The structural basis of the inhibition of human glycosidases by castanospermine analogues

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A series of epimers and deoxy derivatives of castanospermine has been synthesized to investigate the contribution of the different chiral centres to the specificity and potency of inhibition of human liver glycosidases. Castanospermine inhibits all forms of α - and β -D-glucosidases, but alteration to any of the five chiral centres in castanospermine markedly decreases the inhibition. 6-Epicastanospermine, which is related to D-pyranomannose in the same way as castanospermine is to D-pyranoglucose, does not inhibit lysosomal (acidic) α -mannosidase, but is a good inhibitor of the cytosolic or neutral α -mannosidase. Conversely, 1-deoxy-6-epicastanospermine inhibits acidic α -mannosidase strongly, but not the neutral α -mannosidase. An explanation of this different inhibition based on preferential recognition of different configurations of mannose by the different forms of α -mannosidase is postulated. All derivatives of 6-epicastanospermine also have the minimum structural feature for the inhibition of α -L-fucosidase, but those with a β -anomeric substituent do not inhibit the enzyme, or do so very weakly. 1-Deoxy-6,8a-diepicastanospermine, which has four chiral centres identical with α -L-fucose, is, however, a potent inhibitor of α -L-fucosidase (K_i 1.3 μ M).

INTRODUCTION

Castanospermine, [(1S,6S,7R,8R,8aR)-1,6,7,8-tetrahydroxy-octahydroindolizine] is a potent and specific inhibitor of mammalian and plant α - and β -D-glucosidases *in vitro* (Hohenschutz *et al.*, 1981; Saul *et al.*, 1983) and *in vivo* (Pan *et al.*, 1983; Saul *et al.*, 1985; Cenci di Bello *et al.*, 1988). It was first isolated from seeds of the Australian legume *Castanospermum australe* (Hohenschutz *et al.*, 1981) and has subsequently been identified in eight species of another legume, *Alexa* (Nash *et al.*, 1988). It has also been synthesized chemically (Bernotas & Ganem, 1984; Setoi *et al.*, 1985). The inhibition of the different types of cellular α - or β -glucosidases by castanospermine *in vivo* produces a wide range of biological effects, which have suggested applications for this compound (Fellows *et al.*, 1989). Inhibition of mammalian lysosomal α - and β -glucosidases results in the disturbance of the metabolism of glycogen (Saul *et al.*, 1985) and glycolipids (Cenci di Bello *et al.*, 1988) respectively. Other observations have been attributed to the alteration of glycoprotein processing by inhibition of the processing enzymes α -glucosidase I and II in the endoplasmic reticulum. They include affecting the growth and infectivity of human immunodeficiency virus (Gruters *et al.*, 1987; Tyms *et al.*, 1987) and other retroviruses (Sunkara *et al.*, 1987), inhibition of metastasis (Humphries *et al.*, 1986) and tumour growth in mice (Ostrander *et al.*, 1988) and modulation of the immune response (Wall *et al.*, 1988). Castanospermine delays the hyperglycaemic response to oral carbohydrates in rats, probably by inhibiting intestinal disaccharidases (Rhinehart *et al.*, 1987). It is a feeding deterrent to some insects (Dreyer *et al.*, 1985) and is toxic to others, perhaps by blocking the binding of phagostimulatory sugars to receptors (Nash *et al.*, 1986). The inhibitory properties of castanospermine have been attributed to the close structural resemblance between the structure and chirality of the four substituents at C-6, C-7, C-8 and C-8a on the piperidine ring of castanospermine with the four substituents on C-2, C-3, C-4 and C-5 of the pyranose ring of D-glucose (Saul *et al.*, 1984; Cenci di Bello *et al.*, 1988). To gain some insight into the contribution of the chiral centres and substituents to its

potency and specificity, several analogues of castanospermine have been synthesized and their specificity of inhibition towards a panel of human glycosidases investigated.

