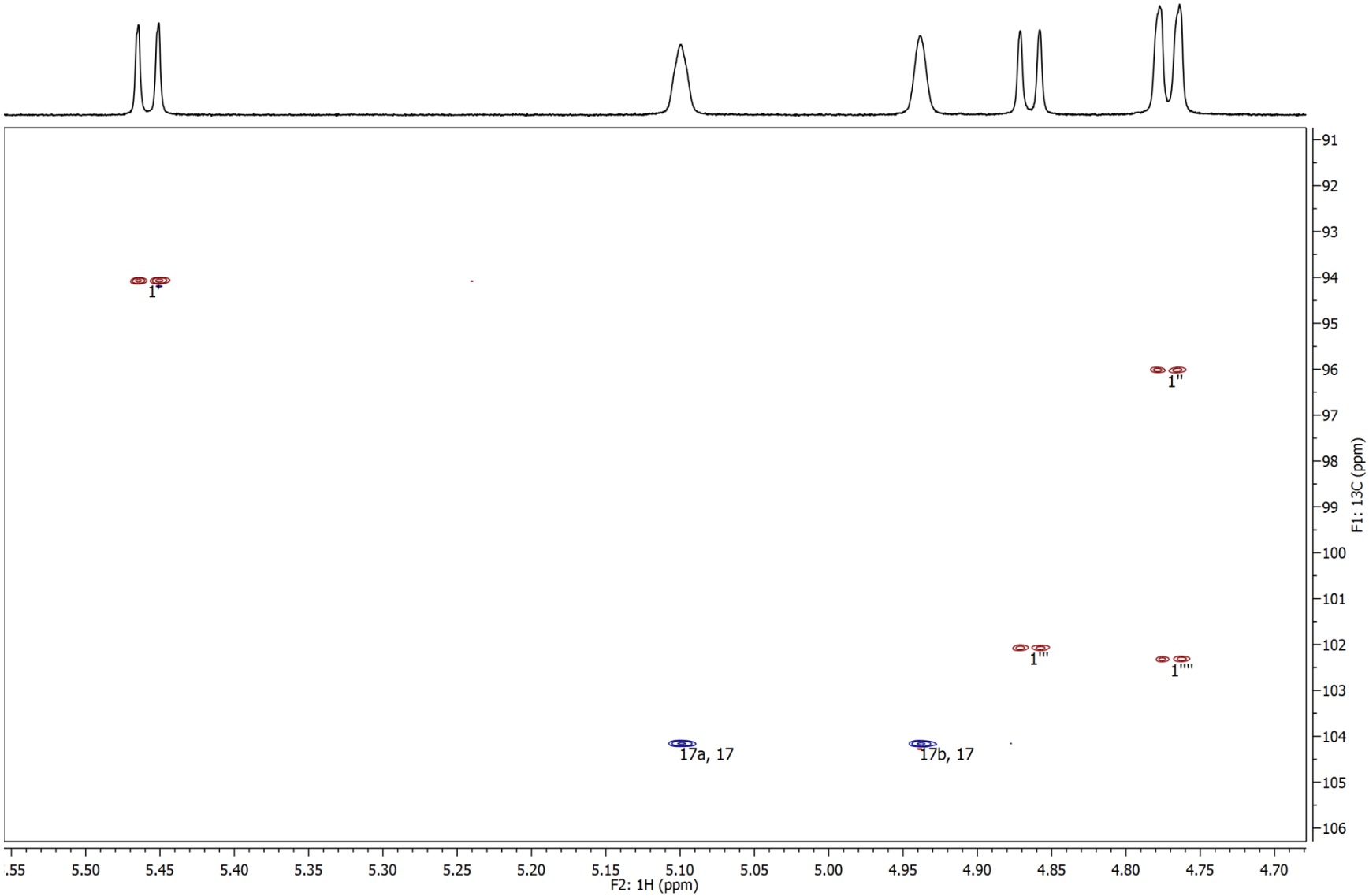

Fig. A. 5. Expansion of the anomeric/vinylic region of multiplicity-edited HSQCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Red contours indicate methines and methyls, blue contours indicate methylenes. Numbers indicate assignments.

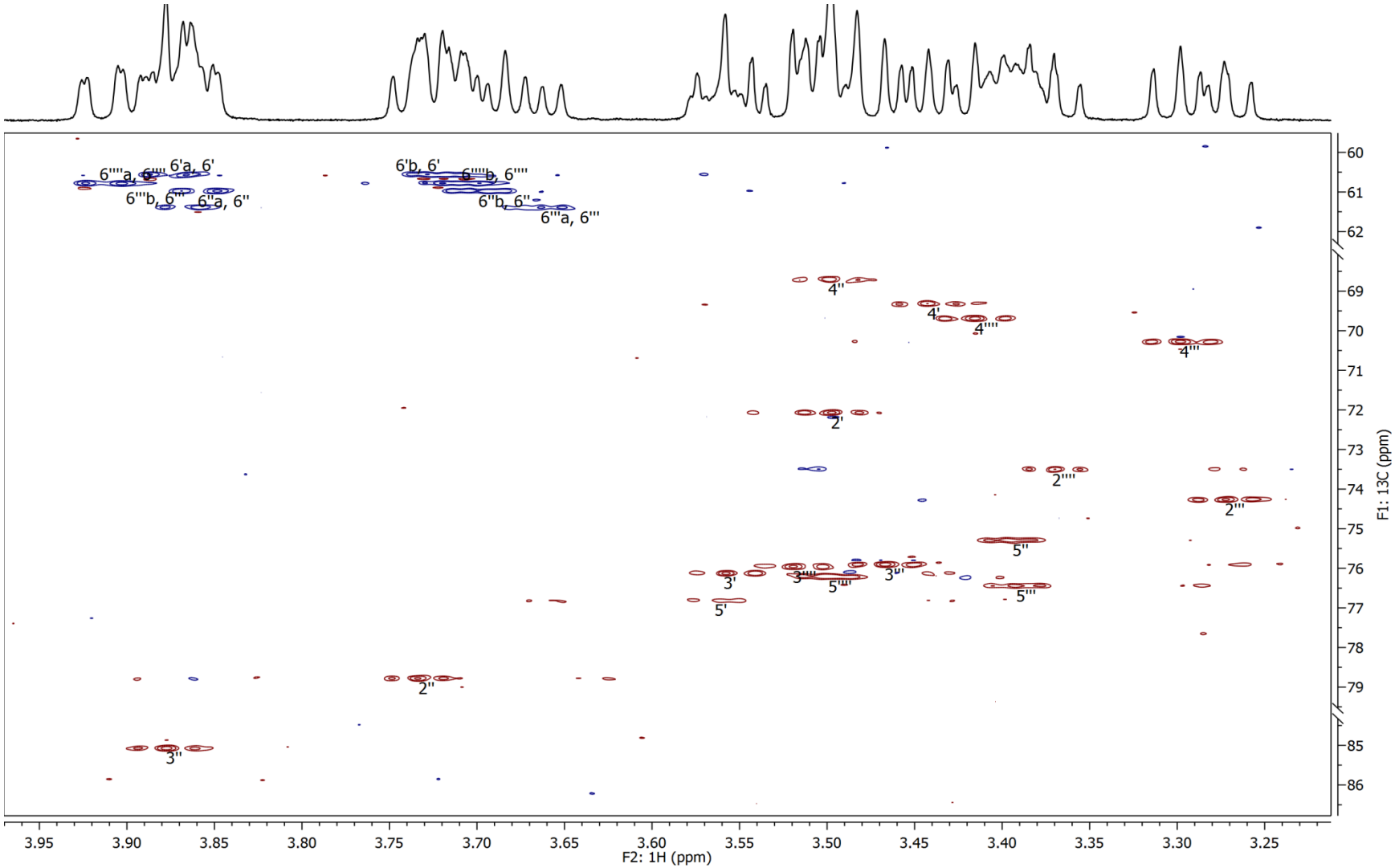

Fig. A. 6. Expansion of the carbohydrate region of multiplicity-edited HSQCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C with display cut to regions of interest. Red contours indicate methines and methyls, blue contours indicate methylenes. Numbers indicate assignments.

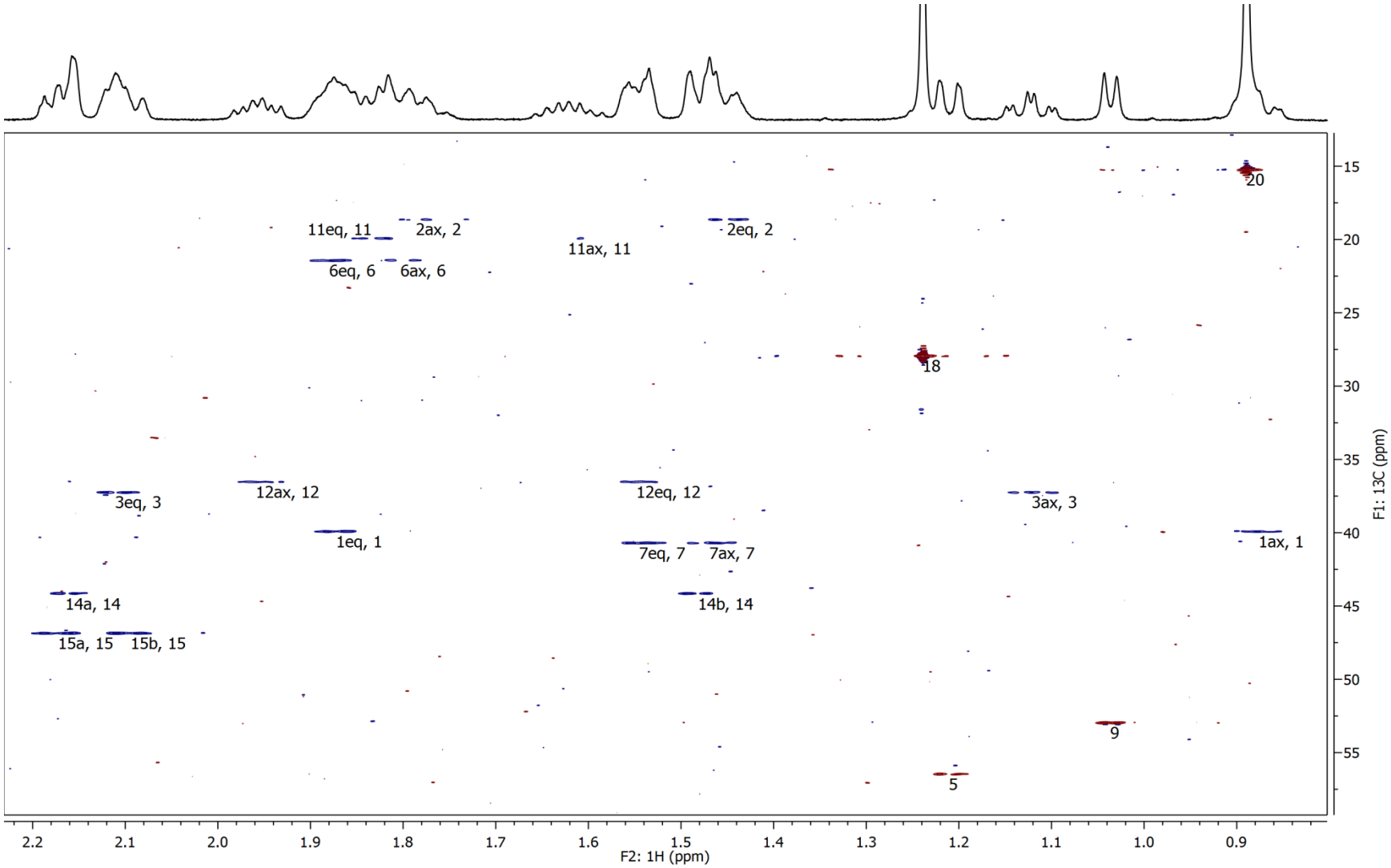

Fig. A. 7. Expansion of the aliphatic region of multiplicity-edited HSQCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Red contours indicate methines and methyls, blue contours indicate methylenes. Numbers indicate assignments.

