

# Chemistry and biology of calystegines

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The review covers structures, chemical properties and synthesis of calystegines. Occurrence in the plant kingdom, analysis in plant material and biological properties are also summarised. An emphasis is laid on the biosynthesis of the nortropane alkaloids and on the enzymes and genes that are involved. The literature from 1988 up to August 2003 is reviewed, and 150 references are cited.

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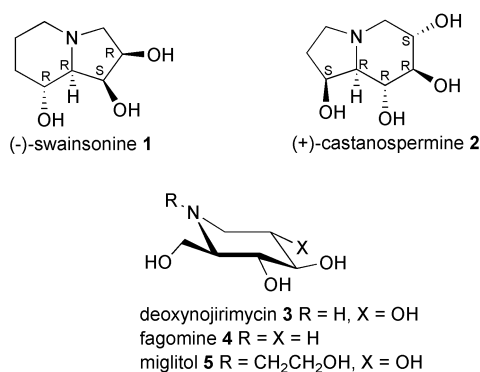
## 1 Introduction

Although tropane alkaloids from plant sources have been known for more than 170 years,<sup>1,2</sup> it was only in 1990 that the structures of the first calystegines were published. Calystegines are selective glycosidase inhibitors; they share this activity with other hydroxylated nitrogen containing bicyclic compounds like swainsonine **1** or castanospermine **2** or monocycles like deoxy-nojirimycin **3** or fagomine **4** (Fig. 1). Classical tropane alkaloids, in contrast, are either parasympatholytic, as with atropine and scopolamine, or anaesthetic and sympathomimetic, as with cocaine. When calystegines are isolated, analysed, and tested for therapeutic potential, reference is made to other alkaloidal glycosidase inhibitors, employing the same methods and comparing biological activity. The biosynthesis of calystegines, however, shares metabolic steps and enzymes of the classical tropane alkaloid formation. Biosynthesis of calystegines and their occurrence in the plant kingdom are emphasised in the present review.

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**Fig. 1** Other naturally occurring glycosidase inhibitors.

## 2 Chemistry

### 2.1 Structures of naturally occurring calystegines and nomenclature

Calystegines are nortropane alkaloids with three to five hydroxyl groups in various positions. The name of the group of

alkaloids derives from the first detection in roots of *Calystegia sepium* (Convolvulaceae).<sup>3</sup> Three compounds were identified, and structures were elucidated as one trihydroxylated and two tetrahydroxylated nortropanes, calystegine A<sub>3</sub> **6** (8-azabicyclo[3.2.1]octane-1*R*,2*S*,3*R*-triol or 1*R*,2*S*,3*R*-trihydroxynortropane), calystegine B<sub>1</sub> **10** and calystegine B<sub>2</sub> **11** (Fig. 2). A fraction containing four different trihydroxynortropanes, *i.e.* calystegine A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub>, and A<sub>4</sub>, was obtained from *C. sepium* roots, but only calystegine A<sub>3</sub> was isolated in sufficient amount and purity to be structurally elucidated.<sup>4</sup> The three other trihydroxylated calystegines were not further characterised in the course of the investigation, but later the indices A<sub>1</sub>, A<sub>2</sub>, and A<sub>4</sub> were considered as occupied and not used for further trihydroxynortropanes. Therefore, when *Physalis alkekengi* roots, Solanaceae, were investigated, a new trihydroxylated nortropane **7** was named calystegine A<sub>5</sub>, and a new tetrahydroxylated structure **12** received the assignment calystegine B<sub>3</sub>.<sup>5</sup> A pentahydroxynortropane **15** from *Morus alba* root bark, Moraceae, was consequently named calystegine C<sub>1</sub>.<sup>6</sup> Calystegine B<sub>4</sub> **13** was isolated from the roots of another Solanaceae, *Scopolia japonica*,<sup>7</sup> and calystegine C<sub>2</sub> **16** with five hydroxyl groups was reported from the roots of *Lycium chinense*, Solanaceae.<sup>8</sup> A novel nortropane alkaloid **17** with a bridgehead amino group was isolated from *Hyoscyamus niger*, Solanaceae, and denoted as calystegine N<sub>1</sub>.

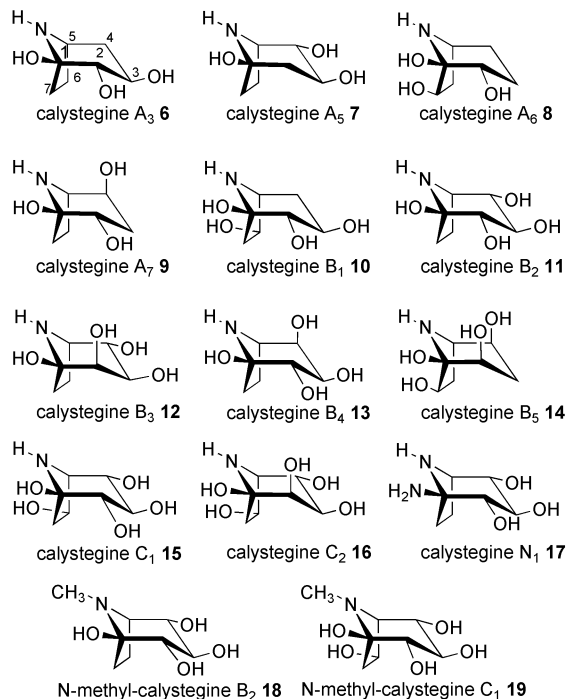


Fig. 2 Naturally occurring calystegines.

But the calystegine nomenclature is far from being consistent and depends to some extent on the species from which the compound has been isolated and on the preferences of the authors. Tropane alkaloids with three, four (*e.g.* **18**) or five (*e.g.* **19**) hydroxyl groups were called *N*-methylcalystegines, when they were found in the course of a calystegine investigation or synthesised by methylation of calystegines,<sup>8</sup> but when trihydroxylated nortropane alkaloids were identified in an alkaloid screening in *Erythroxylum* species, they were not named calystegines, but nortropane-3*α*,6*β*,7*β*-triol esters<sup>9</sup> or ditigloyloxynortropane,<sup>10</sup> consistent with other 3,6,7-trihydroxynortropanes from *Erythroxylum* species. Nobody would call teloidine **20** (Fig. 3) from *Datura meteloides*,<sup>11</sup> which has been known for almost 100 years, an *N*-methylcalystegine. New trihydroxylated nortropanes, **21** and **22**, from fruits of *Morus alba*, however, could have followed the calystegine line of names

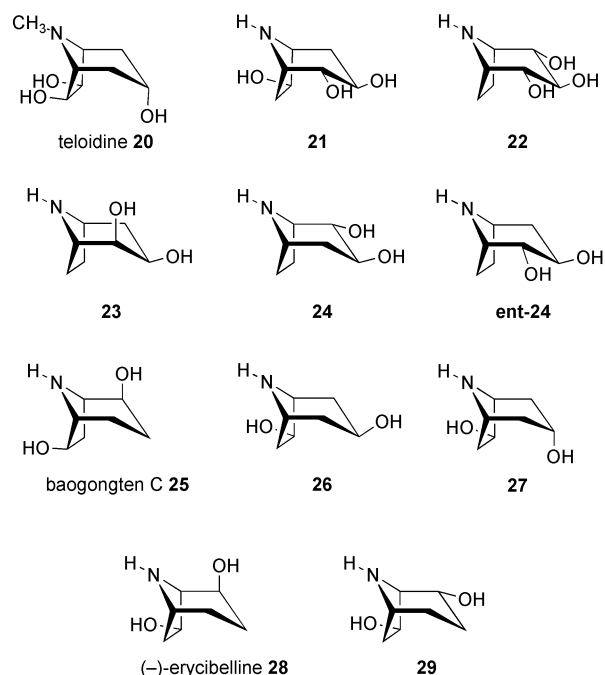


Fig. 3 Naturally occurring compounds related to calystegines.

and thus be calystegines A<sub>8</sub> and A<sub>9</sub>, but the authors chose not to do this.<sup>12</sup> Dihydroxynortropanes, **23** to **28**, are not named calystegines, even though they were identified in calystegine-containing plant families and derive from the same biosynthetic pathway. It could be argued that the aminoketal functionality conferred by the hydroxyl group in the 1-position is the criterion to name a compound calystegine, but then calystegine N<sub>1</sub> would have to be renamed.