MATERIALS AND METHODS

Tissues and sources of enzymes

Post-mortem human liver, which had been stored at -20°C until required, was homogenized in deionized water (50%, w/v) in a Potter–Elvehjem homogenizer and then centrifuged at 37000 g for 30 min in an MSE 18 centrifuge. The resultant supernatant was used as the source of the human glycosidases for the initial screening of glycosidase inhibition by castanospermine and its derivatives. Lysosomal glycosidases with acidic pH optima were separated from the non-lysosomal, with neutral pH optima, by affinity chromatography on concanavalin A–Sephrose-4B (Phillips *et al.*, 1976; Cenci di Bello *et al.*, 1985). Lysosomal glycosidase activities bind to concanavalin A whereas α -glucosidase II, a glycoprotein-processing enzyme in the endoplasmic reticulum, cytosolic β -glucosidase, which also has activity towards α -L-arabinosidase, β -D-galactosidase and β -D-xylosidase (Chester *et al.*, 1976) and cytosolic neutral α -mannosidase (Hirani & Winchester, 1979) do not bind. After dialysis against the appropriate assay buffer to remove methyl α -D-mannoside, the lysosomal α -glucosidase and α -mannosidase were assayed at their pH optimum of 4.0 and at a higher pH to study the effect of pH on inhibition.

Enzyme assays

Glycosidase activities were assayed at their pH optima by using the appropriate fluorogenic 4-methylumbelliferyl glycoside substrate (Melford Laboratories, Ipswich, Suffolk, U.K.) at a concentration of 0.5 mM in the presence or absence of 1 mM-castanospermine analogue (Cenci di Bello *et al.*, 1989; Winchester *et al.*, 1990). The nature of the inhibition and the value of K_i were determined by using the Dixon graphical procedure.

Castanospermine analogues

Castanospermine was isolated from the seeds of the Australian legume *Castanospermum australe* (I) (Hohenschütz *et al.*, 1981). 1-Deoxycastanospermine (II) (Hendry *et al.*, 1987a), 1-deoxy-6,8-diepicastanospermine (VIII) and 1,8-dideoxy-6-epicastanospermine (V) (Hendry *et al.*, 1987b), 6-epicastanospermine (III) and 1,6-diepicastanospermine (VII) (Fleet *et al.*, 1990), 1-deoxy-6-epicastanospermine (IV) and 1-deoxy-6,8a-diepicastanospermine (VI) (Aamlid *et al.*, 1990) were all synthesized for the first time and characterized in our laboratories as

described in the references cited. Their structures are shown in Fig. 1. Deoxymannojirimycin (DMJ) was synthesized as previously described (Fleet *et al.*, 1989).

RESULTS

Inhibition of human liver glycosidases

The initial screening of the inhibition of the glycosidases in a crude liver supernatant showed that only α - and β -D-glucosidase, α -D-mannosidase and α -L-fucosidase were inhibited significantly by castanospermine or its derivatives. To clarify the effects of the compounds on these glycosidases, the multiple forms of α - and β -D-glucosidase and α -D-mannosidase were separated by affinity chromatography. A summary of their inhibitory effects is shown in Table 1. It can be seen that some inhibitors can discriminate among the different forms of an enzymic activity. The inhibition of lysosomal α -glucosidase and α -mannosidase is always greater at a higher pH than at their pH optima. This has been attributed to an ionization of a carboxy group in the active site of the enzyme, leading to the formation of an ion pair with the protonated inhibitor (Cenci di Bello *et al.*, 1985, 1989; Saul *et al.*, 1985). Deductions about the structure-activity relationships for the individual compounds follow.

1-Deoxycastanospermine (II)

Removal of the 1-OH group from castanospermine, corresponding to the C-6 OH group in glucose, abolishes its inhibition of lysosomal and neutral α -glucosidases and markedly lowers its potency towards β -glucosidases. However, at the higher pH value of 6.0 this compound is a moderate inhibitor of lysosomal α -glucosidase. It is concluded that the C-6 OH group is important for the recognition and binding of substrates and inhibitors with the pyranose configuration by mammalian α - and β -glucosidases. Interestingly, 1-deoxycastanospermine is a weak inhibitor of *N*-acetyl- β -D-glucosaminidase [I_{50} (concn. causing 50% inhibition) 1 mM], whereas castanospermine does not inhibit this activity.