Parameter	Value
Experiment	HMBC
Pulse Sequence	gHMBCAD
Solvent	d2o
Temperature	40.0
Number of Scans	8
Receiver Gain	60
Relaxation Delay	1.0000
Nucleus	(H1, C13)
Spectrometer Frequency	(599.50, 150.76)
Spectral Width	(3188.3, 31658.1)
Acquisition Time	(0.30, 0.06)
Acquired Size	(956, 1800)
Pulse Width	(7.25, 15.25)
Spectral Size	(2048, 4096)

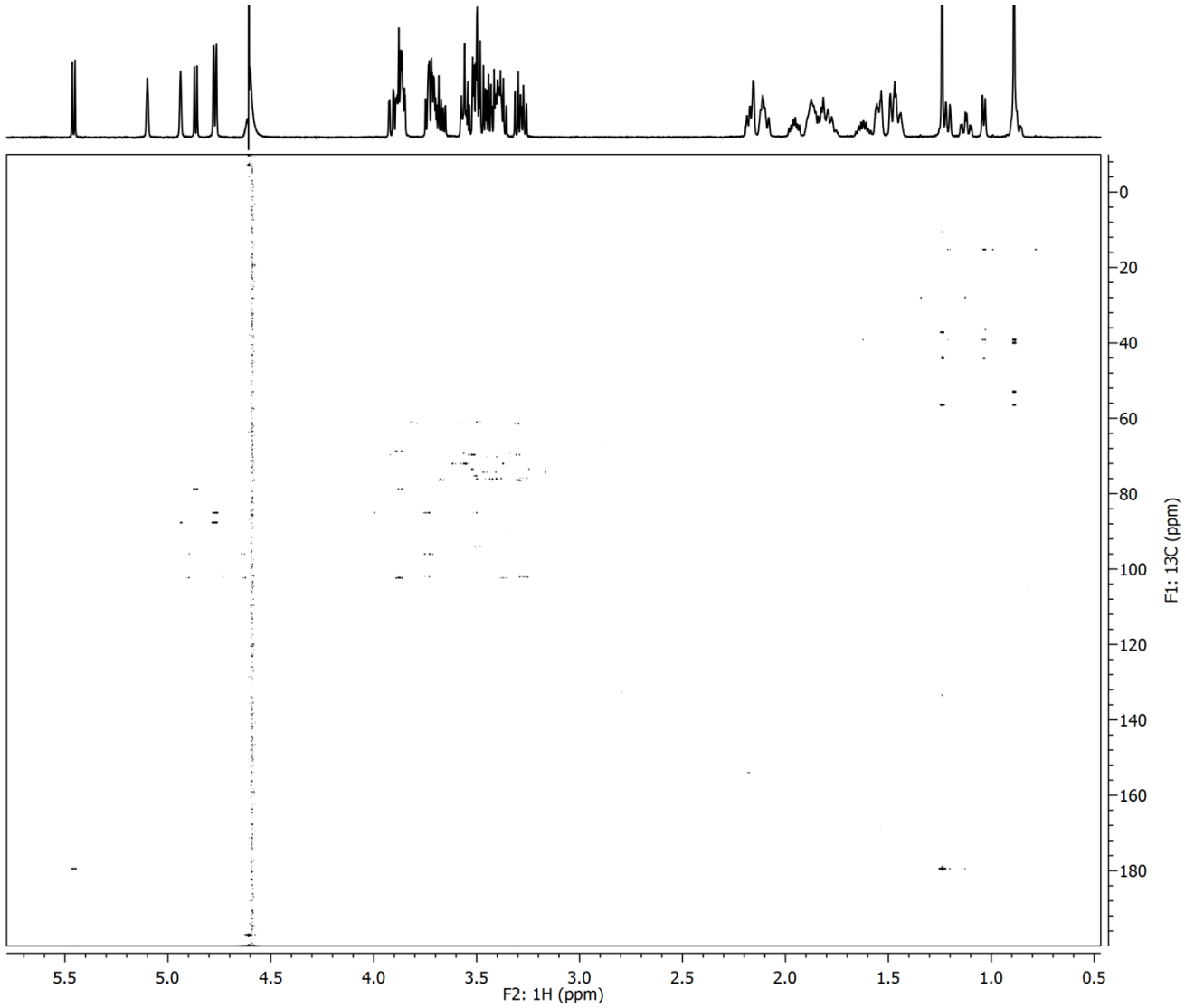

Fig. A. 8. Gradient-selected HMBCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C.

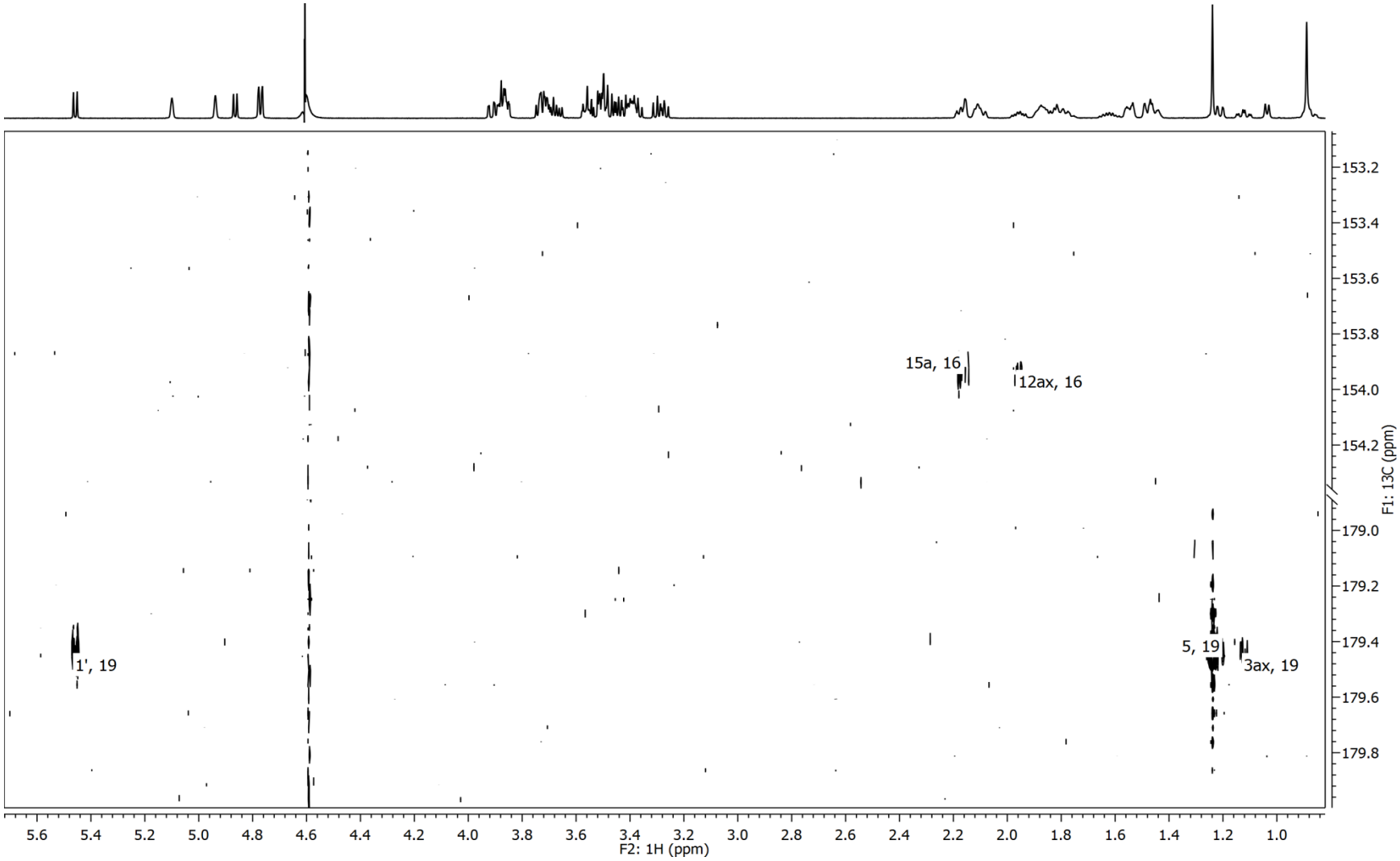

Fig. A. 9. Expansion of the vinylic/carbonyl region of the gHMBCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Display cut to regions of interest. Numbers indicate assignments.

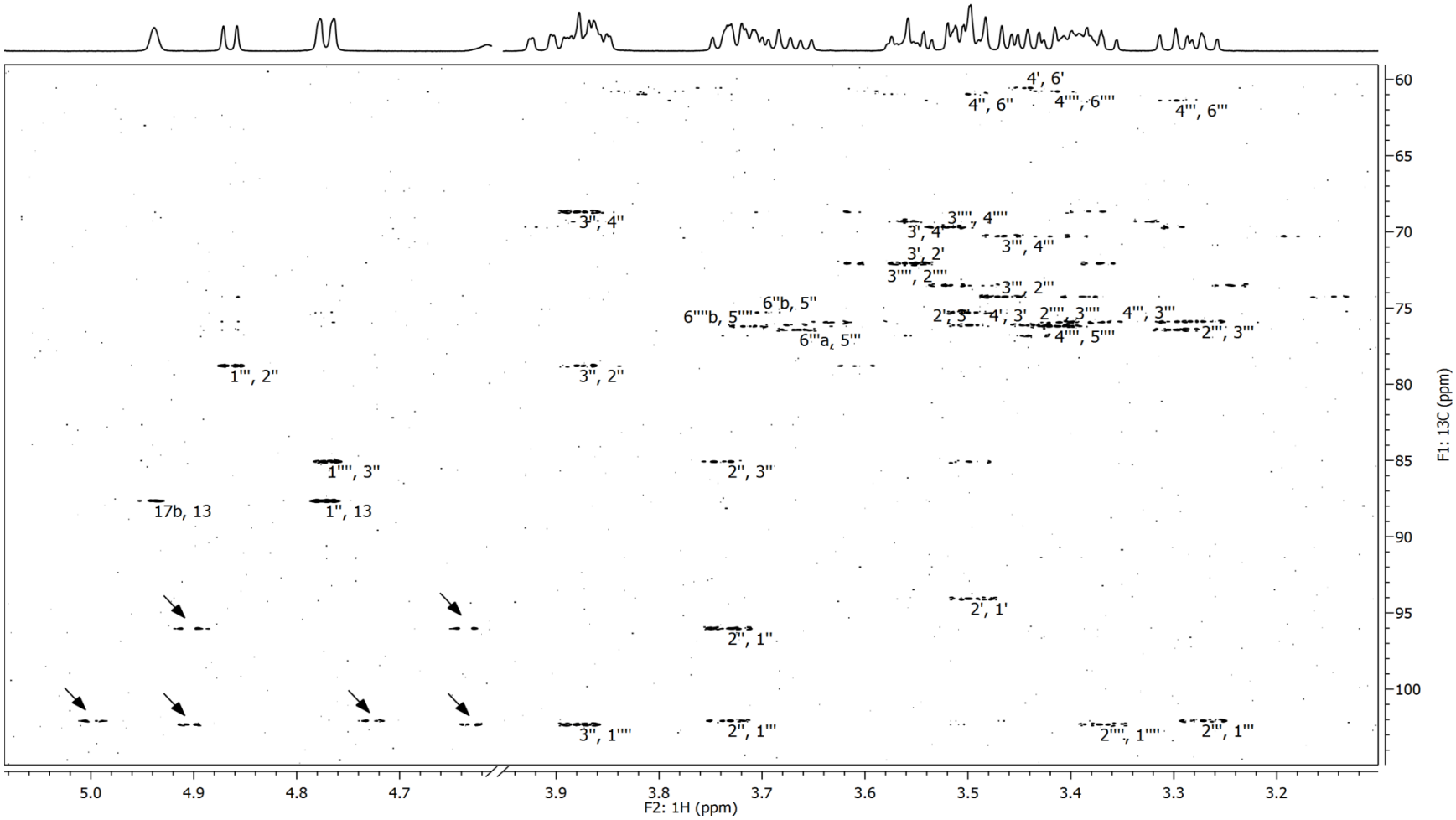

Fig. A. 10. Expansion of the carbohydrate region of the gHMBCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Display cut to regions of interest. Numbers indicate assignments, arrows indicate residual HMQC correlations.