Even more confusing is the  $\alpha$ - and  $\beta$ -distinction used to depict the position of the hydroxyl groups in the nortropane ring structure. Some authors prefer the cycloheptane ring with a 1,5-nitrogen bridge as the basic structure and hydroxyl groups at all positions, 1 to 7, are given the stereochemical descriptors  $\alpha$  or  $\beta$ . Others use the six-membered piperidine ring with an ethylene bridge as the basis and describe hydroxyl groups on carbon 6 and 7 as *endo* or *exo*. For steroids, triterpenes and carbohydrates, for example, different rules for the assignment of  $\alpha$  and  $\beta$  hydroxyl groups are applied. Because of the ambiguity it is preferable to avoid  $\alpha/\beta$ -systems and use for saturated ring systems *a* for axial groups and *e* for equatorial groups. The *R,S*-nomenclature is unambiguous but rather complicated because hydroxyl groups can change their assignment if they have another neighbour. For example, C-3 changes from being *R* in calystegine A<sub>3</sub> **6** to being *S* in calystegine A<sub>5</sub>.

The structures of all known non-esterified nortropanes, if they carry two or more hydroxyl groups, are shown in Figs. 2 and 3. Tropane alkaloids bearing a methyl group on the nitrogen are included only if they are not esterified and were explicitly named *N*-methylcalystegines by the authors who first described them.

## 2.2 Chemical properties

Calystegines are highly hydrophilic, their calculated log *P* values (*P* is the octanol–water partition coefficient) are well below zero, ranging from  $-0.8$  for the diols (*e.g.* **24**) down to  $-1.6$  for the pentahydroxy alkaloids (**15** and **16**). This is one reason why they were detected only recently, although they occur in long-known and extensively examined plant tissues like potato tuber or belladonna leaf. With a typical alkaloid isolation scheme using organic solvent extraction from an alkaline aqueous solution or from an alkalised plant homo-

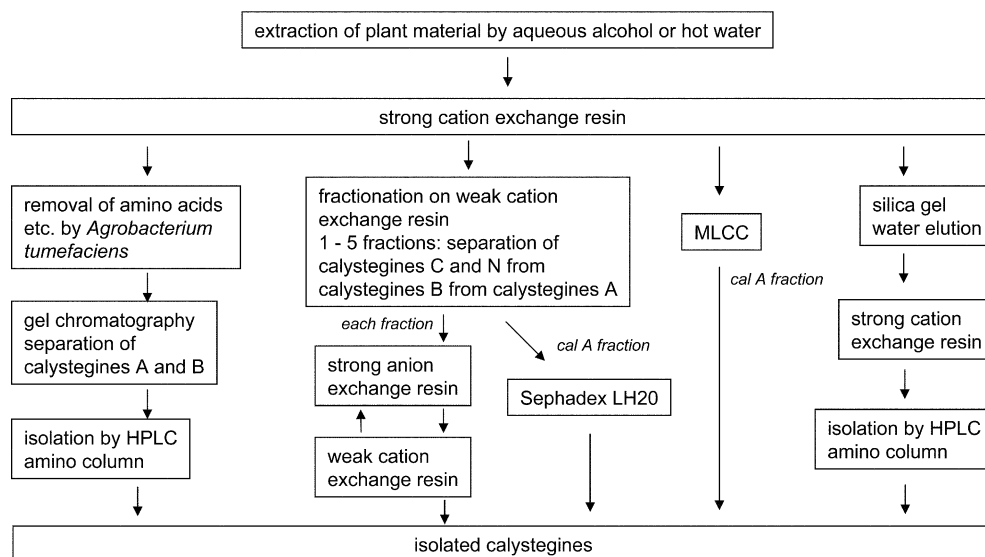


Fig. 4 Major isolation steps for calystegines.

genate, calystegines are discarded with the plant residues or with the water phase.

A typical feature of calystegines is a hydroxyl group on the bridgehead carbon 1, resulting in an aminoketal structure. The compounds **20** to **29** (Fig. 3) do not possess this property, which may be a reason for not including them in the calystegine group. Being in the vicinity of a hydroxyl group causes the nitrogen atom to become less basic, and the  $pK_a$  is lower in calystegines (calculated values 6.3 to 8.3) than in compounds **20** to **29** (calculated values 8.9 to 9.9). It has been questioned whether the bicyclic aminoketal structure is stable or whether it is in equilibrium with the monocyclic 1-aminocycloheptan-4-one structure. Studies on the synthesis of calystegines and analogous compounds have indicated that the number and position of the hydroxyl groups are decisive for the position of the equilibrium between monocyclic and bicyclic structures,<sup>13</sup> the naturally occurring calystegines showing a clear preference for the bicyclic structure. New calystegines or calystegine-like structures are constantly being published, and many more structures can be theoretically designed using the nortropane skeleton and assigning hydroxyl groups in all possible positions and arrangements. It is evident that the naturally occurring calystegines have a preference for equatorial or *exo* hydroxy groups. Calystegines with many axial hydroxyl groups are not found in nature, suggesting that they are less stable. This hypothesis was strengthened by the synthesis of epimers of calystegines **B**<sub>2</sub>, **B**<sub>3</sub> and **B**<sub>4</sub>, which turned out not to form stable compounds.<sup>14</sup> If a monocyclic cycloheptanone structure were to arise in plants, it might be subjected to ketone reduction, leading to a 5-amino-1,2,3-cycloheptanetriol, which can no longer form an aminoketal structure.<sup>8</sup>

In plants, glycosides of calystegines have been described, the first being a 3- $\beta$ -glucoside of calystegine **B**<sub>1</sub>.<sup>15</sup> Later, a 4- $\alpha$ -galactoside of calystegine **B**<sub>2</sub> was reported to occur in potato, in belladonna leaf,<sup>16</sup> and in mulberry.<sup>17</sup> No esters of calystegines **6** to **17** have been reported from natural sources, while, in contrast, hydroxylated tropane rings mostly accumulate esterified with aliphatic and aromatic carboxylic acids.<sup>18</sup> Esterification also is the preferred biotransformation reaction if tropine or other tropane alkaloid alcohols are applied to plant tissues from outside.<sup>19–22</sup> Mono- and di-hydroxynortropans, not calystegines in the strict sense, also occur as esters, baogongten **A** being one example.<sup>23</sup> Glycosylated alkaloids, in contrast, are rare in nature. It is remarkable that calystegines share the feature of being derivatised as glycosides with other alkaloidal glycosidase inhibitors such as deoxyojirimycin and fagomine.<sup>6,17,24</sup>

### 2.3 Isolation procedures from plant material

Isolation and purification of calystegines have been expertly reviewed,<sup>25</sup> therefore, only a short overview on the common approaches is given here. A few alterations of the protocols have been published since 1996 and they will be listed.