6-Epicastanospermine (III)

Inversion of the configuration at C-6 in castanospermine produces the mannose analogue, in which the substituents on the

Table 1. Inhibition of human liver glycosidases by castanospermine (CS) analogues

Values are average inhibitions (%) from two to five determinations using inhibitor at a concentration of 1 mM.

Compound	α -Glucosidases			β -Glucosidases		α -Mannosidases			α -Fucosidase
	Lysosomal		Neutral	Lysosomal	Cytosolic	Lysosomal		Neutral	
	pH 4.0	pH 6.0	pH 6.0	pH 4.0	pH 4.5	pH 4.0	pH 6.5	pH 6.5	pH 5.5
CS (I)	100 (K_i 0.1 μ M)	99	97 (K_i 10 μ M)	96 (K_i 7 μ M)	90 (K_i 40 μ M)	0	10	13	0
1-Deoxy-CS (II)	7	65	0	31	36	21	53	2	0
6-Epi-CS (III)	28	86	59	14	8	0	10	76	15
1-Deoxy-6-epi-CS (IV)	7	59	0	12	9	75 (K_i 0.5 mM)	97	15	55
1,8-Dideoxy-6-epi-CS (V)	0	6	0	29	48	10	19	4	0
1-Deoxy-6,8a-diepi-CS (VI)	20	31	0	44	70	22	40	0	100 (K_i 1.3 μ M)
1,6-Diepi-CS (VII)	3	4	0	9	0	0	0	7	15
1-Deoxy-6,8-diepi-CS (VIII)	6	14	0	9	16	0	6	5	0

Fig. 2. Structural basis for inhibition of α -fucosidase by castanospermine (CS) analogues

piperidine ring have the same chirality as those in the pyranose form of D-mannose. However, 6-epicastanospermine does not inhibit lysosomal α -mannosidase, but it is a good inhibitor of the neutral form of the enzyme. It is a weak inhibitor of the lysosomal and neutral α -glucosidase but, again, its effect is more notable on the acidic enzyme at higher pH. 6-Epicastanospermine, which had been isolated from *Castanospermum australe*, has been shown previously to be a potent inhibitor of a fungal α -glucosidase, a poor inhibitor of plant processing α -glucosidases and to have no effect on jack-bean (*Canavalia ensiformis*) α -mannosidase (Molyneux *et al.*, 1986).

1-Deoxy-6-epicastanospermine (IV)

This compound, which incorporates both structural alterations in inhibitors II and III, does not inhibit any forms of α - and β -glucosidases except the lysosomal α -glucosidase at pH 6.0. In contrast with 6-epicastanospermine, it inhibits lysosomal α -mannosidase in a competitive manner (K_i 0.5 mM), but has no effect on neutral α -mannosidase (15%). The moderate inhibition of α -fucosidase can be explained by considering it as an analogue of deoxyfuconojirimycin (Fig. 2).

1,6-Diepicastanospermine (VII)

The inversion of configuration at C-1 destroys all the inhibitory properties shown by 6-epicastanospermine. Comparison

with 1-deoxy-6-epicastanospermine suggests that incorrect configuration at C-1 is more deleterious than the absence of the OH group.

1-Deoxy-6,8a-diepicastanospermine (VI)

The three ring OH substituents and the anomeric substituent in 1-deoxy-6,8a-diepicastanospermine have the same chirality as the corresponding ones in α -L-fucose (Fig. 2). The change from an α -L to a β -L substituent at C-1 probably accounts for the increased inhibition of α -fucosidases by this compound (competitive inhibition; K_i 1.3 μ M) relative to 1-deoxy-6-epicastanospermine (I_{50} 1 mM). In addition, it is a moderate inhibitor of both lysosomal and neutral β -glucosidases.