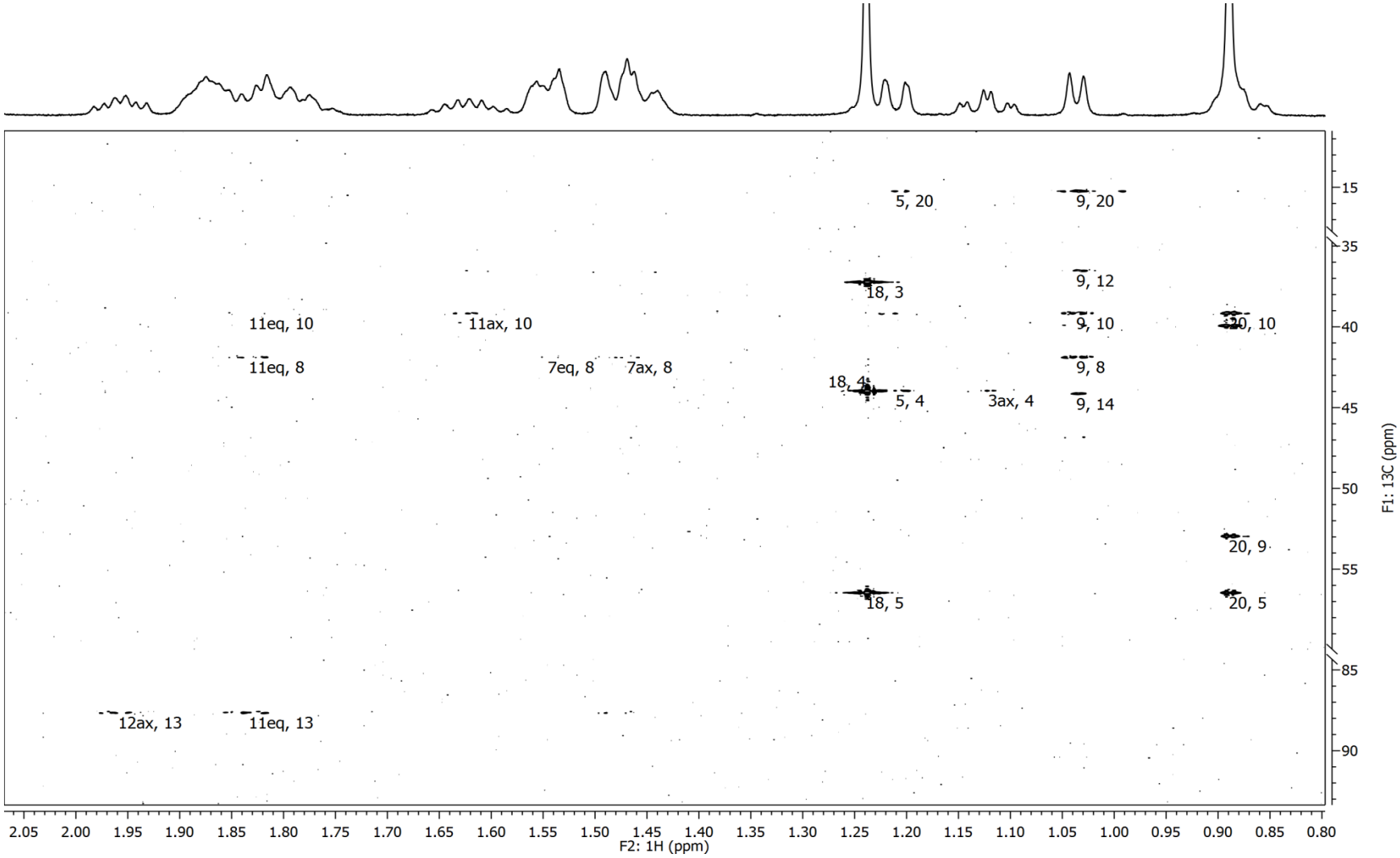

Fig. A. 11. Expansion of the aliphatic region of the gHMBCAD spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Display cut to regions of interest. Numbers indicate assignments.

Parameter	Value
Experiment	COSY
Pulse Sequence	gCOSY
Solvent	d2o
Temperature	40.0
Number of Scans	1
Receiver Gain	46
Relaxation Delay	1.0000
Pulse Width	7.2500
Nucleus	(H1, H1)
Spectrometer Frequency	(599.50, 599.50)
Spectral Width	(3188.3, 3188.3)
Acquisition Time	(0.60, 0.16)
Acquired Size	(1913, 512)
Spectral Size	(4096, 2048)

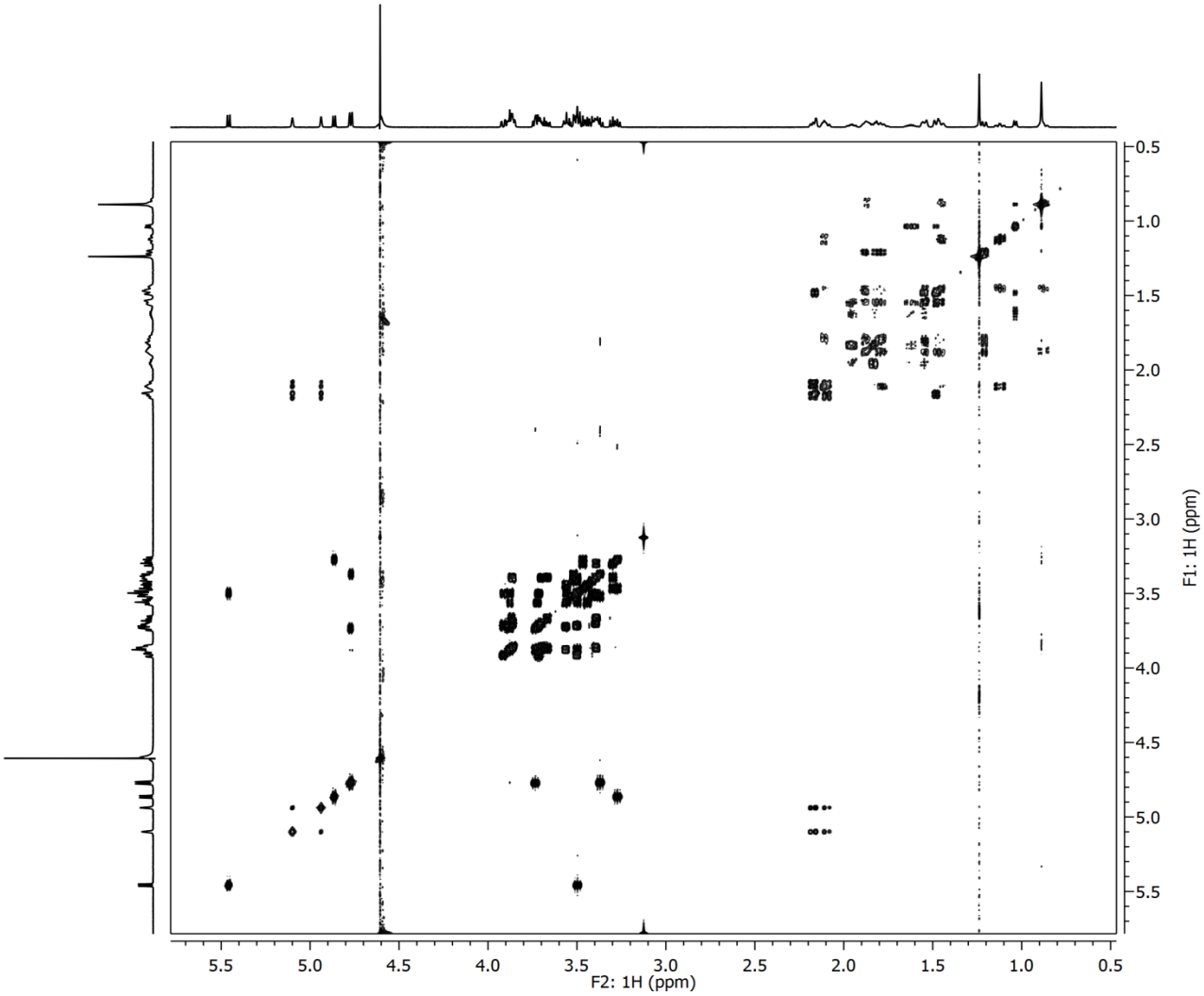

Fig. A. 12. 600 MHz gradient-selected COSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. HOD signal was suppressed by presaturation.

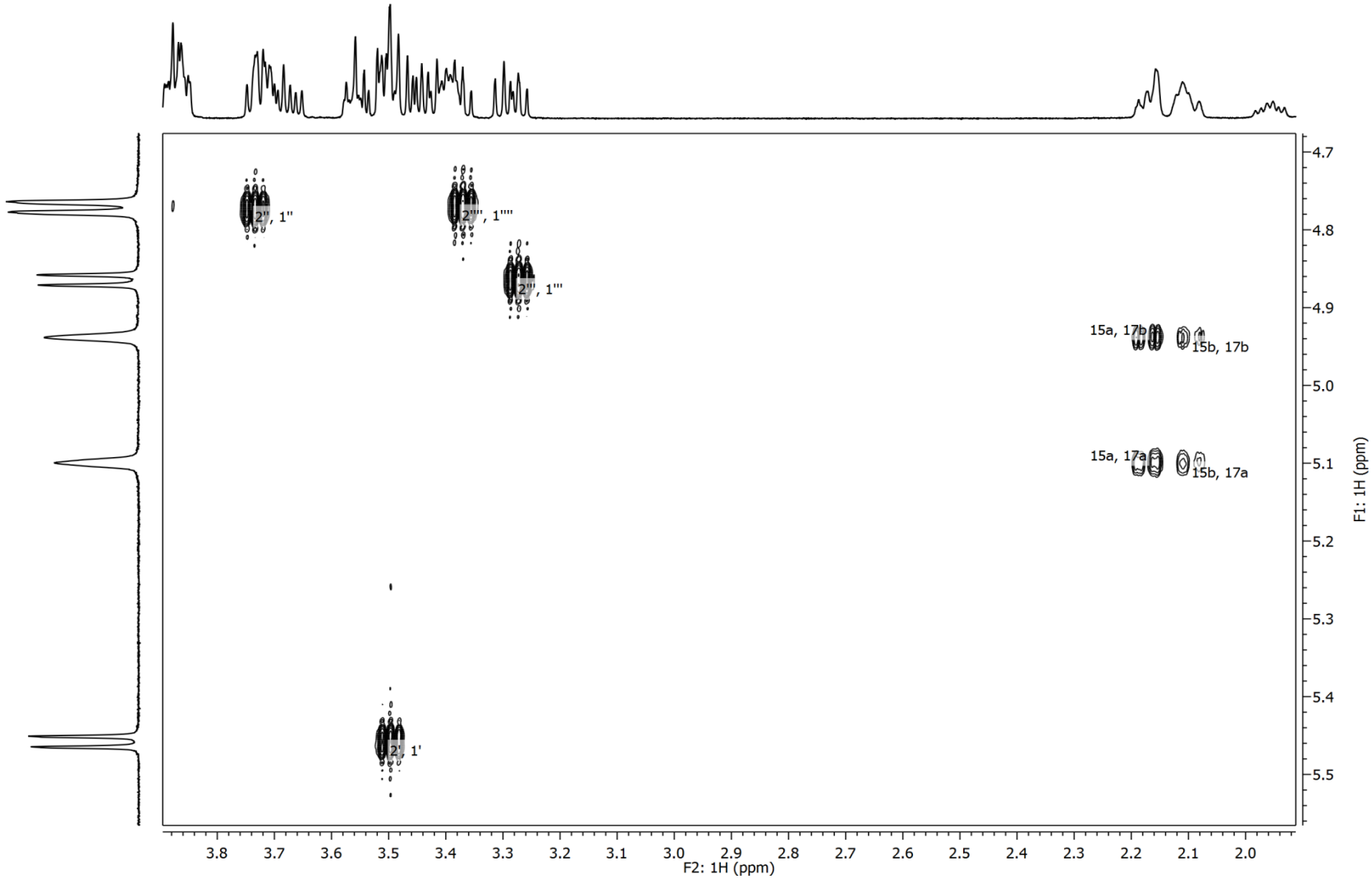

Fig. A. 13. Expansion plot of gCOSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C showing correlations to the anomeric and vinylic hydrogens. Numbers indicate assignments.

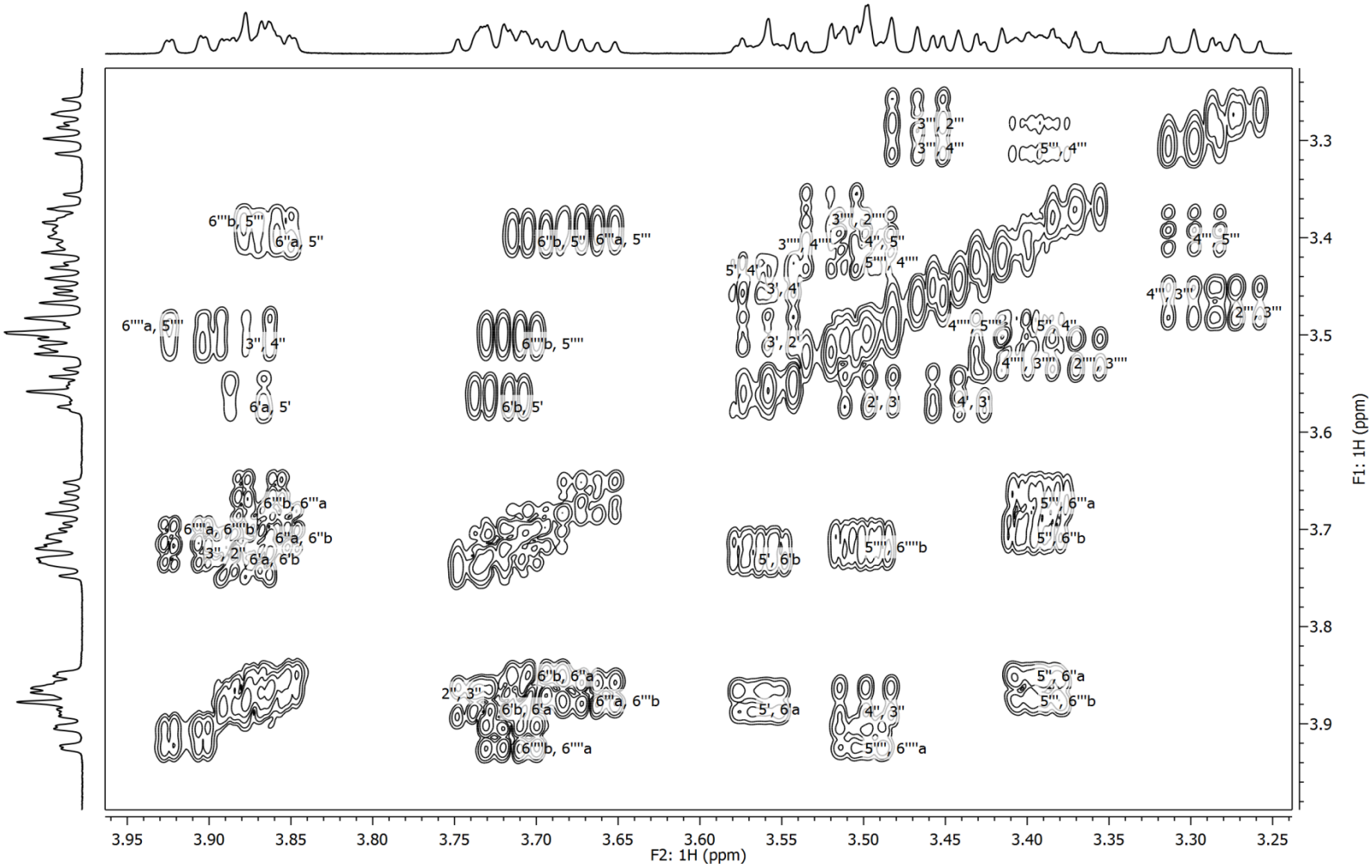

Fig. A. 14. Expansion of the carbohydrate region of gCOSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Numbers indicate assignments.

