2012第三届甜菊糖360°产业论坛

甜菊糖的酶法改性及其生物活性研究进展

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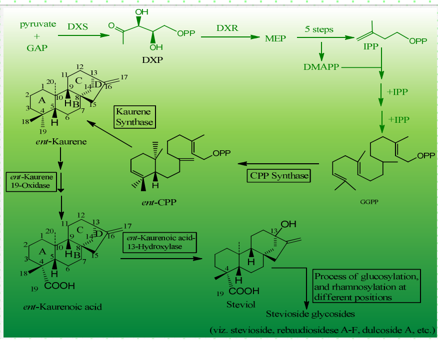
- 概述
- 甜菊糖的酶法改性
- 生物活性研究进展
- 展望

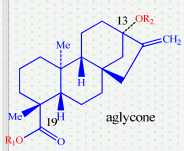
Stevia rebaudiana

甜菊糖生物体内合成—甲羟戊酸途径合成贝壳杉烯



Brahmachari G., Mandal L. C., Roy R. et al. Stevioside and Related Compounds – Molecules of Pharmaceutical Promise: A Critical Overview. 2011

甜叶菊中主要的糖苷



Major glycoside components and characteristics of *Stevia rebaudiana* leaves

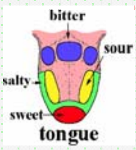
组分	简写	R_1-19位	R_2-13位	分子式	甜度
甜菊苷 Stevioside	St	-Glu	-Glu-Glu(2-1)	$C_{38}H_{60}O_{18}$	250-300
莱鲍迪苷 ARebaudioside A	R_A	-Glu	-Glu(Glu(2-1))(Glu(3-1))	$C_{44}H_{70}O_{23}$	350-450
莱鲍迪甙B Rebaudioside B	R_B	H	-Glu(Rhm(2-1))(Glu(3-1))	$C_{38}H_{60}O_{18}$	300-350
莱鲍迪甙C Rebaudioside C	R_C	-Glu	-Glu(Glu(2-1))(Glu(3-1))	$C_{44}H_{70}O_{22}$	50-120
莱鲍迪甙D Rebaudioside D	R_D	-Glu-Glu(2-1)	-Glu(Glu(2-1))(Glu(3-1))	$C_{50}H_{80}O_{28}$	200-300
莱鲍迪甙E RebaudiosideE	R_E	-Glu-Glu(2-1)	-Glu-Glu(2-1)	$C_{44}H_{70}O_{23}$	250-300
杜克甙A DulcosideA	D_A	-Glu	-Glu-Rhm(2-1)	$C_{38}H_{60}O_{17}$	50-120
甜菊双糖苷 Steviobioside	S-Bio	H	-Glu-Glu(2-1)	$C_{32}H_{50}O_{13}$	100-125
甜菊醇 Steviol		H	H	$C_{20}H_{30}O_3$	0

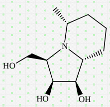
Glu =葡萄糖基　Rhm =鼠李糖基

Xu ZW, Li YQ, Wang YH. et al. Production of β-fructofuranosidase by Arthrobacter sp. and its application in the modification of stevioside and rebaudioside A, 2009

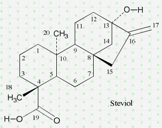
甜菊糖致命缺点——后苦涩味

- 非糖部分；单宁、类黄酮、生物碱等都系疏水性呈苦味；
- 甜菊糖本身苷元的苦味；





Steviamine

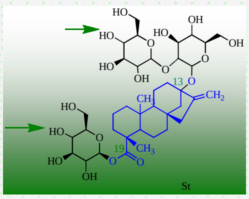


Steviol

Michalik A., Hollinshead J., Jones L., et al. Steviamine, a new indolizidine alkaloid from Stevia rebaudiana. 2010

酶法改性甜菊糖—转苷和水解

- 转糖基酶：呋喃果糖苷酶、环糊精葡萄糖基转移酶(CGTase)、葡萄糖苷酶、半乳糖苷酶等



CGTase催化甜菊糖转苷

CGTase酶改性产物结构

组分	a 13位, R_2	19位, R_1
1-1a	-G	-H
1-1b	-H	-G
1-2a	-G-G	-H
1-2b	-G	-G
1-2c	-H	-G-G
1-3a	-G-G-G	-H
1-3b	-G-G	-G
1-3c	-G	-G-G
1-3d	-H	-G-G-G