1-Deoxy-6,8-diepi- (VIII) and 1,8-dideoxy-6-epicastanospermine (V)

The inversion of the configuration (D-talo) or removal of the C-8 OH substituent abolishes the inhibition of α -mannosidase and α -fucosidase shown by 1-deoxy-6-epicastanospermine. These observations support our previous conclusions about the number of correct chiral centres necessary for the inhibition of these two activities (Cenci di Bello *et al.*, 1989; Winchester *et al.*, 1990).

DISCUSSION

The specificity of inhibition of the castanospermine derivatives permits general deductions about the structural requirement of an inhibitor for specific glycosidases. Any alteration of the five chiral centres in castanospermine markedly decreased the inhibition of α - and β -glucosidases. The lack of inhibition of α -glucosidases at their pH optima by 1-deoxycastanospermine suggests the C-6 OH group of pyrano analogues of glucose is important for recognition and strong binding by the active site of these enzymes. This may not be true for analogues in the furano form, because 2-epiwainsonine (Elbein *et al.*, 1987) and 1,4-dideoxy-1,4-imino-D-arabinitol (Fleet *et al.*, 1985), which lack the equivalent of the C-6 OH group and can be considered as furanogluco structures, are both strong inhibitors of α -glucosidases. The only analogue that retained inhibitory activity towards the α -glucosidases was 6-epicastanospermine (or mannocastanospermine), which was a relatively strong inhibitor of the lysosomal α -glucosidase at higher pH values and of the neutral α -glucosidase. This suggests that the three chiral substituents at C-3 C-4 and C-5 in a pyrano configuration are sufficient to bind to α -glucosidase. 1,6-Diepicastanospermine was non-inhibitory, even at higher pH values, suggesting that the pro-chiral C-6 OH group in D-glucose binds in a specific way, the *S* configuration, to the substrate-recognition site. The increased inhibition of lysosomal α -glucosidase at higher pH values by amino sugars had been reported previously (Saul *et al.*, 1984). The strong inhibition of lysosomal α -glucosidase at pH 6 by 6-epicastanospermine indicates that the preferred ring structure for inhibition may compensate for the incorrect chirality of a substituent.

None of the derivatives was a potent inhibitor of either β -glucosidase, emphasizing the rigorous structural features necessary for inhibition of these activities. It is interesting that castanospermine, but not deoxynojirimycin (B. Winchester, unpublished work), which it resembles closely, inhibits the β -glucosidases, again suggesting that the configuration of the C-6 atom contributes significantly to the binding process.

The differential effect of 6-epicastanospermine and 1-deoxy-6-epicastanospermine towards the α -mannosidases supports our conclusion that the number of chiral centres and the configuration of the ring in a mannose analogue determine the relative potency of inhibition of lysosomal (acidic) α -mannosidase and neutral α -mannosidase (Cenci di Bello *et al.*, 1989). Thus 6-epicastanospermine, with five chiral centres, selectively inhibits neutral α -mannosidase, whereas 1-deoxy-6-epicastanospermine, which lacks the equivalent of the C-6 OH group in mannose, only inhibits the lysosomal α -mannosidase. The lack of inhibition of acidic α -mannosidase by 1,6-diepicastanospermine suggests that, although the C-6 OH group does not appear to be essential for binding, the *R* configuration for this substituent is prohibited. It also abolishes the inhibition of the neutral α -mannosidase, showing that the configuration of C-6 in the pyrano analogues is important in binding to the active site of α -mannosidases as well as the glucosidases.