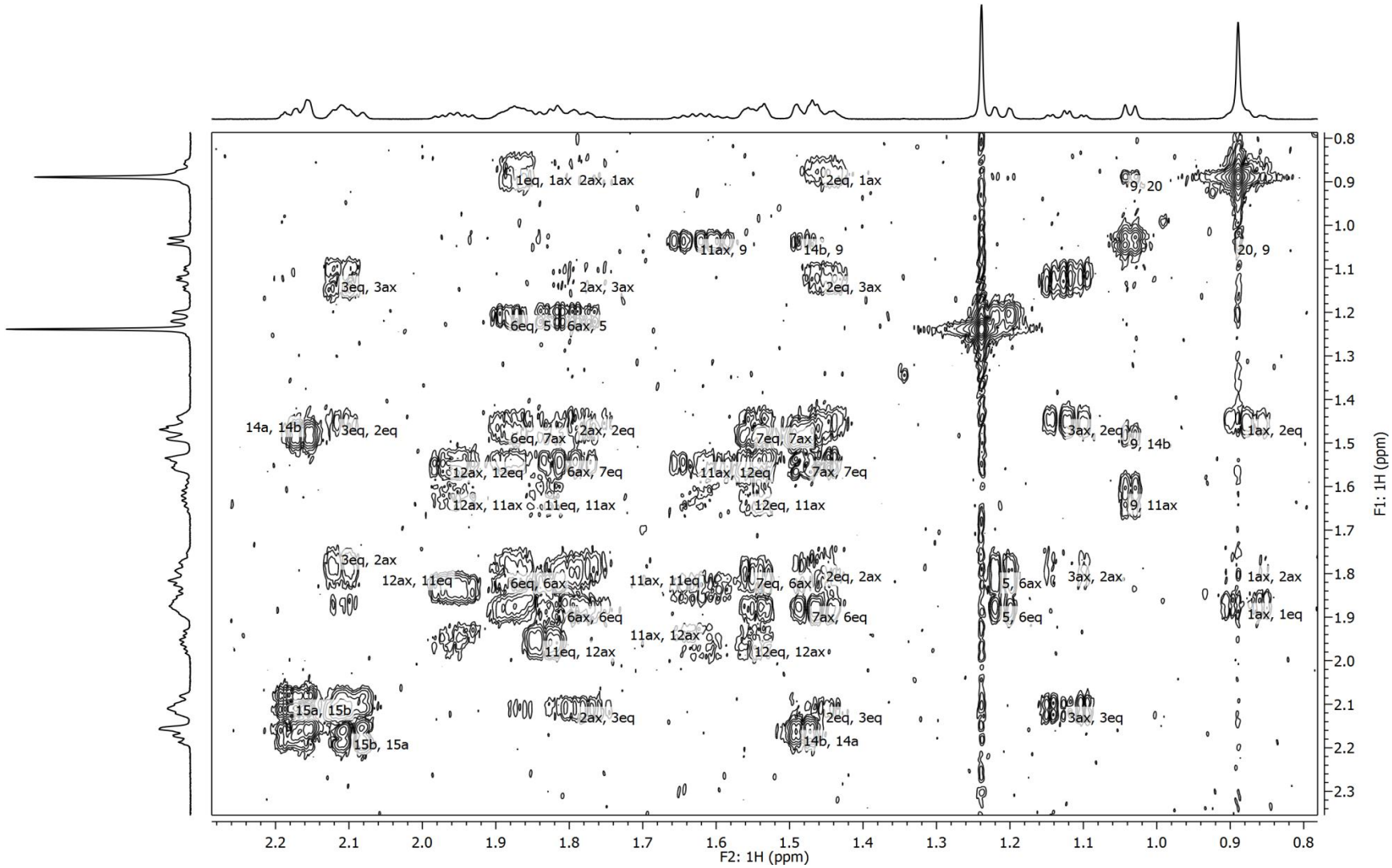

Fig. A. 15. Expansion of the aliphatic region of gCOSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Numbers indicate assignments.

Parameter	Value
Experiment	TOCSY
Pulse Sequence	TOCSY
Solvent	d2o
Temperature	40.0
Number of Scans	2
Receiver Gain	46
Relaxation Delay	1.0000
Pulse Width	7.2500
Nucleus	(H1, H1)
Spectrometer Frequency	(599.50, 599.50)
Spectral Width	(3188.3, 3188.3)
Acquisition Time	(0.50, 0.16)
Acquired Size	(1594, 512)
Spectral Size	(4096, 2048)

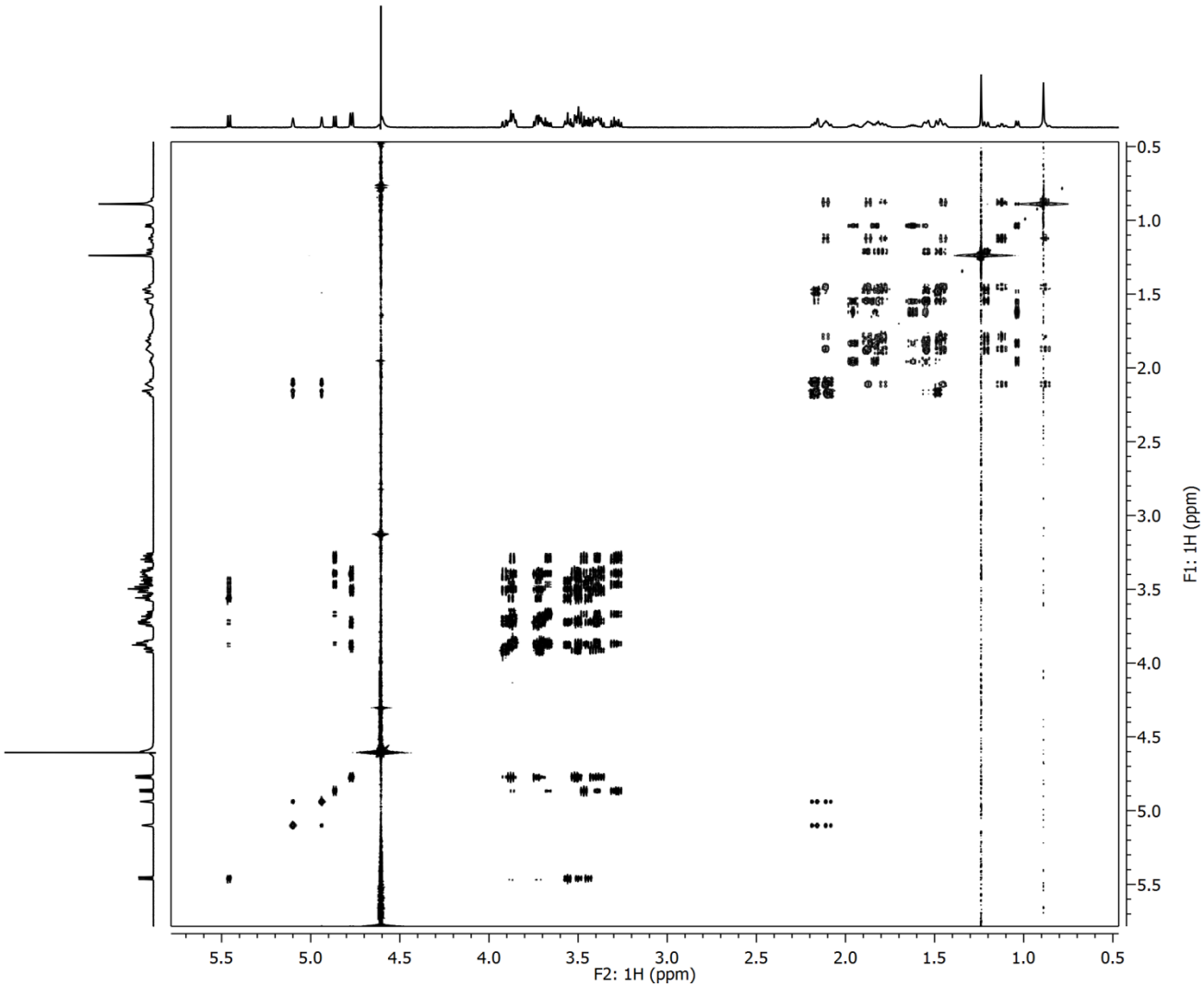

Fig. A. 16. 600 MHz TOCSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. HOD signal was suppressed by presaturation.

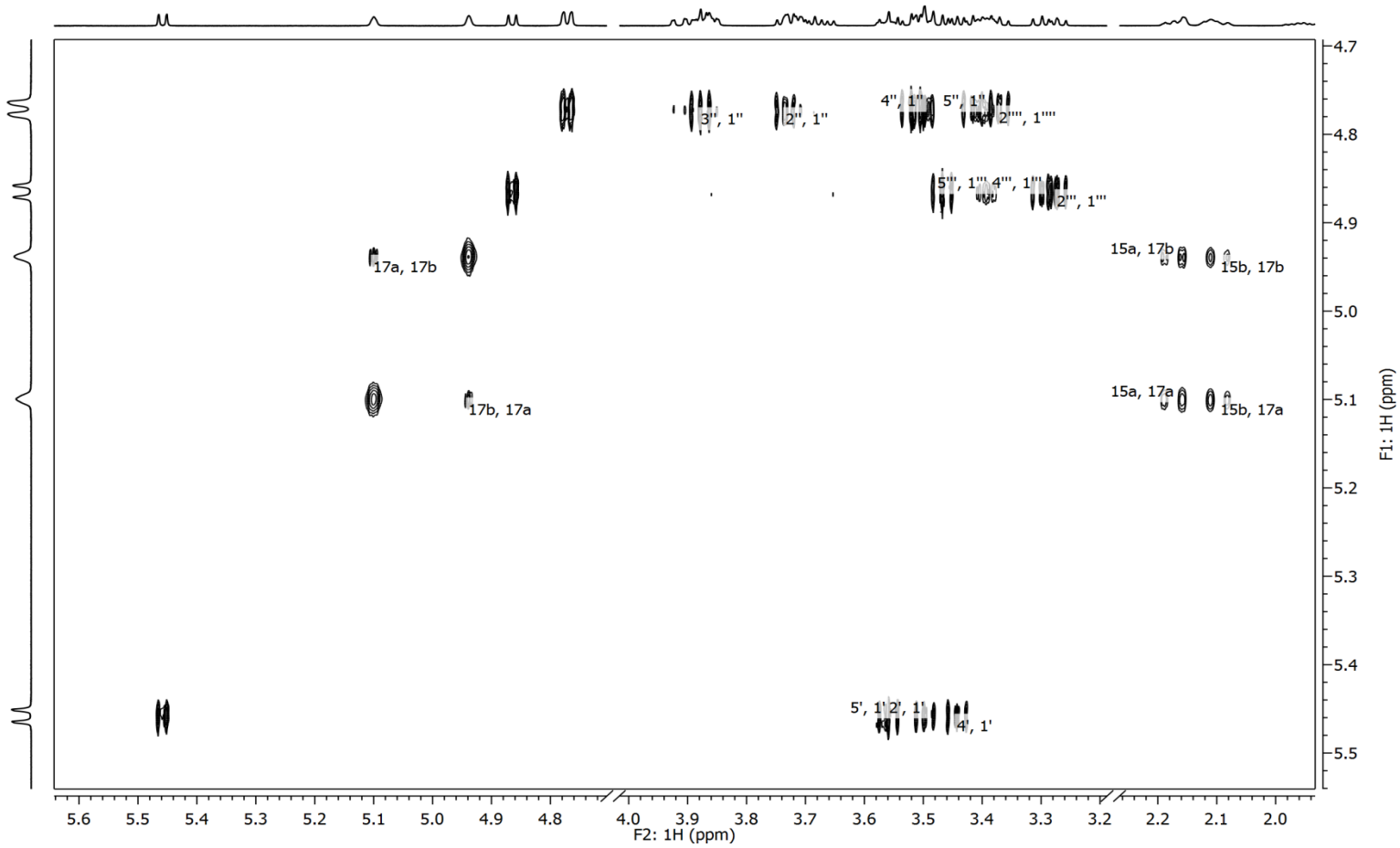

Fig. A. 17. Expansion plot of TOCSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C showing correlations to the anomeric and vinylic hydrogens. Display is cut to regions of interest, numbers indicate assignments.