CGTase酶改性产物口感特征

改善 味质 变差

参照物质 R_A(×210; 3: 1) St(×160; 2: 1)

糖基化产物

1-1a (×180; 3: 1; 鲜味)
1-2a (×205; 4: 1; 鲜味)
1-3a (×117; 5: 1; 后味)
1-3d (×121; 2: 4; fresh taste)
1-1b (×133; 2: 2; 后苦味)
1-2b (×136; 3: 2; 余涩味)
1-3c (×150; 3: 3; 余涩味)
1-2c (×136; 2: 3; 后味)
1-3b (×146; 4: 2; 苦味)

注: ( )相对于蔗糖的甜度; m : n, 在13位(m, R_2)和19位(n, R_1)葡糖基数之比; 口感特征

Abelyan, V. H.,Balayan, A. M.,Ghochikyan, V. T..Markosyan, A. A., Transglycosylation of stevioside by cyclodextrin glucanotransferases of various groups of microorganisms. 2004.
Fukunaga, Y., Miyata, T., Nakayasu, N., et al. Enzymic transglucosylation products of stevioside: separation and sweetness-evaluation. 1989

呋喃果糖苷酶催化甜菊糖的果糖苷化

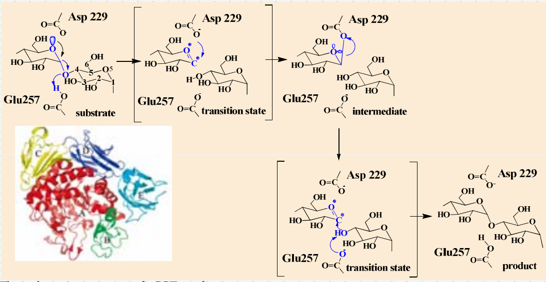
- **呋喃果糖苷酶催化甜菊糖的果糖苷化**起初是很引人注目的课题，因为甜菊糖C13位糖基的果糖衍生物具有较好的味质[13-14]。Ishikawa等[14]用β-呋喃果糖苷酶(*Microbacterium sp.*H-1)改性RA，得到了C19位的配糖体。Suzuki等[15]以β-呋喃果糖苷酶催化蔗糖或者蔗糖和葡萄糖/果糖共存时改性甜菊糖，发现转糖基反应由于蔗糖及转化产物的水解而受到抑制，符合乒乓反应动力学机制。用β-呋喃果糖苷酶(*Arthrobacter sp*.10137)催化St和RA的混合物和蔗糖反应[11]，仅生成C19位的果糖衍生物；而且低浓度蔗糖不倾向转糖基反应[16]；当蔗糖浓度高达糖基受体100倍时，St和RA的最高转化率为65%-70%。所以呋喃果糖苷酶催化甜菊糖的果糖苷化仍然是可以期待但还需要深层次的研究。

CGTase在甜菊糖改性中的应用

- CGTase (EC 2.4.1.19)是糖基水解酶α-淀粉酶家族(family 13)重要成员，能够催化环化、偶合、歧化、水解4类反应

Leemhuis H., Kelly R., Dijkhuizen L. Engineering of cyclodextrin glucanotransferases and the impact for biotechnological applications. 2010.

CGTase 催化转苷机理

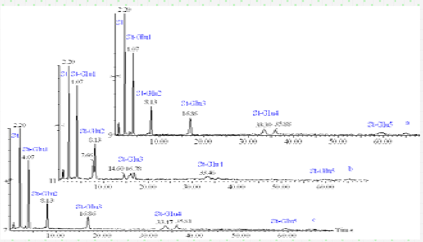


The schematic structure of CGTase from *Bacillus Circulans strain 251*

Mechanism of transglycosylation by CGTase

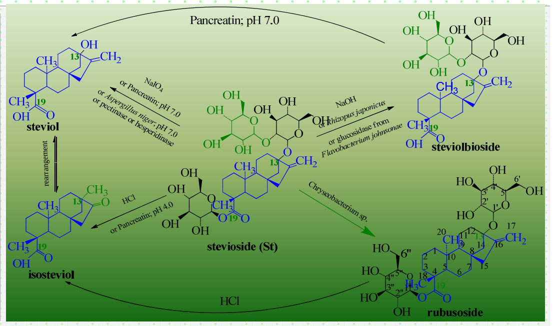
Uitdehaag J. C. M., van der Veen B. A., Dijkhuizen L., et al. Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the α-amylase. 2002.