All derivatives of 6-epicastanospermine have the minimum structural feature for inhibition of α -L-fucosidase, i.e. the three OH groups on C-6, C-7 and C-8 of the piperidine corresponding to C-2, C-3 and C-4 of L-fucose (Winchester *et al.*, 1989) (see Fig. 2). The pyrrolidine ring between the ring nitrogen atom and C-8a of castanospermine is equivalent to β -substitution of C-1 and alkylation of the ring nitrogen atom in deoxyfuconojirimycin. 1-Deoxy-6-epicastanospermine is a much weaker inhibitor of α -fucosidase than 1,5-dideoxy-1,5-imino-D-rhamnitol (a β -1-substituted compound with a K_i of 70 μ M) (N. G. Ramsden, I. Cenci di Bello, G. Fleet & B. Winchester, unpublished work) or *N*-

methyldeoxyfuconojirimycin (K_i 0.05 μ M). However, if the anomeric substitution is α , as in 1-deoxy-6,8a-diepicastanospermine, then potent inhibition occurs. The presence of an OH group in the pyrrolidine ring at C-1 of 6-epicastanospermine in either the *R* (1,6-diepicastanospermine) or *S* (6-epicastanospermine) configuration completely abolishes inhibition of α -fucosidase. In contrast, the closely related structure deoxymannojirimycin is a potent inhibitor of α -fucosidase. These observations suggest that the orientation of the anomeric substituent is important in the binding of inhibitors to the active site of α -fucosidase. Furthermore, the rigidity of the pyrrolidine ring in either the α - or the β -anomeric position decreases the potency of inhibition. Substitution of the ring nitrogen atom does not prevent binding, but it will lower the p*K* of the ring nitrogen atom compared with deoxyfuconojirimycin. Inhibition of α -fucosidase depends on ion-pair formation between the protonated amino sugar and a putative carboxy group in the active site (Winchester *et al.*, 1990). The lower p*K* of castanospermine ($\sim$ 6.1) may contribute to its showing a weaker inhibition than deoxymannojirimycin (p*K* 7.2) or *N*-methyldeoxyfuconojirimycin (p*K* 7.8).

The results presented here indicate that several different structural features contribute to the specificity of inhibition of glycosidases by castanospermine derivatives. These include the number and chirality of the piperidine-ring C-OH substituents, the presence and configuration of the substituent at the bridge C atom corresponding to the anomeric substituent or C-5 substituent, the presence of an OH group on C-1 on the pyrrolidine ring and whether the amino sugar is a pyranose or furanose analogue. Finally, it is important to note that different multiple forms of an activity may be susceptible to selective inhibition by compounds with different structural features. By studying a range of inhibitors for an individual enzymic activity, prediction of the inhibitory effect of a new potential inhibitor becomes more reliable.

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REFERENCES

- Aamlid, K. H., Hough, L. & Richardson, A. C. (1990) Carbohydr. Res., in the press
- Bernotas, R. C. & Ganem B. (1984) Tetrahedron Lett. **25**, 165–168
- Cenci di Bello, I., Dorling, P., Evans, S., Fellows, L. & Winchester, B. (1985) Biochem. Soc. Trans. **13**, 1127–1128
- Cenci di Bello, I., Mann, D., Nash, R. & Winchester, B. (1988) in Lipid Storage Disorders (Salvayre, R., Douste-Blazy, L. & Gatt, S., eds), pp. 635–641, Plenum Publishing Corp. New York
- Cenci di Bello, I., Fleet, G., Namgoong, S. K., Tadano, K.-I. & Winchester, B. (1989) Biochem. J. **259**, 855–861
- Chester, M. A., Hultberg, B. & Ockerman, P. A. (1976) Biochim. Biophys. Acta **429**, 517–526
- Dreyer, D. L., Jones, K. C. & Molyneux, R. J. (1985) J. Chem. Ecol. **11**, 1045–1051
- Elbein, A. D., Szumilo, T., Sandorf, B. A., Sharpless, B. K. & Adams, C. (1987) Biochemistry **26**, 1573–1576
- Fellows, L. E., Kite, G. C., Nash, R. J., Simmonds, M. S. J. & Scofield, A. M. (1989) in Plant Nitrogen Metabolism (Poulton, J. E., Romeo, J. T. & Conn, E. E., eds), pp. 395–427, Plenum Publishing Corp. New York
- Fleet, G. W. J., Nicholas, S. J., Smith, P. W., Evans, S. V., Fellows, L. E. & Nash R. J. (1985) Tetrahedron Lett. **26**, 3127–3130
- Fleet, G. W. J., Ramsden, N. G. & Witty, D. R. (1989) Tetrahedron Lett. **45**, 319–326
- Fleet, G. W. J., Ramsden, N. G., Nash, R. J., Fellows, L. E., Jacob, G. S., Cenci di Bello, I. & Winchester, B. (1990) Carbohydr. Res., in the press
- Gruters, R. A., Neffjes, J. J., Tersmette, M., de Goede, R. E. Y., Tulp, A., Huisman, H. G., Miedema, R. & Ploegh, H. L. (1987) Nature (London) **330**, 74–77