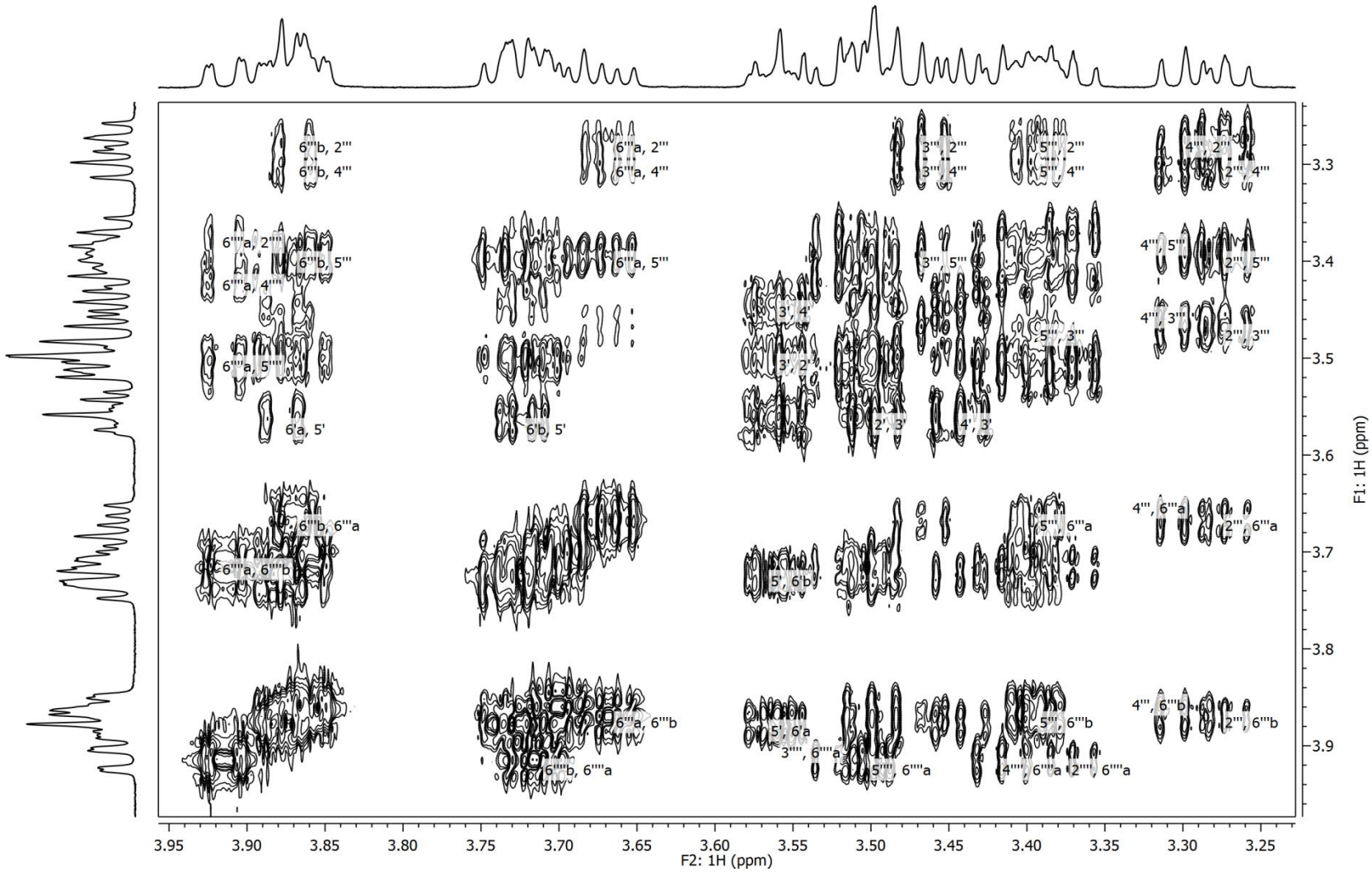

Fig. A. 18. Expansion of the carbohydrate region of TOCSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Numbers indicate assignments.

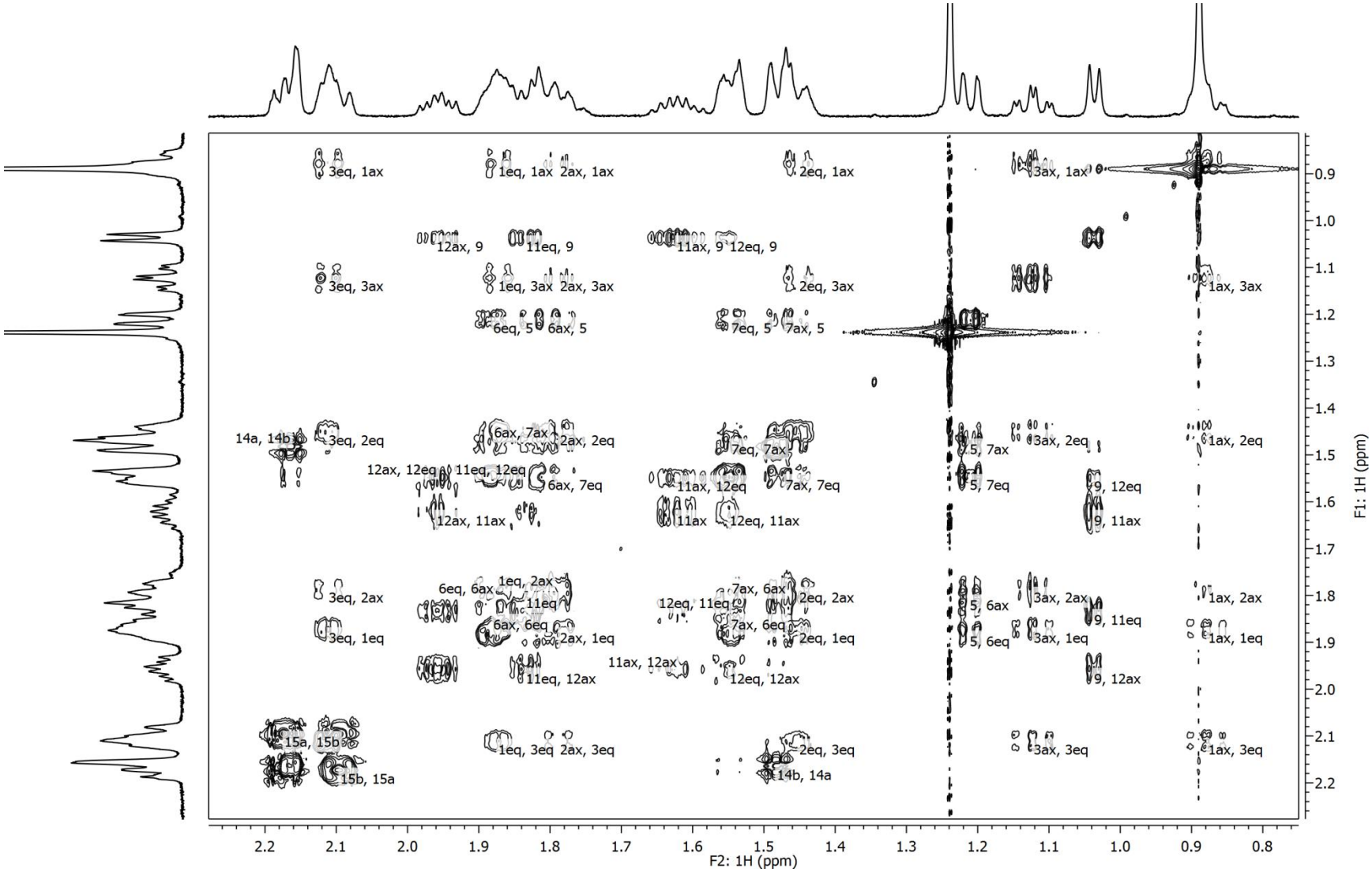

Fig. A. 19. Expansion of the aliphatic region of TOCSY spectrum of 0.5 mM Rebaudioside A in D2O at pH = 3 at 40°C. Numbers indicate assignments.

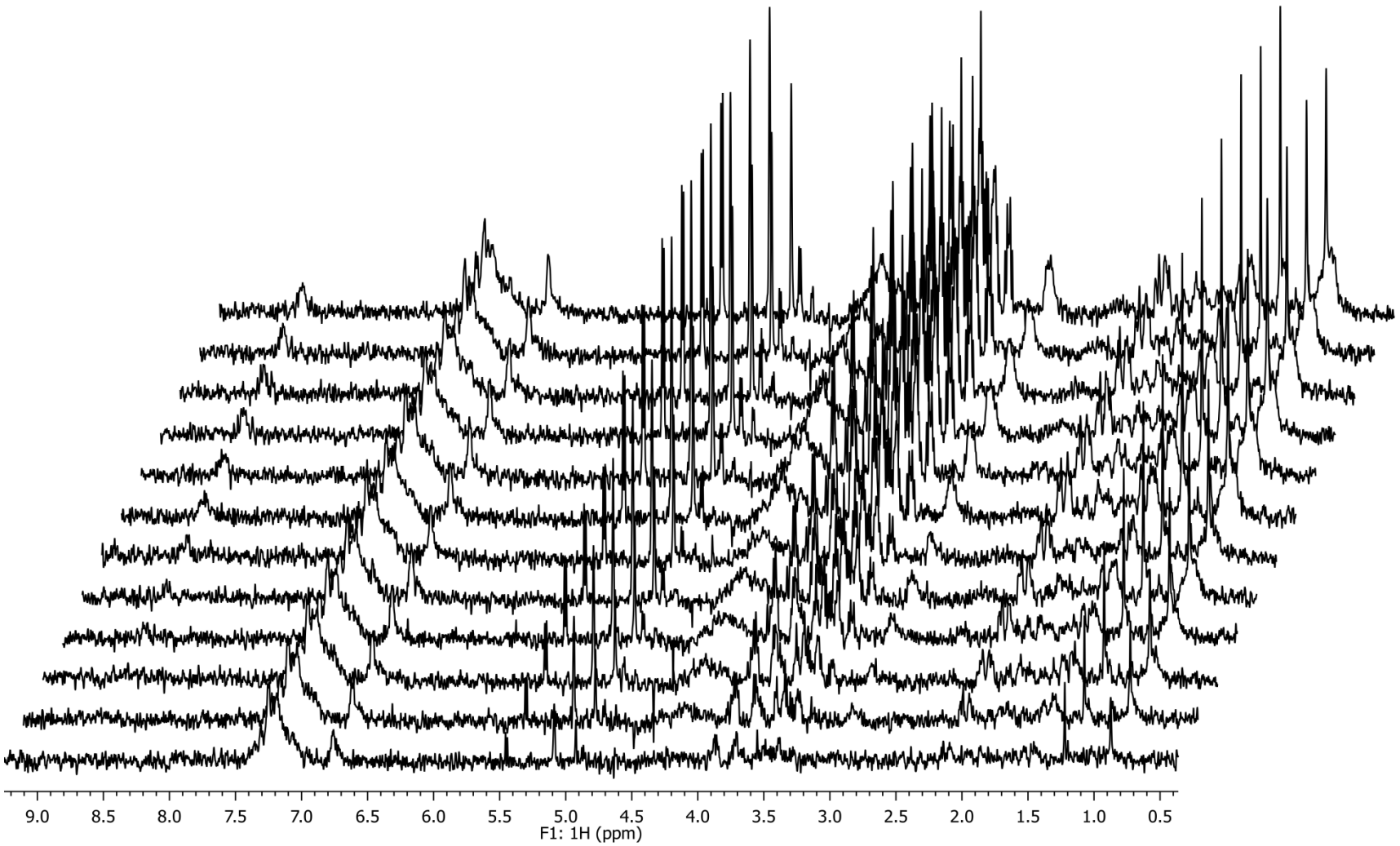

Fig. A. 20. Stack plot of STD experiments on a sample containing 1 mM Reb A and 20 µM BSA at 40°C and pH 3. Aromatic protein resonances at 7.19 ppm were used for saturation and difference spectra were generated internally with off-resonance saturation at 30 ppm. Saturation times were varied from 0.1 to 3.5 s to generate build-up curves.