Calystegines are extracted from plant tissues either with 30–75% aqueous methanol or ethanol or with hot water. The heat is useful to destroy enzymatic activity and to coagulate and precipitate proteins. The aqueous solvents ensure the solubility of the hydrophilic alkaloids and prevent excessive extraction of contaminants.

In all protocols that have been developed for calystegine isolation, the first purification step is adsorption to a strong cation exchange resin, *e.g.* Dowex 50X8 or Amberlite IR-120B (Fig. 4). The alkaline nature of the molecules is utilised to separate organic acids and neutral compounds like carbohydrates by elution of the resin with water. Calystegines are thereafter eluted with dilute ammonia. The eluate will contain other alkaline organic compounds like certain amino acids and amino sugars. An elegant method for the removal of those was applied in the initial isolation procedures from *Calystegia sepium* roots. An *Agrobacterium* strain, *A. tumefaciens* B6 806, that was shown before not to catabolise calystegines was employed to remove amino acids and other organic material.<sup>3</sup> Subsequent gel chromatography separated the pools of calystegines **A** and **B**, which were subjected to paper electrophoresis and high performance liquid chromatography (HPLC) on an amino-column for isolation of single compounds.<sup>4</sup>

Isolation of calystegines can also be achieved by extensive ion exchange chromatography. Initial purification on a strong cation exchange column is followed by fractionation on a weak cation exchange column (Amberlite CG-50) in the  $NH_4^+$  form. Elution with water must be monitored by thin layer chromatography (TLC) for each fraction.<sup>26</sup> Calystegines like other alkaloidal glycosidase inhibitors possess no UV-light absorption. Fractions are pooled into two to five batches according to their appearance on TLC. A last fraction from the weak cation exchange column is obtained by elution with diluted ammonia. The elution order for calystegines is roughly correlated with their  $\log P$  values, with the calystegines **N**<sub>1</sub>, **C**<sub>1</sub>, and **C**<sub>2</sub> eluting in the first batch, followed by calystegines of the **B**-group, and finally the calystegines of the **A**-group.<sup>5,6,8</sup> *N*-Methylcalystegines **B**<sub>2</sub> **18** and **C**<sub>1</sub> **19** elute together with their non-methylated parent compounds.<sup>8</sup> Glucosylated calystegine **B**<sub>1</sub> was obtained from the weak cation exchange column by elution with aqueous pyridine.<sup>15</sup> The pooled fractions from the weak

cation exchange column are subjected individually to repeated ion exchange chromatography on a strong anion exchange column (Dowex 1-X2) in the OH<sup>-</sup> form and on the same weak cation exchange resin (Amberlite CG-50, NH<sub>4</sub><sup>+</sup> form), eluting with water only. Calystegines will pass through anion exchange columns almost without retention, but many contaminants, e.g. amino acids are retained due to their carboxylic group. Sometimes, an additional weak cation exchange column (CM-Sephadex C-25) eluted with 0.01 M ammonia is applied to the last fraction, which elutes from the Amberlite CG-50 column only with dilute ammonia. Pure dihydroxynortropans were obtained by this step.<sup>27</sup> These procedures for isolation are successful, but tedious and prone to high losses.

Calystegines of the A-group can alternatively also be isolated from their fraction after weak cation exchange using Sephadex LH-20 in the reversed phase mode with elution by n-butanol-acetic acid-water-methanol (8 : 8 : 72 : 12).<sup>5</sup> Sephadex LH 20 and elution by ethanol have also been used for purification of calystegine synthesis products.<sup>14</sup> Calystegine A<sub>3</sub> is sufficiently non-polar to be extracted from alkaline water (0.1 M ammonia) into an organic phase consisting of n-butanol. A multilayer coil chromatography (MLCC) apparatus is needed for the procedure to be efficient, but it still takes 20 hours.<sup>28</sup> A modified approach for isolation of calystegines and similar compounds from *Morus alba* was successfully applied by combining cation ion exchange chromatography with silica gel column chromatography.<sup>12</sup> After initial concentration of hydroxylated alkaloids on a strong cation exchange column, a silica gel column was eluted with a stepwise gradient starting with pure chloroform, adding methanol, then switching to methanol-water and ending with pure water elution. Calystegines eluted with the aqueous fractions. They were concentrated by absorption onto a cation exchange column (Dowex 50W-X4) adjusted to pH 5.7 with a formic acid-ammonia buffer and gradient elution by increasing ammonia (0% to 2.8%) in water. Isolation of individual calystegines was achieved by a final preparative HPLC step on an amino column eluted with 80% acetonitrile in water. Silica gel flash chromatography again is often used for purification of calystegine synthesis products or intermediates.

## 2.4 Structure elucidation

The main methods for structure elucidation of calystegines are high resolution mass spectrometry (MS) and proton and carbon nuclear magnetic resonance (NMR), including homonuclear and heteronuclear decoupling and two-dimensional techniques. Nuclear Overhauser effect (NOE) enhancements, heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) experiments are evaluated for the determination of interconnectivities and the stereochemistry of the alkaloids. Circular dichroism (CD) is necessary to determine the absolute configuration of the molecules. Mostly, optical rotation of a solution in water is also given for calystegines, but this does not define the absolute configuration. An excellent review in 1996<sup>25</sup> lists the nine calystegines whose structures had been elucidated by that time (A<sub>3</sub>, A<sub>5</sub>, A<sub>6</sub>, B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>4</sub>, C<sub>1</sub>, N<sub>1</sub>) with their mass spectrometric and spectroscopic data.

The calystegines A<sub>7</sub>, B<sub>5</sub> and the *N*-methylcalystegines were isolated from *Lycium chinense*, Solanaceae, and their structure elucidated in 1997,<sup>8</sup> and the same methods outlined above were adopted for their structure determination. Only two of the five *N*-methylcalystegines that were listed in the publication are of natural origin; *N*-methylcalystegines A<sub>3</sub>, B<sub>3</sub> and B<sub>4</sub> were obtained by methylation of the corresponding nortropans. Calystegine C<sub>2</sub> was isolated from *Duboisia leichhardtii* in 1997 and again structurally elucidated by high-resolution fast atom bombardment MS and a series of NMR experiments.<sup>29</sup> Dihydroxynortropans from *Erycibe* species, Convolvulaceae, were described earlier than calystegines and named erycibel-

line<sup>30</sup> **28** and baogongten C<sup>31</sup> **25** in 1986. Their structures were elucidated and their absolute configurations were determined by the [Pr(dpm)<sub>3</sub>]-induced CD split Cotton effect method, the exciton chirality method and the Horeau method.<sup>32</sup> Calystegine producing plants were examined for dihydroxynortropans, and two compounds were found in Convolvulaceae, (+)-**24** and (+)-**29**. 3*R*,6*R*-Dihydroxynortropane† **27** (optically inactive) was isolated from *Duboisia leichhardtii*, Solanaceae, aerial parts.<sup>27</sup> MS- and NMR-data were given, but the absolute configuration was not determined. *Morus alba* (white mulberry) was re-examined and yielded (-)-**26** from the fruits.<sup>34</sup> A thorough investigation of *Morus alba* fruits produced two new dihydroxynortropans: (-)-**23** and (-)-*ent*-**24**.<sup>12</sup> Infra red absorption bands, MS and NMR data were given for all compounds, and the absolute configurations were determined by CD experiments. In addition, the already known 3*S*,6*R*-dihydroxynortropane **26** was confirmed and its absolute configuration determined. This investigation also produced two further trihydroxynortropans, **21** and the symmetrical **22**. As a result of these studies, the absolute configurations of all the nortropans (Fig. 3) other than **27** and **29** have been determined but for the calystegines (Fig. 2) only B<sub>2</sub>, B<sub>3</sub> and B<sub>4</sub> have had their absolute configurations determined.<sup>14,33</sup> The MS and NMR data of the 3-O-β-D-glucoside of calystegine B<sub>2</sub> that served for structure elucidation were reported<sup>15</sup> as well as those of the 4-α-galactoside of calystegine B<sub>2</sub>.<sup>34</sup> Calystegines A<sub>3</sub>, B<sub>1</sub> and B<sub>2</sub>, 2,7-dihydroxynortropane **29** and in addition pseudotropine have been monitored by <sup>15</sup>N NMR after enrichment by tropinone application. The resonances for the respective nitrogen atoms were resolved by inverse-detected 2D NMR.<sup>35</sup>