3种CGTase催化淀粉水解液和St的反应



- Total ion chromatogram of the transglucosylation products of stevioside catalyzed by CGTase from: a. *bacillus macerans*; b. *Thermoanaerobacter*; c. *Paenibacillus macerans* JFB05-01（St-Glu1 is the single substituted stevioside, etc.）

甜菊糖苷的水解



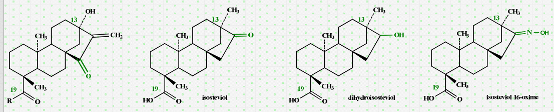
Hydrolysis of stevioside using different catalysts

Milagre H. M. S., Martins L. R., Takahashi J. A. Novel Agents for Enzymatic and Fungal Hydrolysis of Stevioside. 2009

水解

甜菊醇，再引入*exo*-亚甲基环戊酮结构，同时对其C19位羧基进行衍生修饰；具有抗癌效果

- 异甜菊醇：心肌保护、抗高血压以及抗糖尿病；
- 二氢异甜菊醇和16-肟异甜菊醇：抗腹泻作用；
- Ru: 悬钩子苷,主要来源于广西甜茶，多种生物活性
- ……………………



Shi L.-Y., Wu J.-Q., Zhang D.-Y., et al. Efficient Synthesis of Novel Jolkinolides and Related Derivatives Starting from Stevioside. 2011.
Pariwat P., Homvisasevongsa S., Muanprasat C., et al. A natural plant-derived dihydroisosteviol prevents cholera toxin-induced intestinal fluid secretion. 2008.

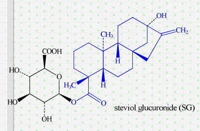
- 安全性？毒理和药代
- 生物活性：抗高血糖、免疫、抗氧化、抗癌？

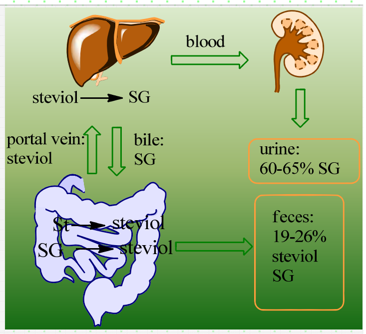
the role of gut microbiota in the metabolism of the steviol glycosides, stevioside and rebaudioside A

- they are not absorbed intact but undergo hydrolysis by the intestinal microflora to steviol.
- Steviol is not metabolized by the intestinal flora and is absorbed from the intestine.
- The rate of hydrolysis for stevioside is greater than for rebaudioside A.
- Bacteroides species are primarily responsible for hydrolysis via their beta-glucosidase activity.
- Fecal incubation studies with both human and animal mixed flora provide similar results, and this indicates that the rat is an appropriate model for studies on steviol glycosides.
- Given the similarity in the microbial metabolism of stevioside and rebaudioside A with the formation of steviol as the single hydrolysis product that is absorbed from the intestinal tract, **the toxicological data on stevioside are relevant to the risk assessment of rebaudioside A.**

Renwick, A. G. and S. M. Tarka (2008). "Microbial hydrolysis of steviol glycosides

甜菊糖药代动力学





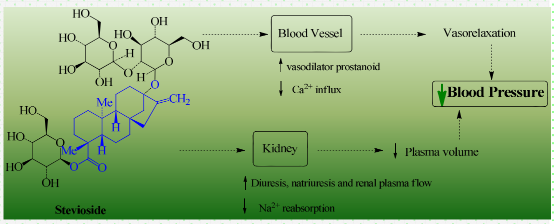
Maier V., Huber C. Stevia - natural sweetener with healthy benefit. 2010
Renwick A. G., Tarka S. M. Microbial hydrolysis of steviol glycosides. 2008