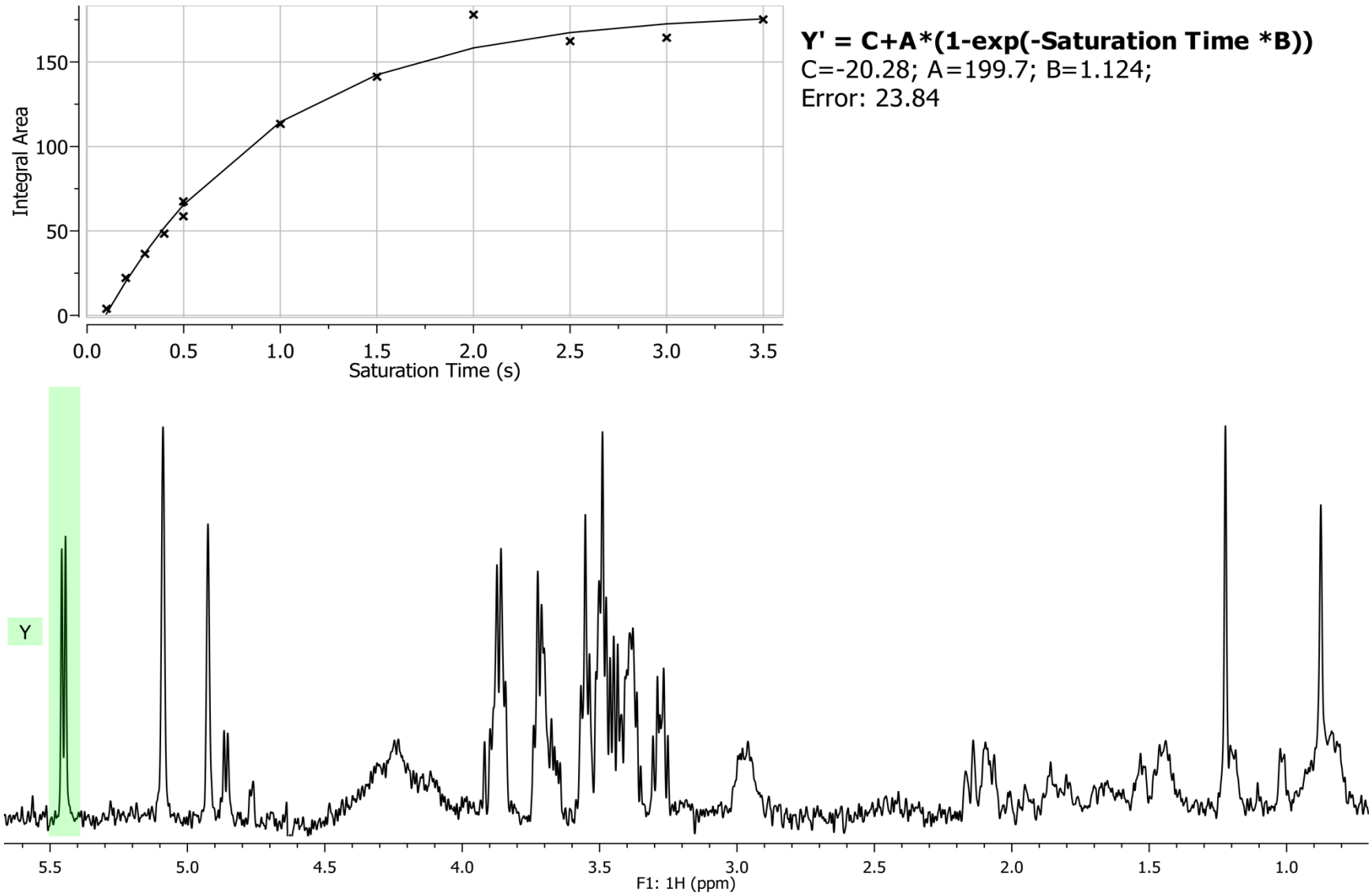

Fig. A. 21. Typical build-up curve for a carbohydrate resonance based on the integral area of the highlighted region. Solid line represents best fit to a single exponential approach function.

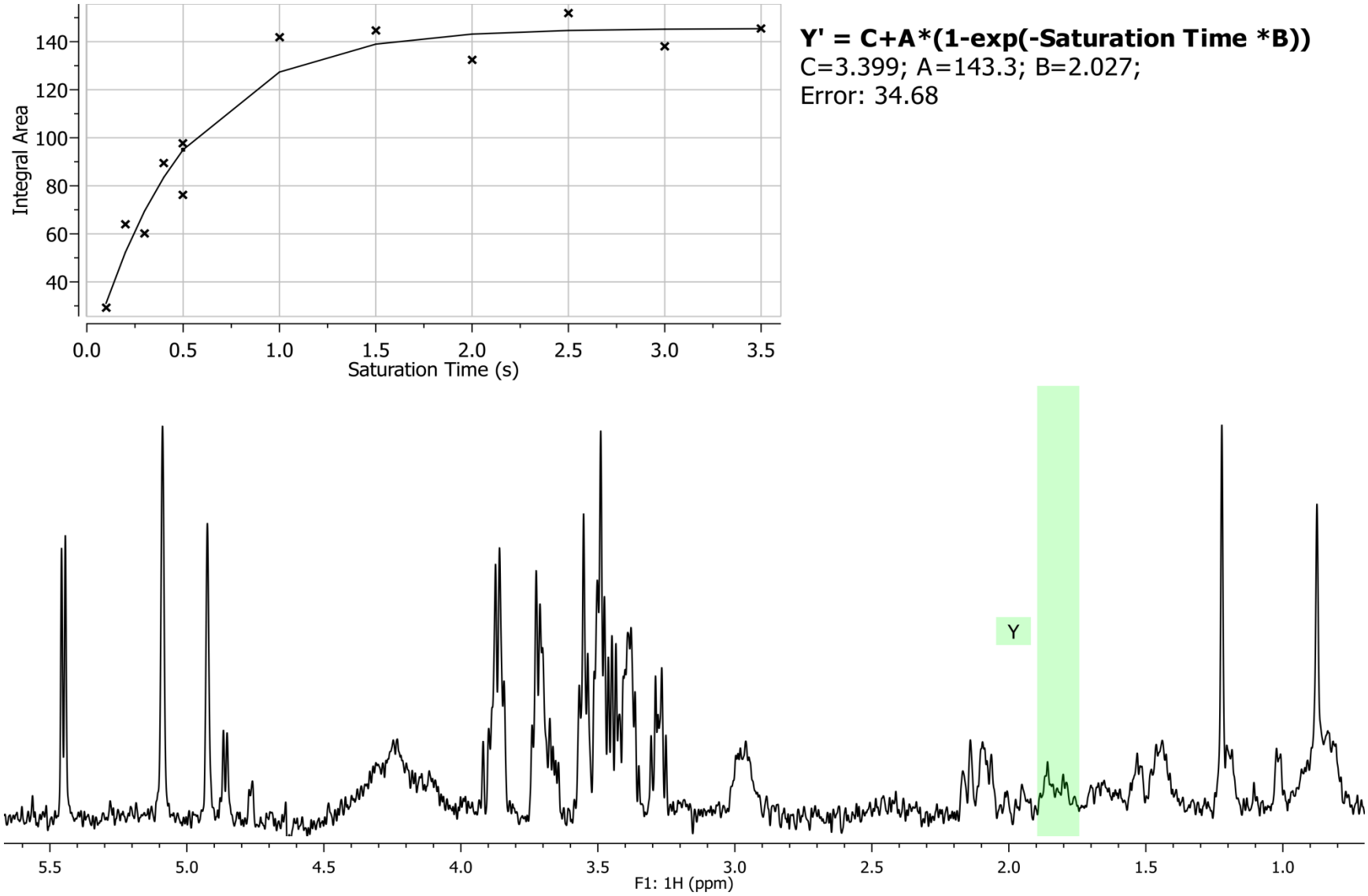

Fig. A. 22. Typical build-up curve for an aliphatic resonance based on the integral area of the highlighted region. Solid line represents best fit to a single exponential approach function.

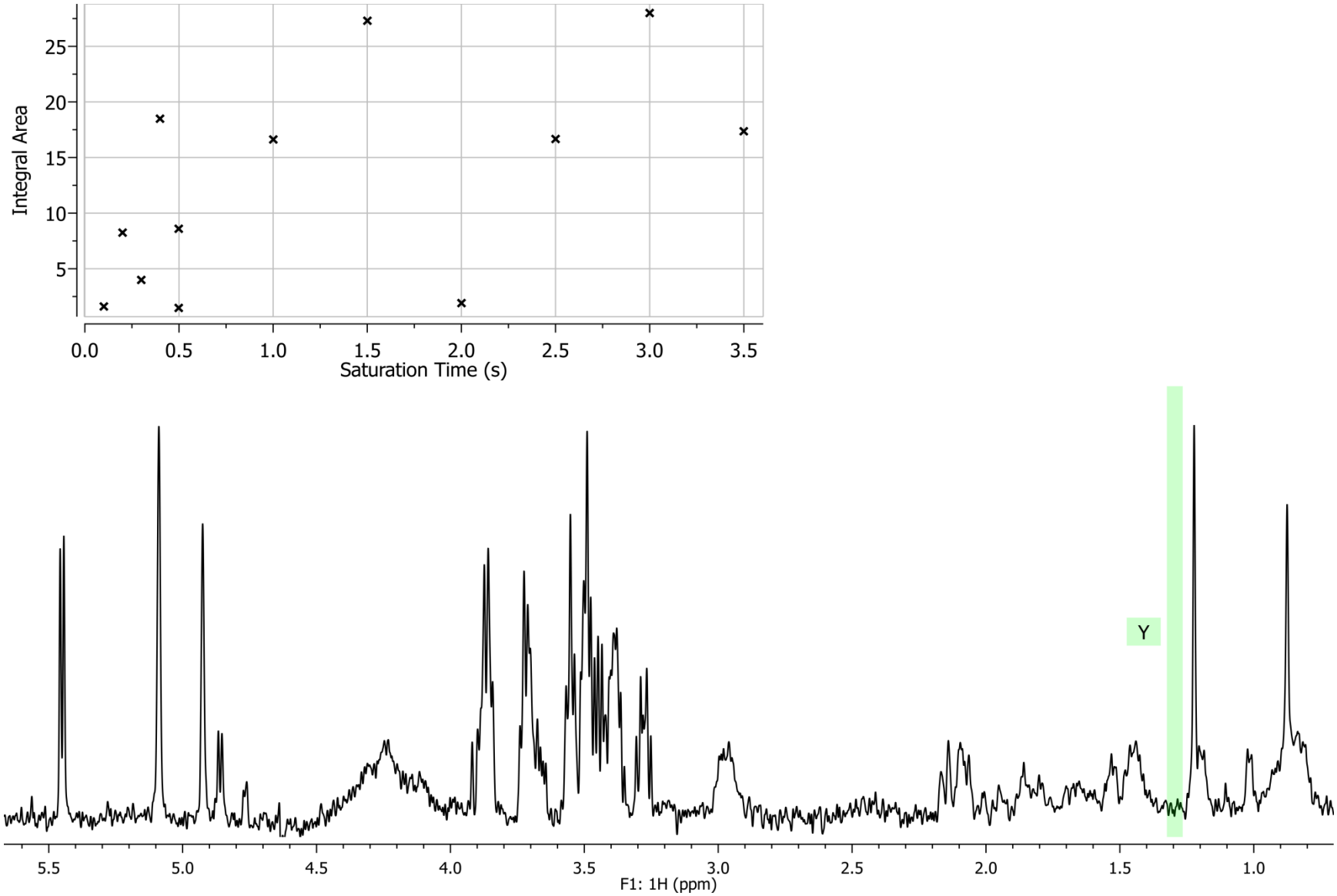

Fig. A. 23. Typical integrals for the baseline in the aliphatic region based on the highlighted area.

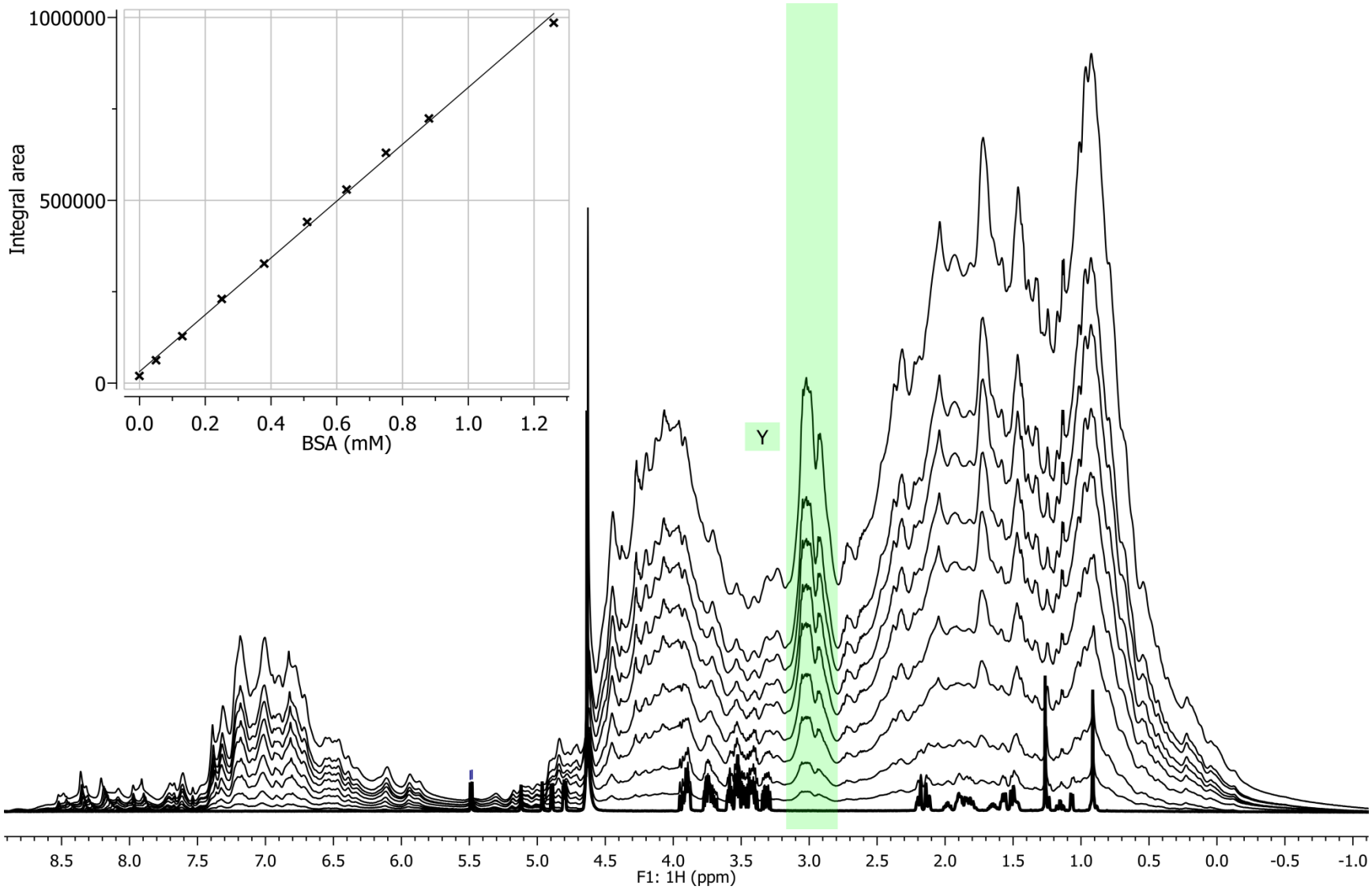

Fig. A. 24. Superimposed spectra from the titration of a 0.5 mM solution of Reb A with BSA at pH 6.7 and 40°C. Control spectrum of Reb A is highlighted in black. Graph shows a plot of integral area for BSA resonances vs. nominal BSA concentrations.