## 2.5 Chemical synthesis

Since the elucidation of calystegine structures there has been considerable interest in the synthesis of these compounds. Enantioselective preparation, however, poses high demands, and the first synthetic approaches went through many steps, which resulted in a low overall yield. Only recently, more straightforward approaches have been published for the tetrahydroxylated calystegines of the B-group.<sup>36,37</sup> Of the known calystegines of natural origin, calystegines A<sub>3</sub>, B<sub>2</sub>, B<sub>3</sub>, and B<sub>4</sub> have been synthesised.

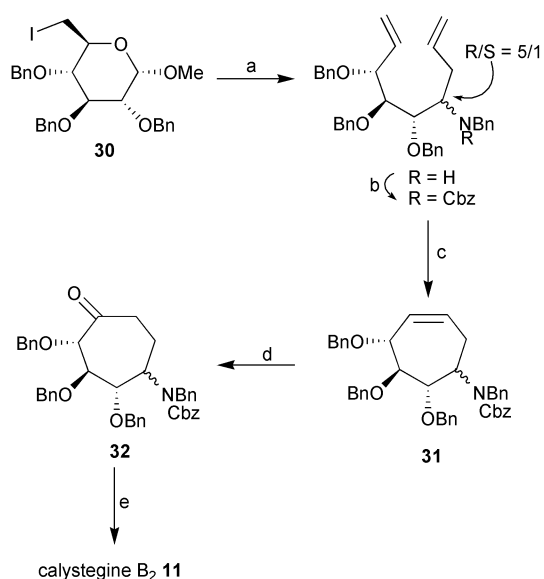
Two synthetic routes for calystegines A<sub>3</sub> have been published. The first approach used 3-aminocyclohexanone as starting material.<sup>13</sup> Ring enlargement into 5-amino-2-cycloheptenone was achieved by a three-step procedure: silyl enol ether formation, Simmons-Smith cyclopropanation, and finally ring-opening with iron(III) chloride. Subsequent *trans*-dihydroxylation of the double bond and cyclisation to the nortropane skeleton yielded a racemic mixture of calystegine A<sub>3</sub>. Both enantiomers of calystegine A<sub>3</sub> were produced by a different synthetic route starting from cycloheptatriene and going *via* the 6-azido derivatives of *meso*-2-cycloheptene-1,4-diol.<sup>38</sup> The amine group was obtained by reduction of the azido function and protected as benzyl carbamate. Asymmetrisation was achieved by using a *Pseudomonas cepacia* lipase, that selectively acylated one of the two hydroxyl groups of the aminocycloheptene ring. The remaining alcohol was substituted with phenyl selenide which after oxidation to the selenoxide produced a sigmatropic rearrangement to give the corresponding 6-aminocycloheptene-3,4-diol. After stereospecific introduction of a keto group, the aminocycloheptanone-diols were converted into the bicyclic aminoketal structures of the respective enantiomers of calystegine A<sub>3</sub>. The specific rotations of the calystegine A<sub>3</sub> enantiomers were +12.4° and -12.4° (*c* 0.63, water). As yet, however, it is not clear which is the

† The authors named the compound 3α,7β-dihydroxynortropane, but according to the IUPAC rules the numeration of carbons carrying hydroxyl groups as 3 and 6 is preferable.

absolute configuration of the naturally occurring calystegine A<sub>3</sub>. It is only assumed that it may be a precursor of calystegine B<sub>2</sub> and thus have the same configuration as the natural enantiomer (+)-calystegine B<sub>2</sub>.

Enantioselective synthetic routes for calystegine B<sub>2</sub> have been published. All start from D-glucose for the enantioselective preparation of the (–) and (+) enantiomers of calystegine B<sub>2</sub><sup>33,37,39–42</sup> or, in addition, from other aldohexose epimers, D-galactose for calystegine B<sub>3</sub> and D-mannose for calystegine B<sub>4</sub>.<sup>36</sup> The monosaccharide precursors introduced three hydroxyl groups in the particular desired configuration. Ring enlargement of the aldohexose was performed by various strategies and led to a protected polyhydroxyaminocycloheptanone, which after deprotection underwent spontaneous cyclisation to the bicyclic nortropane. The ring enlargement was achieved after formation of a cyclohexanone intermediate from glucose by a Ferrier reaction.<sup>33,40</sup> An alternative way to the aminocycloheptanone was the cycloaddition of chiral nitroso derivatives with cyclohepta-1,3-diene, which selectively provided one single stereoisomer.<sup>41</sup> Intramolecular cycloaddition of an olefinic nitrile oxide obtained from D-glucose was used in the first approach to achieve the hydroxylated aminocycloheptanone.<sup>39</sup> These methods suffered from low overall yields, being more than ten reaction steps in each case.

Ring closing olefin metathesis with Grubb's catalyst after a zinc-mediated tandem reaction on a methyl 6-iodoglycoside **30** is an elegant method leading to a protected trihydroxylated aminocycloheptene **31** (Fig. 5). Subsequent regioselective oxidation of the olefin then affords the corresponding aminocycloheptanone **32**. The method is much shorter and more efficient than older procedures. The formation of the bicyclic nortropane structure occurs spontaneously upon reductive deprotection. The synthesis of calystegine B<sub>2</sub> has been achieved by this method<sup>37,42</sup> and calystegines B<sub>3</sub> and B<sub>4</sub> were synthetically prepared for the first time starting from the respective D-galactose and D-mannose derivatives.<sup>14,36</sup>



**Fig. 5** Synthesis of calystegine B<sub>2</sub> by ring-closing metathesis starting from protected glucose.<sup>14</sup> *Reagents and conditions:* (a) Zn, BnNH<sub>2</sub>, CH<sub>2</sub>=CHCH<sub>2</sub>Br, THF, sonication, 40 °C; (b) CbzCl, KHCO<sub>3</sub>, EtOAc, H<sub>2</sub>O; (c) 2 mol% Grubb's catalyst, CH<sub>2</sub>Cl<sub>2</sub>, rt; (d) BH<sub>3</sub>·THF, THF, –40 to 0 °C, then H<sub>2</sub>O<sub>2</sub>, NaOH, H<sub>2</sub>O, 0 °C, then Dess–Martin periodinane, CH<sub>2</sub>Cl<sub>2</sub>, rt, separation of ketone isomers; (e) H<sub>2</sub>, Pd(OH)<sub>2</sub>/C, THF, H<sub>2</sub>O.