毒理学研究

- 通过对老鼠喂食不同剂量的甜菊糖来研究其急性和慢性毒性，甜菊糖摄入量达到15g/kg时并未产生急性毒性，然而甜菊醇的ID_{50}为5-20g/kg。给老鼠喂食St及其同系物(纯度为95.2%，摄入量0.1%-1%)进行慢性毒性实验，2年以后老鼠未发生异常变化；
- 口服甜菊糖不会影响老鼠的生育能力、交配性能、怀孕和胚胎发育；对鸡蛋注射甜菊糖或甜菊醇之后，胚胎发育正常，不会增加雏鸡死亡率和畸形；
- 遗传学分析表明St和RA不具有诱变性；人体血液淋巴细胞培养实验显示甜菊糖和甜菊醇并未引起染色体突变，小鼠体内实验同样证明St不会致癌；
- ADI值：4mg/kg/d；

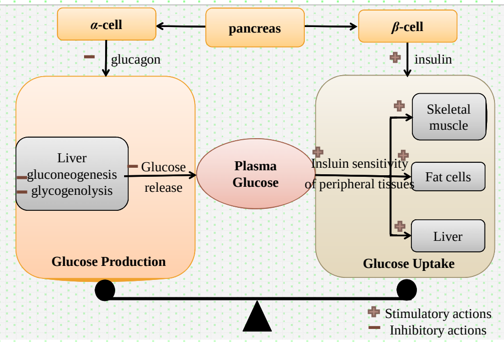
Chatsudthipong V., Muanprasat C. Stevioside and related compounds: Therapeutic benefits beyond sweetness. 2009.
Xili L., Chengjiany B., Eryi X., et al. Chronic oral toxicity and carcinogenicity study of stevioside in rats. 1992.
Geuns J. M. C., Bruggeman V., Buyse J. G. Effect of Stevioside and Steviol on the Developing Broiler. 2003.
Toyoda K., Matsui H., Shoda T., et al. Assessment of the carcinogenicity of stevioside in F344. 1997.

降血压作用



Tirapelli C. R., Ambrosio S. R., de Oliveira A. M., et al. Hypotensive action of naturally occurring diterpenes: A therapeutic promise for the treatment of hypertension. Fitoterapia. 2010.

甜菊糖的降血糖作用



Chatsudthipong V., Muanprasat C. Stevioside and related compounds: Therapeutic benefits beyond sweetness. 2009.

抗炎、抗癌等活性

- 甜菊醇：抗高血糖、抗突变、抗癌、抑制TPA生成（TPA引起局部炎症和皮肤癌）；
- 异甜菊醇:能够阻碍某些人体癌细胞的生长，通过抑制DNA聚合酶和拓扑异构酶II；
- 甜菊糖(6.25-25 mg/kg)：喂食老鼠，增强其噬菌细胞功能和体内免疫应答
- Stevia leaves are also in use for their medicinal benefits in hypertension, obesity, topical dressing for wounds, and other skin disorders
- …………

Nakamura Y., Sakiyama S., Takenaga K. Suppression of syntheses of high molecular weight nonmuscle tropomyosins in macrophages.1995. ; Mizushina Y., Akihisa T., Ukiya M., et al. Structural analysis of isosteviol and related compounds as DNA polymerase and DNA topoisomerase inhibitors. 2005. , Sehar I., Kaul A., Bani S., et al. Immune up regulatory response of a non-caloric natural sweetener, stevioside. 2008.

挖掘，从产品到性能

Table 1. *ent*-Kaurene diterpenoids from *Stevia* plants.