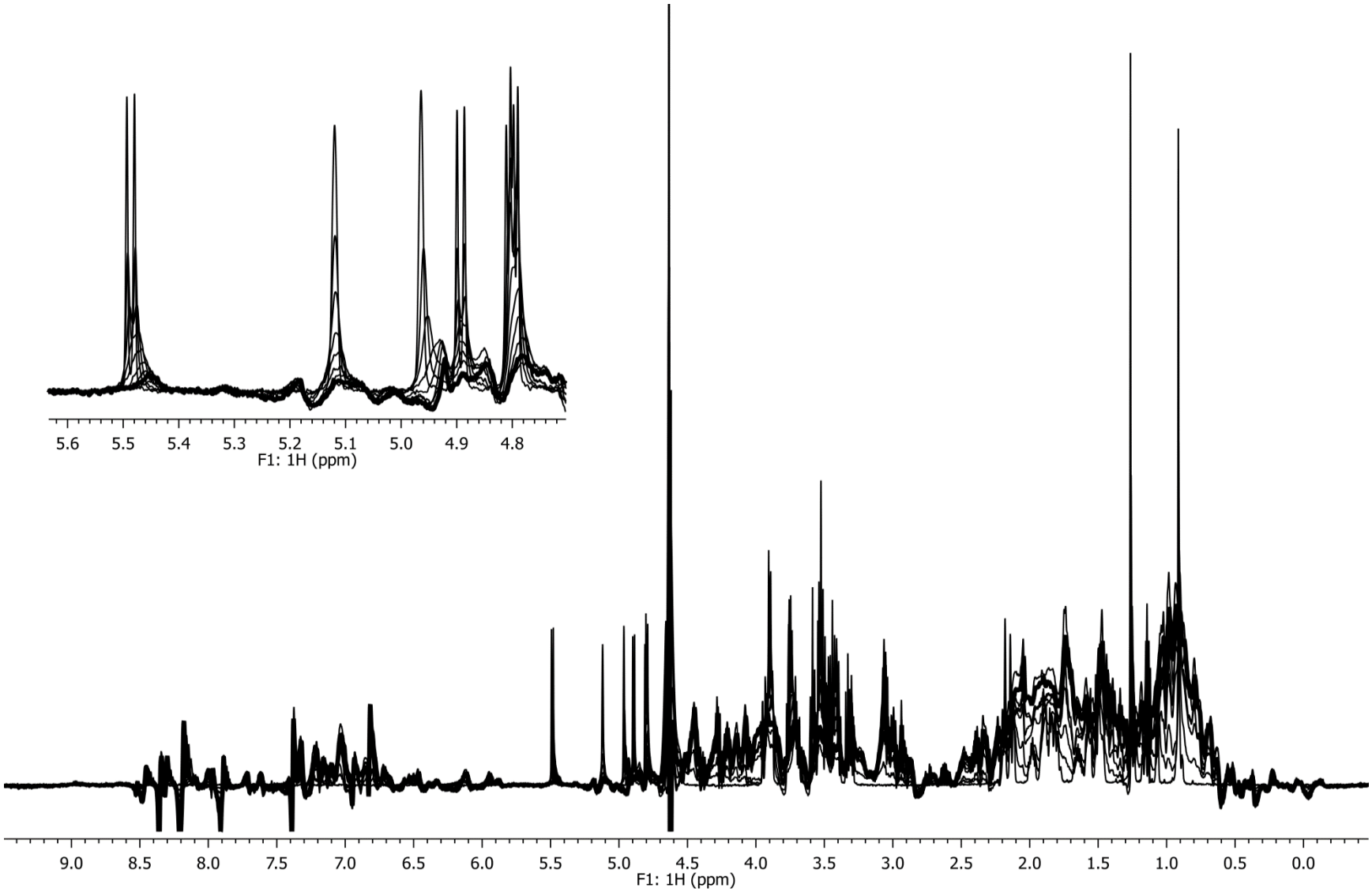

Fig. A. 25. Superimposed spectra from the titration of a 0.5 mM solution of Reb A with BSA at pH 6.7 and 40°C after subtraction of the appropriately scaled 1.26 mM BSA spectrum to minimize protein background. Insert shows an expansion of the anomeric/vinylic region.

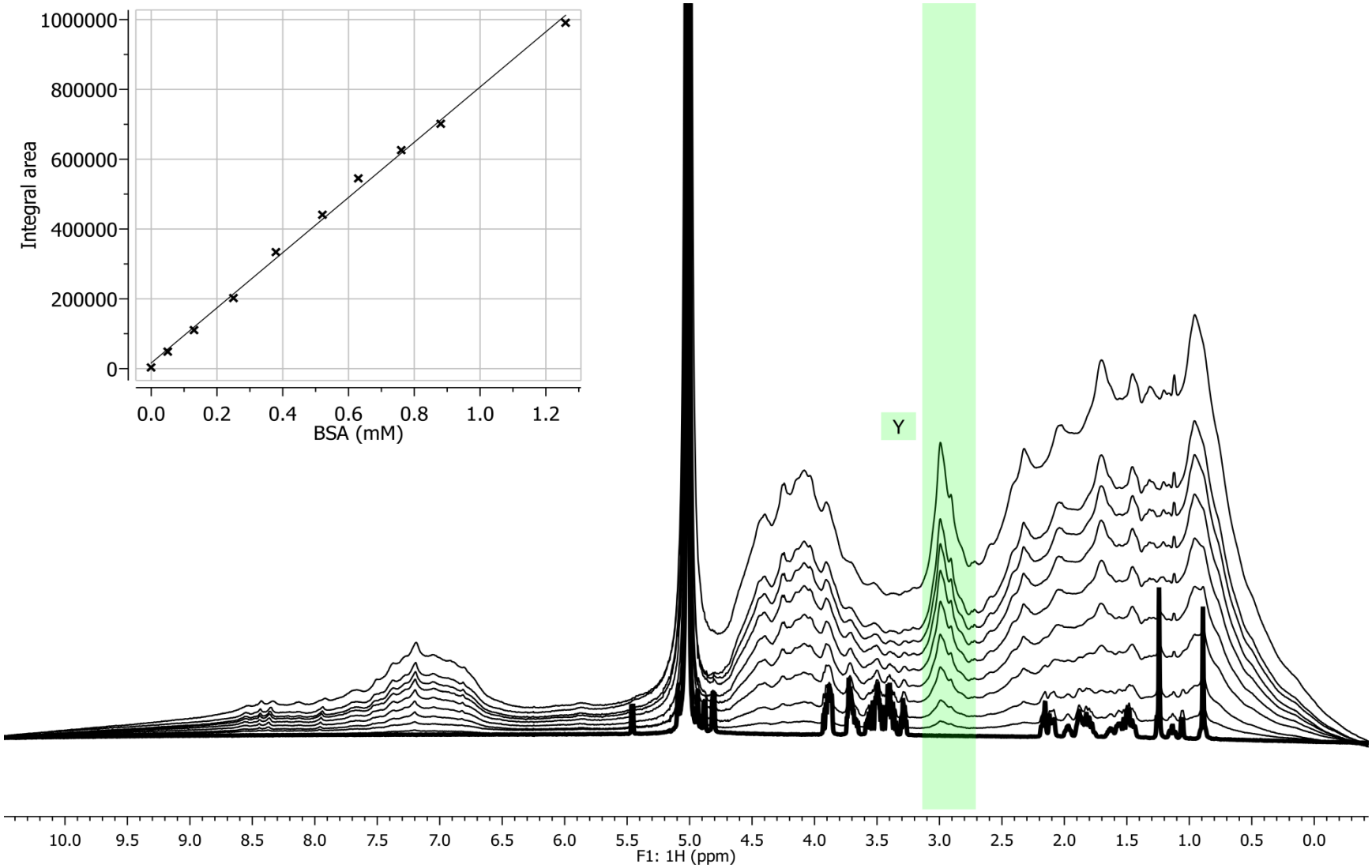

Fig. A. 26. Superimposed spectra from the titration of a 0.5 mM solution of Reb A with BSA at pH 6.7 and 4°C. Control spectrum of Reb A is highlighted in black. Graph shows a plot of integral area for BSA resonances vs. nominal BSA concentrations.

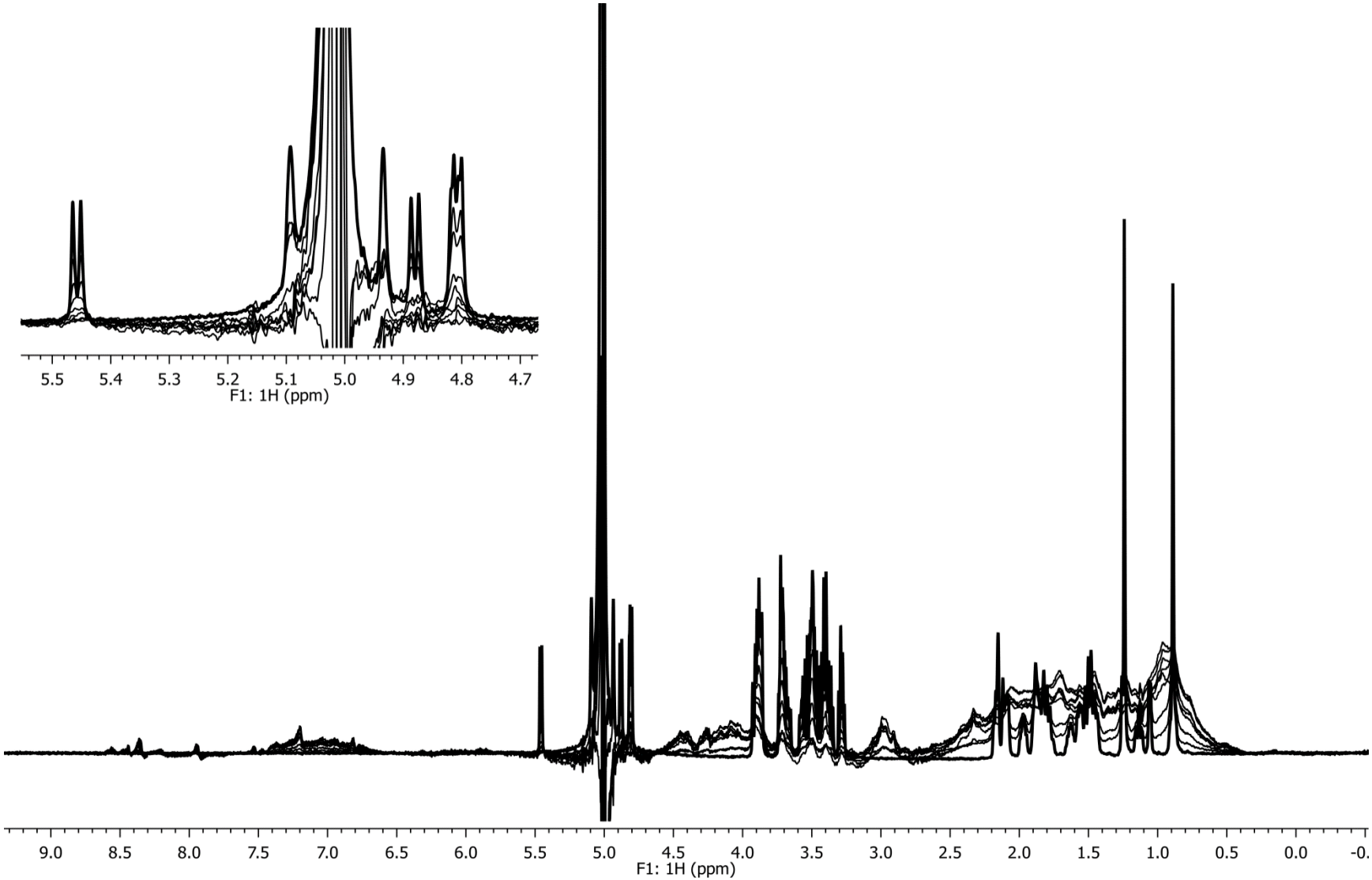

Fig. A. 27. Superimposed spectra from the titration of a 0.5 mM solution of Reb A with BSA at pH 6.7 and 4°C after subtraction of the appropriately scaled 1.26 mM BSA spectrum to minimize protein background. Insert shows an expansion of the anomeric/vinylic region.