### 3 Biology

#### 3.1 Occurrence in the plant kingdom

For calystegines we are confronted with the same questions that are currently most intriguing for all secondary metabolites in

plant biology: what is the role of secondary products in the plant, and how has the specialised biosynthesis of these metabolites evolved during plant species diversification? As precise as possible an inventory of calystegine occurrence is essential to tackle questions of physiological impact and evolution. Authoritative reviews in recent years on polyhydroxy alkaloids include listings of calystegine occurrence in the plant kingdom,<sup>43–45</sup> but since then more structures and findings have been reported. For hypotheses on physiological roles, information on specific organ- and development-dependent concentrations is a prerequisite. It is attempted here to give a comprehensive overview on calystegine occurrence in plant species and plant organs and on quantities, as far as they have been reported.

#### 3.1.1 Identification and measurement in plant materials

Reports on calystegines occurrence in plants can be placed in three categories: 1) isolation from plant material, 2) quantitation (Table 1), and 3) further reports on calystegine occurrence. NMR spectroscopy in combination with high resolution MS of isolated compounds is the most reliable means of identification (see section 2.4). These reports usually give no indication of the quantities of calystegines in the plant source material. Concentrations of calystegines are determined by gas chromatography (GC) or GC-MS after derivatisation. For nortropane alkaloids a silylation procedure with mild reagents that leaves the secondary amino group non-derivatised leads to quantitative derivative formation. An initial screening of plant material for calystegine occurrence can be readily performed by thin layer chromatography. Confirmation of individual calystegine identities is achieved by GC-MS. Detailed methods for sample preparation and chromatographic analysis of calystegines from plant materials have been reviewed recently.<sup>26,46</sup>

Apart from the species listed in Table 1, calystegines have been reported from a few further Solanaceae without indication of quantities. Leaves of *Datura wrightii*, *Solanum dulcamara*, *S. melongena*, *S. dimidiatum* and *S. kwebense* were found to contain calystegine B<sub>2</sub>.<sup>47</sup> Roots and leaves of *Datura stramonium* contain calystegines in minute quantities, predominantly calystegine B<sub>2</sub>.<sup>48</sup> A considerable number of Convolvulaceae were submitted to a systematic screening for calystegines; 65 species were examined by GC-MS.<sup>49</sup> For many species, roots, leaves, flowers, and seeds were analysed separately. Thirty species contained at least one calystegine and most contained two or more different compounds. The calystegine-positive species belong to 15 genera: *Argyreia*, *Bonania*, *Calystegia*, *Convolvulus*, *Dichondra*, *Evolvulus*, *Erycibe*, *Falkia*, *Ipomoea*, *Iseia*, *Maripa*, *Merremia*, *Parona*, *Strictocardia*, and *Turbinia*. Calystegines A<sub>3</sub>, A<sub>5</sub>, B<sub>1</sub>, B<sub>2</sub>, B<sub>4</sub>, and C<sub>1</sub> were included in the screening and, as in Solanaceae, calystegine B<sub>2</sub> was the most frequently found. Calystegine C<sub>1</sub> was found in *Anagyrea hookeri* and *Ipomoea aquatica* only. Organ-specific calystegine patterns were recorded, and aerial parts of the plants were positive for calystegines as often as roots were. Within the Moraceae, only *Morus albus* fruits, leaves and root bark and *Morus bombycis* leaves have been reported as sources for calystegines (Table 1).

#### 3.1.2 Chemotaxonomy

Calystegines have been reported from three families of vascular plants but not from fungi or micro-organisms. Solanaceae and Convolvulaceae are closely related, both are in the order Solanales. Moraceae are more distant in the order Urticales. Tropane alkaloids in general are more widespread within the angiosperm plants.<sup>18,61</sup> They have been reported from eight mostly unrelated families of dicotyledonous plants (Magnoliopsida). No tropane alkaloids have been reported from monocots (Liliopsida) so far. A centre of tropane alkaloid occurrence appears to be in the family of Solanaceae. Calystegines