- Hendry, D., Hough, L. & Richardson, A. C. (1987a) *Tetrahedron Lett.* **28**, 4597–4600
- Hendry, D., Hough, L. & Richardson, A. C. (1987b) *Tetrahedron Lett.* **28**, 4601–4604
- Hirani, S. & Winchester, B. G. (1979) *Biochem. J.* **179**, 583–592
- Hohenschutz, L. D., Bell, E. A., Jewess, P. J., Leworthy, D. P., Pryce, R. J., Arnold, E. & Clardy, J. (1981) *Phytochemistry* **20**, 811–814
- Humphries, M. J., Matsumoto, K., White, S. L. & Olden, K. (1986) *Cancer Res.* **46**, 5215–5222
- Molyneux, R. J., Roitman, J. N., Dunnheim, G., Szumilo, T. & Elbein, A. D. (1986) *Arch. Biochem. Biophys.* **251**, 405–457
- Nash, R. J., Fenton, K. A., Gatehouse, A. M. R. & Bell, E. A. (1986) *Entomol. Exp. Appl.* **42**, 71–77
- Nash, R. J., Fellows, L. E., Dring, J. V., Stirton, C. H., Carter, D., Hegarty, M. P. & Bell, E. A. (1988) *Phytochemistry* **27**, 1403–1404
- Ostrander, G. K., Scribner, N. K. & Rohrschneider, L. R. (1988) *Cancer Res.* **48**, 1091–1094
- Pan, Y. T., Hori, H., Saul, R., Sanford, B. A., Molyneux, R. J. & Elbein, A. D. (1983) *Biochemistry* **22**, 3976–3984
- Phillips, N. C., Robinson, D. & Winchester, B. G. (1976) *Biochem. J.* **153**, 579–587
- Rhinehart, G. L., Robinson, K. M., Payne, A. J., Wheatley, M. E., Fisher, J. L., Liu, P. S. & Cheng, W. (1987) *Life Sci.* **41**, 2325–2331
- Saul, R., Chambers, J. P., Molyneux, R. J. & Elbein, A. D. (1983) *Arch. Biochem. Biophys.* **221**, 593–597
- Saul, R., Molyneux, R. J. & Elbein, A. D. (1984) *Arch. Biochem. Biophys.* **230**, 668–675
- Saul, R., Ghidoni, J. J., Molyneux, R. J. & Elbein, A. D. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 93–97
- Setoi, H., Takeno, H. & Hashimoto, M. (1985) *Tetrahedron Lett.* **26**, 4617–4620
- Sunkara, P. S., Bolin, T. L., Liu, P. S. & Sjoerdsma, A. A. (1987) *Biochem. Biophys. Res. Commun.* **148**, 206–210
- Tyms, A. S., Berrie, E. M., Ryder, T. A., Nash, R. J., Hegarty, M. P., Taylor, D. L., Mobberley, M. A., Davis, J. M., Bell, E. A., Jeffries, D. J., Taylor-Robinson, D. & Fellows, L. E. (1987) *Lancet* **ii** 1025–1026
- Wall, K. A., Pierce, J. D. & Elbein, A. D. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5644–5648
- Winchester, B., Barker, C., Baines, S., Jacob, G. S., Namgoong, S. K. & Fleet, G. (1990) *Biochem. J.* **265**, 277–282

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