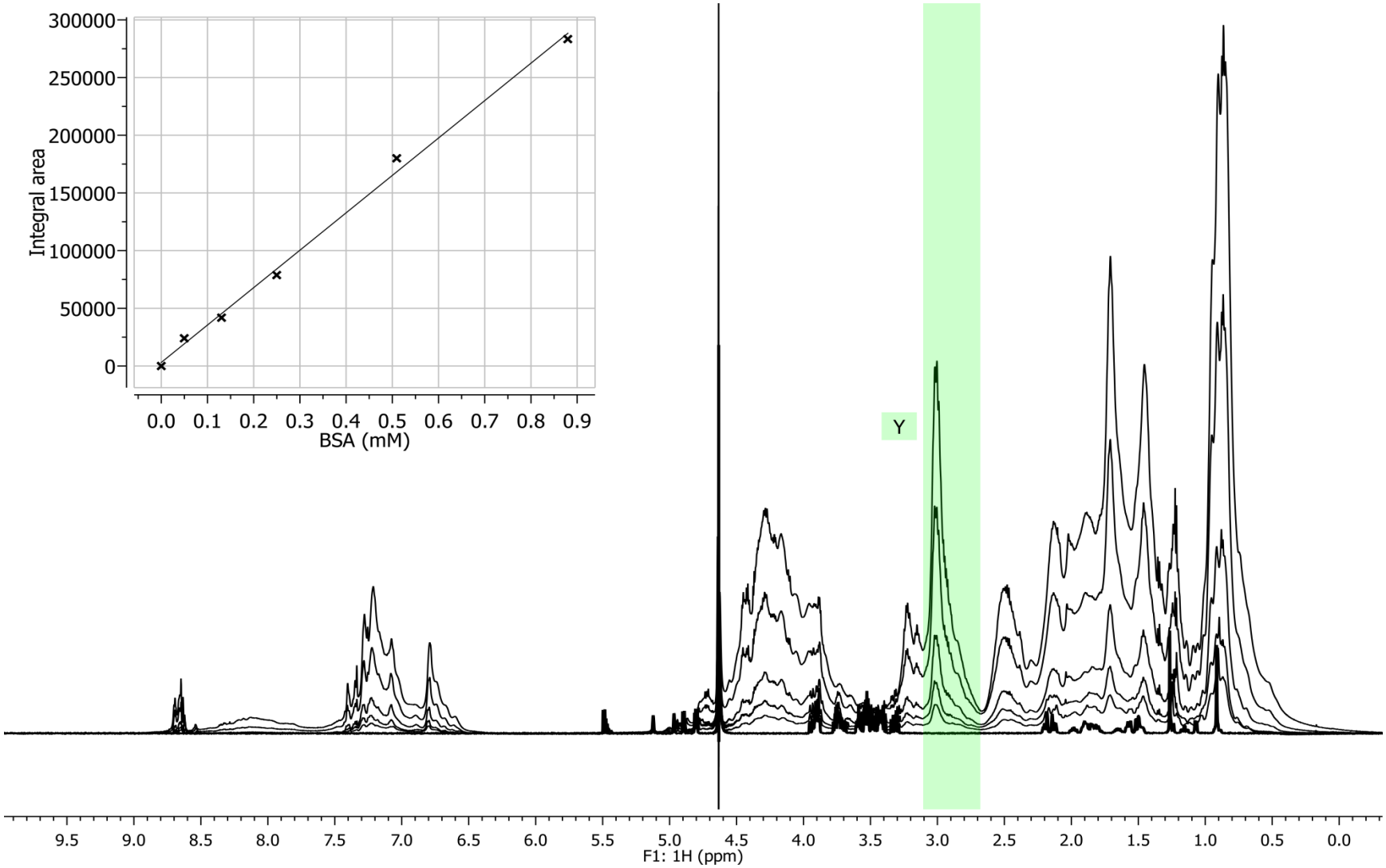

Fig. A. 28. Superimposed spectra from the titration of a 0.5 mM solution of Reb A with BSA at pH 3.0 and 40°C. Control spectrum of Reb A is highlighted in black. Graph shows a plot of integral area for BSA resonances vs. nominal BSA concentrations.

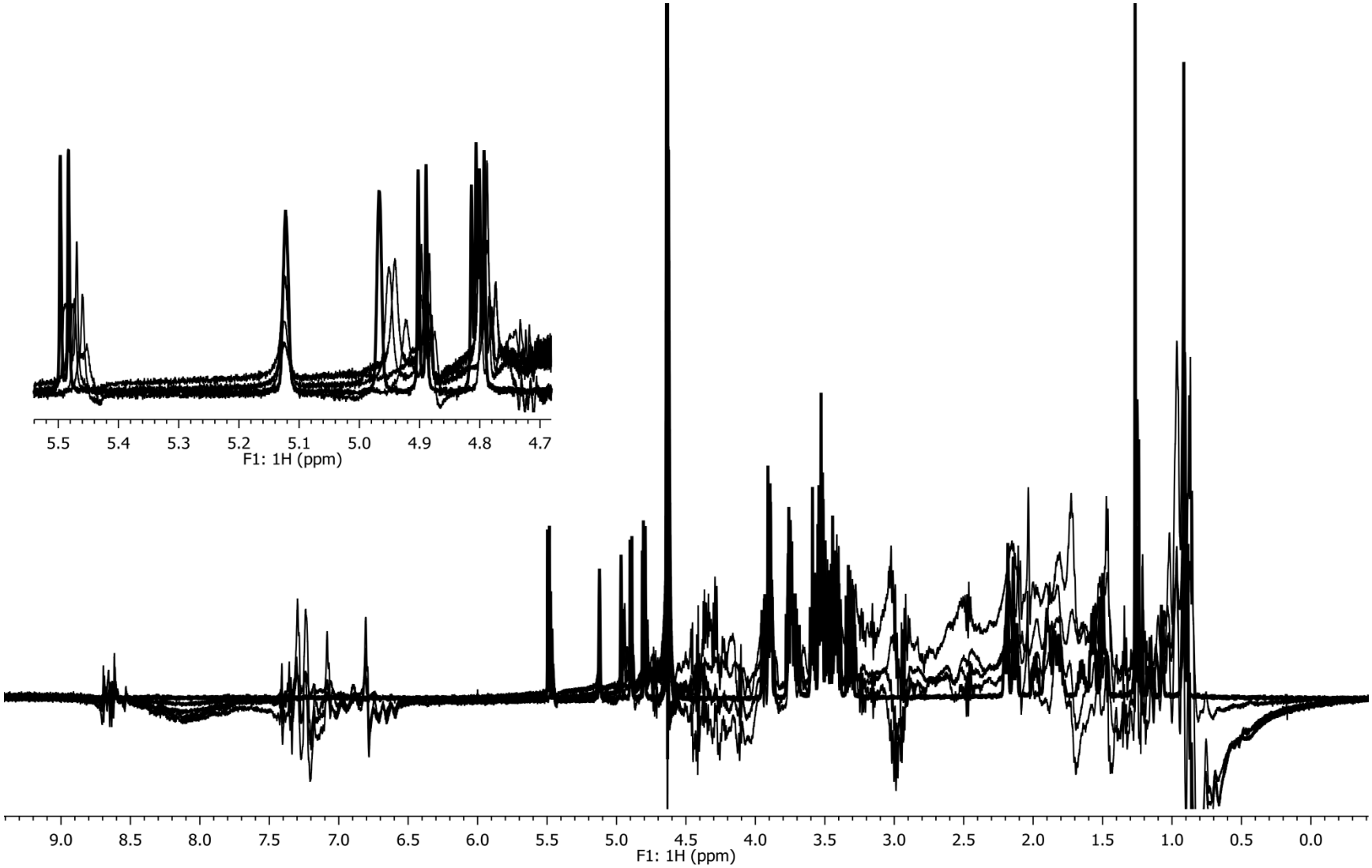

Fig. A. 29. Superimposed spectra from the titration of a 0.5 mM solution of Reb A with BSA at pH 3 and 40°C after subtraction of the appropriately scaled 0.88 mM BSA spectrum to minimize protein background. Insert shows an expansion of the anomeric/vinylic region.

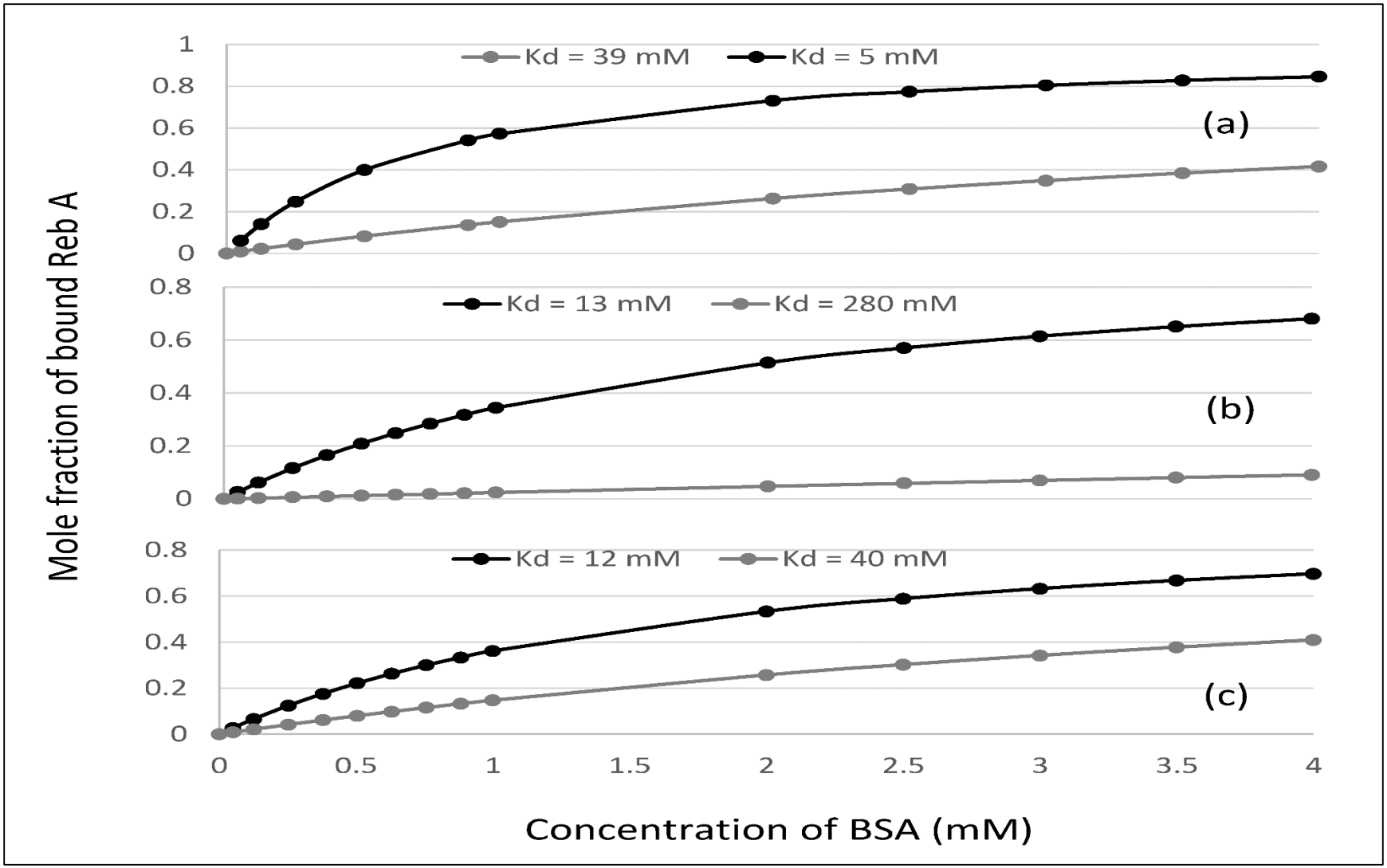

Fig. A. 30. Plot showing mole fraction of bound Reb A (concentration fixed at 0.5 mM) with increasing BSA concentration at (a) pH 3 and 40°C, (b) pH 6.7 and 40°C and (c) pH 6.7 and 4°C.