**Table 1** Occurrence of calystegines and related compounds

	Isolation	Concentration in µg/g fresh mass <sup>a</sup>
<b>calystegines A<sub>3</sub> (6), B<sub>1</sub> (10) and B<sub>2</sub> (11)</b>	<b>Convolvulaceae</b>	
	<i>Calystegia sepium</i> root culture A <sub>3</sub> , B <sub>1</sub> , B <sub>2</sub> <sup>4,50</sup>	<i>Calystegia sepium</i> root A <sub>3</sub> 800–190 (dm); B <sub>2</sub> 400–640 (dm) <sup>51</sup>
	<i>C. soldanella</i> whole plant A <sub>3</sub> , B <sub>1</sub> , B <sub>2</sub> <sup>27</sup>	<i>C. sepium</i> leaf A <sub>3</sub> 100–1340 (dm); B <sub>2</sub> 40–966 (dm) <sup>51</sup>
	<i>C. japonica</i> root, A <sub>3</sub> , B <sub>1</sub> , B <sub>2</sub> <sup>27</sup>	<i>C. sepium</i> seedling A <sub>3</sub> 3900 (dm); B <sub>2</sub> 1800 (dm) <sup>51</sup>
	<i>Ipomoea batatas</i> aerial parts B <sub>1</sub> , B <sub>2</sub> <sup>27</sup>	<i>C. sepium</i> flower A <sub>3</sub> 214 (dm); B <sub>2</sub> 244 (dm) <sup>51</sup>
	<i>I. carnea</i> aerial parts B <sub>1</sub> , B <sub>2</sub> <sup>27,52</sup>	<i>C. sepium</i> root culture A <sub>3</sub> 780 (dm); B <sub>1</sub> 300 (dm); B <sub>2</sub> 500 (dm) <sup>51</sup>
	<i>I. carnea</i> seed B <sub>1</sub> , B <sub>2</sub> <sup>52</sup>	<i>I. batatas</i> tuber A <sub>3</sub> 0.1; B <sub>1</sub> 2.4–16; B <sub>2</sub> 1.1–19 <sup>53</sup>
	<i>I. obscura</i> whole plant B <sub>1</sub> , B <sub>2</sub> <sup>27</sup>	<i>I. carnea</i> leaf B <sub>1</sub> 7; B <sub>2</sub> 21 <sup>52</sup>
	<i>I. pes-caprae</i> aerial parts B <sub>2</sub> <sup>27</sup>	<i>I. carnea</i> flower B <sub>1</sub> 4; B <sub>2</sub> 7 <sup>52</sup>
	<i>Quamoclit angulata</i> aerial parts B <sub>1</sub> , B <sub>2</sub> <sup>27</sup>	<i>I. carnea</i> seed B <sub>1</sub> 52; B <sub>2</sub> 213 <sup>52</sup>
	<b>Moraceae</b>	
	<i>Morus alba</i> root bark B <sub>1</sub> , B <sub>2</sub> <sup>6,17</sup>	<i>Morus alba</i> fruit B <sub>2</sub> <sup>7,53</sup>
	<i>M. alba</i> fruit B <sub>2</sub> <sup>17</sup>	
	<i>M. alba</i> leaf B <sub>2</sub> <sup>17</sup>	
	<i>M. bombycis</i> leaf B <sub>2</sub> <sup>24</sup>	
	<b>Solanaceae</b>	
	<i>Duboisia leichhardtii</i> leaf B <sub>1</sub> , B <sub>2</sub> <sup>29</sup>	<i>Atropa belladonna</i> root A <sub>3</sub> 14, B <sub>1</sub> 4, B <sub>2</sub> 13 <sup>48</sup>
	<i>Hyoscyamus niger</i> whole plant A <sub>3</sub> , B <sub>1</sub> , B <sub>2</sub> <sup>54</sup>	<i>A. belladonna</i> leaf A <sub>3</sub> 62, B <sub>1</sub> 16, B <sub>2</sub> 70 <sup>48</sup>
	<i>Lycium chinense</i> root A <sub>3</sub> , B <sub>1</sub> , B <sub>2</sub> <sup>8</sup>	<i>A. belladonna</i> young leaf A <sub>3</sub> 280, B <sub>1</sub> 80, B <sub>2</sub> 380 <sup>48</sup>
	<i>Nicandra physalodes</i> fruit B <sub>1</sub> <sup>15</sup>	<i>A. belladonna</i> flower A <sub>3</sub> 146, B <sub>1</sub> 57, B <sub>2</sub> 263 <sup>48</sup>
	<i>Physalis alkekengi</i> var. <i>francheti</i> root A <sub>3</sub> , B <sub>1</sub> , B <sub>2</sub> <sup>5</sup>	<i>A. belladonna</i> fruit A <sub>3</sub> 5, B <sub>2</sub> 16 <sup>48</sup>
	<i>Solanum dulcamara</i> whole plant A <sub>3</sub> , B <sub>1</sub> , B <sub>2</sub> <sup>27</sup>	<i>A. belladonna</i> root culture A <sub>3</sub> 1200 (dm), B <sub>2</sub> 320 (dm) <sup>55</sup>
		<i>Brunfelsia nitida</i> leaf A <sub>3</sub> 14 (dm), B <sub>1</sub> 10 (dm), B <sub>2</sub> 70 (dm) <sup>56</sup>
		<i>H. aureus</i> root culture A <sub>3</sub> 79 <sup>57</sup>
		<i>H. muticus</i> leaf A <sub>3</sub> 4 (dm); B <sub>2</sub> 11 (dm) <sup>58</sup>
		<i>H. muticus</i> root culture A <sub>3</sub> 112 <sup>57</sup>
		<i>H. niger</i> root A <sub>3</sub> 19–23; B <sub>1</sub> 22; B <sub>2</sub> 45 <sup>48,57</sup>
	<i>H. niger</i> leaf A <sub>3</sub> 2–14; B <sub>1</sub> 12; B <sub>2</sub> 15 <sup>48,57</sup>	
	<i>H. niger</i> young leaf A <sub>3</sub> 28; B <sub>1</sub> 35; B <sub>2</sub> 57 <sup>48</sup>	
	<i>H. niger</i> flower A <sub>3</sub> 7; B <sub>2</sub> 3 <sup>48</sup>	
	<i>H. niger</i> root culture A <sub>3</sub> 100–120 <sup>57</sup>	
	<i>H. pusillus</i> root culture A <sub>3</sub> 43 <sup>57</sup>	
	<i>Lycopersicon esculentum</i> fruit A <sub>3</sub> 1.1; B <sub>2</sub> 4.5 <sup>53</sup>	
	<i>Mandragora autumnalis</i> root A <sub>3</sub> 30 (dm); B <sub>2</sub> 20 (dm) <sup>56</sup>	
	<i>M. officinarum</i> root A <sub>3</sub> 48; B <sub>1</sub> 35; B <sub>2</sub> 39 <sup>48</sup>	
	<i>M. officinarum</i> leaf A <sub>3</sub> 87; B <sub>1</sub> 44; B <sub>2</sub> 80 <sup>48</sup>	
	<i>M. officinarum</i> young leaf A <sub>3</sub> 200; B <sub>1</sub> 105; B <sub>2</sub> 138 <sup>48</sup>	
	<i>M. officinarum</i> flower A <sub>3</sub> 76; B <sub>1</sub> 40; B <sub>2</sub> 69 <sup>48</sup>	
	<i>M. officinarum</i> fruit A <sub>3</sub> 20; B <sub>1</sub> 17; B <sub>2</sub> 30 <sup>48</sup>	
	<i>Scopolia carniolica</i> root A <sub>3</sub> 72; B <sub>1</sub> 78; B <sub>2</sub> 60 <sup>48</sup>	
	<i>S. carniolica</i> leaf A <sub>3</sub> 27; B <sub>1</sub> 22; B <sub>2</sub> 23 <sup>48</sup>	
	<i>S. carniolica</i> young leaf A <sub>3</sub> 260; B <sub>1</sub> 144; B <sub>2</sub> 194 <sup>48</sup>	
	<i>S. carniolica</i> flower A <sub>3</sub> 150; B <sub>1</sub> 72; B <sub>2</sub> 140 <sup>48</sup>	
	<i>Physalis peruviana</i> fruit A <sub>3</sub> 0.003; B <sub>1</sub> 0.038; B <sub>2</sub> 0.048 <sup>53</sup>	
	<i>Solanum melongena</i> fruit A <sub>3</sub> 0.312; B <sub>2</sub> 0.473 <sup>53</sup>	
	<i>S. sodomaeum</i> B <sub>2</sub> 100 (dm) <sup>56</sup>	
	<i>S. tuberosum</i> root A <sub>3</sub> 26; B <sub>2</sub> 50 <sup>59</sup>	
	<i>S. tuberosum</i> leaf A <sub>3</sub> 18; B <sub>2</sub> 13–34 <sup>59</sup>	
	<i>S. tuberosum</i> flower A <sub>3</sub> 37; B <sub>2</sub> 103 <sup>59</sup>	
	<i>S. tuberosum</i> tuber flesh A <sub>3</sub> 0.2–11; B <sub>2</sub> 1.7–68 <sup>59,60</sup>	
	<i>S. tuberosum</i> tuber peel A <sub>3</sub> 6.3–390; B <sub>2</sub> 24–450 <sup>59,60</sup>	
	<i>S. tuberosum</i> tuber sprout A <sub>3</sub> 70; B <sub>2</sub> 410 <sup>48</sup>	
	<i>S. tuberosum</i> young t. sprout A <sub>3</sub> 800; B <sub>2</sub> 2300 <sup>59</sup>	
	<i>Withania frutescens</i> leaf A <sub>3</sub> 35 (dm); B <sub>1</sub> 100 (dm); B <sub>2</sub> 40 (dm) <sup>56</sup>	
	<i>W. somnifera</i> leaf B <sub>2</sub> 11 (dm) <sup>56</sup>	
<b>calystegine A<sub>5</sub> (7)</b>	<b>Solanaceae</b>	
	<i>Duboisia leichhardtii</i> aerial parts <sup>27</sup>	<i>A. belladonna</i> root culture 400 (dm) <sup>55</sup>
	<i>H. niger</i> whole plant <sup>54</sup>	<i>C. sepium</i> root culture 6 <sup>55</sup>
	<i>Lycium chinense</i> root <sup>8</sup>	
	<i>P. alkekengi</i> root <sup>5</sup>	
<b>calystegine A<sub>6</sub> (8)</b>	<i>Solanum dulcamara</i> whole plant <sup>27</sup>	
	<i>Scopolia japonica</i> root <sup>7</sup>	
	<b>Solanaceae</b>	
<b>calystegine A<sub>7</sub> (9)</b>	<i>L. chinense</i> root <sup>8</sup>	
	<i>H. niger</i> whole plant <sup>54</sup>	
<b>calystegine B<sub>3</sub> (12)</b>	<b>Solanaceae</b>	
	<i>L. chinense</i> root <sup>8</sup>	
	<b>Convolvulaceae</b>	
	<i>I. carnea</i> leaf <sup>52</sup>	<i>I. carnea</i> leaf 4 <sup>52</sup>
	<i>I. carnea</i> seed <sup>52</sup>	<i>I. carnea</i> seed 6 <sup>52</sup>
	<b>Solanaceae</b>	
	<i>H. niger</i> whole plant <sup>54</sup>	
	<i>L. chinense</i> root <sup>8</sup>	
	<i>P. alkekengi</i> root <sup>5</sup>	
	<i>Scopolia japonica</i> root <sup>7</sup>	

Table 1 (Contd.)