Compound	Source	Parts	Bioactivity	Reference
Steviol (**1**)	*S. rebaudiana*		antihyperglycemic; mutagenic; anticancerous	[71, 79, 85, 100]
Stevioside (Steviol-13-O-β-sophoroside-19-O-β-D-glycopyranosyl ester) (**2**)	*S. rebaudiana*; *S. phlebophylla*	leaves	organoleptic; anti-inflammatory; antihypertensive	[11, 111–113]
Rebaudioside A (**3**)	*S. rebaudiana*	leaves	organoleptic; anti-inflammatory	[111, 112]
Rebaudioside C (dulcoside B) (**4**)	*S. rebaudiana*	leaves	organoleptic; anti-inflammatory	[111, 113]
Dulcoside A (**5**)	*S. rebaudiana*	leaves	organoleptic; anti-inflammatory	[111, 113]
Rebaudioside B (**7**)	*S. rebaudiana*	leaves	–	[114]
Steviolbioside (**8**)	*S. rebaudiana*	leaves	–	[84]
Rebaudioside D (**9**)	*S. rebaudiana*	leaves	–	[114]
Rebaudioside E (**10**)	*S. rebaudiana*	leaves	–	[114]
Rebaudioside F (**11**)	*S. rebaudiana*	leaves	–	[114]
ent-Kaurenoic acid (Fig. 1b)	*S. lucida*	aerial parts, roots		[115]
Stevionolide (**12**)	*S. lucida*	aerial parts	–	[115]
Paniculoside (I-V) (**13–17**)	*S. ovata*	leaves	–	[68]
12β-Ethoxy-*ent*-kaur-9(11),16-dien-19-oic acid (**18**)	*S. eupatoria*	aerial parts	–	[116]
11β,16-Oxo-*ent*-kauran-19-oic acid 19-O-[β-D-glucopyranosyl (1 → 2)]-[β-D-glucopyranosyl (1 → 3)]β-D-glucopyranosyl (subpubescensoside; **19**)	*S. subpubescens*	aerial parts, roots	–	[117]

取之不尽的生物活性资源

- Valdez-Calderon, A., J. M. Torres-Valencia, et al. (2011). "A New Bisabolene from Stevia tomentosa." Natural Product Communications **6**(9): 1225-1228.

  The **new sesquiterpene** (1R,2R,3R,6R,7S)-1-acetoxy-2,3-dihydroxy-2,3-dihydrobisabolene (3) together with **ten known terpenes** and three known **flavonoids** were isolated from the aerial parts and from the roots.

展望

- 甜菊糖产品分级；
- 性能发掘；
- 产品创新；
- 安全性评价。

别人在干嘛？

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Critical issues to be discussed include:

- •**Successfully incorporating stevia into your productline**: hear case studies covering the development ofproducts ranging from ice-cream to preserves and how they overcame challenges in developing and launching their products
- •**Analysing the position of stevia** in the first few months of European regulatory approval- from successful product launches to consumer uptake, has it met expectations at this early stage?
- •**Overcoming the technical challenges** involved in working with stevia as a sugar replacement, **tackling taste and other organoleptic inconsistencies**
- •Discussing the concerns of the industry about the stevia supply **chain-can it cope with increased demand and will it become a cost-neutral alternativeto sugar?**
- •Replacing sugar with naturally derived sweeteners– don't miss a half-day seminar focusing on the technical challenges, regulatory issues and market research in this niche, butever increasing market

风险在哪里？机遇？RA之外.....产品形式？

- **steviol glycosides** have not been approved as food ingredient in the United States or the European Union
- **the leaves of Stevia or their extracts** are permitted to be sold in the US as dietary supplement, as defined in section [201(ff)(1)] of the Federal Food, Drug, and Cosmetic Act (JECFA)
- JECFA specified that steviol glycoside sweeteners must be composed of **at least 95% of the known steviol glycosides**

中国的甜菊糖产业向哪里走？

感谢基金支持

- 国家自然科学基金（功能性甜菊糖苷的酶法制备及其分子识别机制研究，31171752）；
- 江苏省产学研联合创新基金项目（甜菊糖及其功能性衍生物的制备以及高倍甜味剂安全性研究，BY2010115）；
- 食品科学与技术国家重点实验室自主课题(糖基转移酶催化转化甜菊糖的催化机制，TS2012-04);
- 江南大学博士生科学研究基金（功能性甜菊糖苷的酶法制备，JUDCF10034)。

谢谢

请各位老师批评指正