	Isolation	Concentration in µg/g fresh mass <sup>a</sup>
calystegine B <sub>4</sub> (13)	<b>Solanaceae</b> <i>Duboisia leichhardtii</i> leaf <sup>29</sup> <i>L. chinense</i> root <sup>8</sup> <i>Scopolia japonica</i> root <sup>7</sup>	<i>S. tuberosum</i> young t. sprout 100 <sup>59</sup>
calystegine B <sub>5</sub> (14)	<b>Solanaceae</b> <i>L. chinense</i> root <sup>8</sup>	
calystegine C <sub>1</sub> (15)	<b>Convolvulaceae</b> <i>I. carnea</i> leaf <sup>52</sup> <i>I. carnea</i> seed <sup>52</sup>	<i>I. carnea</i> leaf 13 <sup>52</sup> <i>I. carnea</i> seed 40 <sup>52</sup> <i>I. batatas</i> tuber 0.61–9 <sup>53</sup>
	<b>Moraceae</b> <i>M. alba</i> root bark <sup>6</sup>	
	<b>Solanaceae</b> <i>Duboisia leichhardtii</i> leaf <sup>29</sup> <i>L. chinense</i> root <sup>8</sup> <i>Scopolia japonica</i> root <sup>7</sup>	<i>Brunfelsia nitida</i> leaf 350 (dm) <sup>56</sup> <i>Withania frutescens</i> leaf 20 (dm) <sup>56</sup> <i>W. somnifera</i> leaf 80 (dm) <sup>56</sup>
calystegine C <sub>2</sub> (16)	<b>Solanaceae</b> <i>Duboisia leichhardtii</i> leaf <sup>29</sup> <i>Lycium chinense</i> root <sup>8</sup>	
calystegine N <sub>1</sub> (17)	<b>Solanaceae</b> <i>H. niger</i> whole plant <sup>54</sup> <i>L. chinense</i> root <sup>8</sup> <i>S. dulcamara</i> whole plant <sup>27</sup>	
<i>N</i> -methylcalystegine B <sub>2</sub> (18) and C <sub>1</sub> (19)	<b>Solanaceae</b> <i>L. chinense</i> root <sup>8</sup>	
21, 22, 23, <i>ent</i> -24, 26	<b>Moraceae</b> <i>M. alba</i> fruit <sup>12,17</sup>	
24	<b>Convolvulaceae</b> <i>Calystegia soldanella</i> whole plant <sup>27</sup>	
baogongten C (25)	<b>Convolvulaceae</b> <i>Erycibe obtusifolia</i> <sup>30</sup>	
27	<b>Solanaceae</b> <i>D. leichhardtii</i> aerial parts <sup>27</sup>	
erycibelline (28)	<b>Convolvulaceae</b> <i>Erycibe elliptilimba</i> stem <sup>30</sup>	
29	<b>Convolvulaceae</b> <i>C. japonica</i> root <sup>27</sup> <i>C. sepium</i> root culture <sup>27</sup> <i>C. soldanella</i> whole plant <sup>27</sup> <i>I. batatas</i> aerial parts <sup>27</sup> <i>I. carnea</i> aerial parts <sup>27</sup> <i>Quamoclit angulata</i> aerial parts B <sub>1</sub> , B <sub>2</sub> <sup>27</sup>	<i>C. sepium</i> root culture < 10 (dm) <sup>51</sup>

<sup>a</sup> In most reports, calystegines are given in microgram per gram fresh mass. Some authors relate calystegine quantities to plant dry mass (dm). Typically plant tissues contain 10% dry mass per fresh mass, cultured roots 6–10%,<sup>55</sup> potato tuber tissues 12–22%.<sup>59</sup> Ripe seeds contain 10–15% water only.

are also concentrated in Convolvulaceae and Solanaceae. The two *Morus* species containing calystegines seem to be isolated in the Moraceae. A further ten Moraceae were examined and found negative (Brock and Dräger, unpublished). Negative results of screening for alkaloids are seldom reported, but for chemotaxonomy they would be useful to know. The largest coverage of species examined for calystegines was achieved within the Convolvulaceae, where 65 species in 22 genera have been included.<sup>49</sup> Convolvulaceae contain about 1600 species in 56 genera, 600 species belong to the genus *Ipomoea* alone.<sup>62</sup> The large family of Solanaceae comprises 94 genera and 2950 species. The largest genus is *Solanum* with around 1400 species.<sup>62</sup> Only 25 species from 12 genera have been reported positive for calystegines (Table 1). Negative results are not known, except for tobacco species that were reported to contain neither calystegines nor tropane alkaloids.<sup>22</sup> The numbers of taxa in each plant family are subject to small variations depending on the reference, but they illustrate that in spite of intensive research on calystegines in some species the inventory in the three families is far from being comprehensive. Thus chemotaxonomic conclusions are hard to establish, because a very small fraction of total plants has been tested. The analysis for the polar compounds demands special techniques of extraction and separation, that are time-consuming. Nevertheless other

tropane alkaloid containing families should be investigated for calystegines, because in Convolvulaceae and Solanaceae calystegines derive from the tropane alkaloid pathway (chapter 3.2).

### 3.1.3 Calystegine compositions and concentrations

Calystegines were initially reported to be restricted to roots,<sup>3</sup> but later they were found in all parts of the plants, when these were examined. For most species, reports exist for one or selected tissues only, e.g. roots or fruits. The composition and concentration of calystegines are variable among plants and within one plant, still a few generalisations can be established:

- Calystegine B<sub>2</sub> is the most abundant calystegine. It occurs in almost all plants that were analysed positive for calystegines. There are Convolvulaceae that contain other calystegines but not calystegine B<sub>2</sub>, but this is rare.<sup>49</sup> Calystegine B<sub>2</sub> may be the most stable form due to its four equatorial hydroxyl groups.
- Calystegine A<sub>3</sub> and B<sub>1</sub> are also widespread in occurrence.
- Moraceae do not contain the calystegines of the A-group.
- Young tissues are those with the highest concentrations of calystegines, potato tuber sprouts contain more than 3 mg/g fresh mass. Root cultures often contain higher calystegine concentrations than roots of intact plants, indicating that they

contain a large proportion of young and proliferating root tissue. Tropane alkaloid biosynthetic enzymes were localised in root pericycle and endodermis, tissues specific for young roots.<sup>63,64</sup>

*Atropa belladonna* and *Hyoscyamus* species are known for their content of the tropane alkaloids hyoscyamine and scopolamine. Leaves of *A. belladonna* typically contain 3 mg/g dm<sup>‡</sup> of hyoscyamine,<sup>65</sup> more than calystegines A<sub>3</sub>, B<sub>1</sub> and B<sub>2</sub> together (ca. 1.5 mg/g dm). In roots of the plants, hyoscyamine (2–3 mg/g dm) again is higher than calystegine concentrations (ca. 300 µg/g dm), but in cultured roots, calystegines accumulate to higher ratios when 5% sucrose is applied (up to 4 mg/g dm), while hyoscyamine rises to 2.5 mg/g dm. In leaves of *Hyoscyamus muticus* plants<sup>58</sup> and in root cultures of *Hyoscyamus* species, roughly similar ratios were observed.<sup>57,66</sup> Obviously, a considerable fraction of total tropane skeletons that are formed is devoted to calystegine formation, in particular in young tissues.

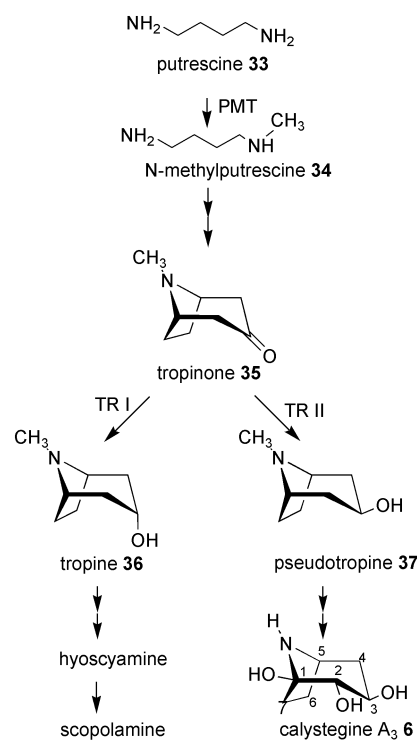
Other plant glycosidase inhibitors typically accumulate in low concentrations. Swainsonine was measured in leaves and flowers of *Swainsona galegifolia* (Fabaceae) as 29 and 28 µg/g fm<sup>‡</sup>, respectively.<sup>52</sup> Transformed root cultures produced higher swainsonine levels (62.3 µg/g dm) than non-transformed root cultures (23.6 µg/g dm) or roots of intact plants (8.7 µg/g dm).<sup>67</sup> *Astragalus* species (Fabaceae) may contain 2.8 mg swainsonine per gram dry mass in flowers, but usual concentrations range from 50 to 300 µg/g dm in leaves.<sup>68</sup> Castanospermine concentrations in *Castanospermum australe* (Fabaceae) were reported as 150–200 µg/g dm in seeds and 130–150 µg/g dm in leaves. These data are comparable to some calystegine concentrations, however, young tissues with extraordinarily high accumulations of glycosidase inhibitors have not been reported for glycosidase inhibitors other than calystegines.

## 3.2 Biosynthesis

Calystegines are the only group of glycosidase inhibitors for which biosynthetic metabolites, enzymes, and genes are known. The investigations have clearly profited from the intensive research performed on tropane alkaloid biosynthesis in Solanaceae.<sup>69–72</sup> In the course of the search for tropinone metabolising enzymes, a reductase was isolated that stereospecifically formed pseudotropine **37** from tropinone **35**.<sup>73</sup> Initially it was a “wrong enzyme”, because tropane alkaloid formation demands a reductase that produces tropine, which is exclusively incorporated into hyoscyamine (Fig. 6). The pseudotropine-forming tropinone reductase (TRII, EC 1.1.1.236) was subsequently found in several Solanaceae in combination with the desired tropine-forming tropinone reductase (TRI, EC 1.1.1.206).<sup>74–77</sup> Both enzymes in all cases proved specific for their respective alcohol isomeric product. As pseudotropine does not accumulate in the root tissues from which TRII was isolated, other unknown alkaloid products were postulated to be formed from pseudotropine, but this was unsatisfactory. The first calystegine structures to be published were isolated from *Calystegia sepium* and *Atropa belladonna* in 1990.<sup>4</sup> They displayed an equatorial hydroxyl group on carbon 3 of a nortropane skeleton. It was therefore hypothesised that calystegines were the sought-after products from pseudotropine. The biosynthesis of calystegines was summarised in 1996<sup>25</sup> and since then some progress has been made.

### 3.2.1 Co-occurrence of calystegines with tropane alkaloids

All Solanaceae species that were known for tropane alkaloid formation and included in a screening also contained calystegines.<sup>48</sup> An initial search for tropinone-reducing enzyme activity revealed TRI and TRII in all Solanaceae that contained tropane alkaloids. No such activity was found in *Nicotiana*



**Fig. 6** Some steps of the tropane alkaloid biosynthetic pathway (PMT putrescine *N*-methyltransferase; TRII tropine-forming tropinone reductase; TRII pseudotropine-forming tropinone reductase).

*tabacum* and in *Brassica campestris* (Brassicaceae, negative control), while *Calystegia sepium* showed only TRII activity.<sup>74</sup> Tropane esters and nortropine esters with vanillic acid and veratric acid had been isolated from *Convolvulus* species, *C. erinacius*, *C. krauseanus*, and *C. lineatus*, and reported between 1965 and 1977.<sup>78–80</sup> Several Convolvulaceae root tissues, *C. sepium* included, were reported in 1973 not to contain tropane alkaloids, but cuscohygrine, a putative by-product of tropane alkaloid formation, was detected in roots.<sup>81</sup> In a later study in 1995, tropinone, tropine and pseudotropine were isolated from aerial parts of *Convolvulus arvensis*.<sup>82</sup> From *Convolvulus siculus* and *C. sabatius* ssp. *mauritanicus* three esters of tropine were isolated.<sup>83</sup> Thus there is evidence for tropane alkaloid formation in *Convolvulus* species, which strengthens the assumption of a common biosynthetic pathway for calystegines and tropane alkaloids in Convolvulaceae as well.

Sprouting potato tubers contain calystegines, but not tropane alkaloids. When tropinone was applied from outside to sprouts or leaves of potato, however, pseudotropine was detected repeatedly, sometimes together with tropine. The capacity to reduce tropinone is specific for tropane alkaloid forming tissues and was found to be absent in *Nicotiana* leaves.<sup>59</sup> Two *Morus* species, from which calystegines were isolated, have never been reported to contain any tropane alkaloids. Application of tropinone to *Morus alba* leaves again yielded pseudotropine (Brock and Dräger, unpublished). Norpseudotropine was recently isolated from *Morus alba* fruits.<sup>12</sup>

### 3.2.2 Tropinone reductases

In order to establish the descent of calystegines from the tropane alkaloid pathway, research focused on distribution and specificities of tropinone reductases in those plants that accumulate calystegines but not classical tropane alkaloids. Characterisation and cloning of TRs from *Datura stramonium*<sup>84</sup> and *Hyoscyamus niger*<sup>85,86</sup> had revealed a high degree of sequence identity and many common features. All enzymes belong to the family of short-chain dehydrogenases (SDR) with a molecular mass around 29 kDa and typical amino acid motifs (Table 2).<sup>87</sup> They all require NADPH as

‡ dm means dry mass (of plant) and fm means fresh mass.

**Table 2** Tropinone reductases, genes and proteins

Reductase	EMBL accession number	Protein size and pI value
<i>Calystegia sepium</i> TRI	AJ427397	263 AA, 28.5 kDa, 6.4
<i>Datura stramonium</i> TRI	L20473	273 AA, 29.6 kDa, 6.1
<i>Hyoscyamus niger</i> TRI	D88156/AB026544	274 AA, 29.6 kDa, 5.9
<i>Solanum tuberosum</i> TRI	AJ307584	264 AA, 28.7 kDa, 5.9
<i>Datura stramonium</i> TRII	L20474	260 AA, 28.3 kDa, 5.8
<i>Hyoscyamus niger</i> TRII	L20485/AB026545	260 AA, 28.4 kDa, 5.9
<i>Solanum tuberosum</i> TRII	AJ292343	261 AA, 28.4 kDa, 5.3
<i>Calystegia sepium</i> putative TR	AJ540305	258 AA, 27.6 kDa, 5.9
<i>Datura stramonium</i> putative TR	L20475	268 AA, 28.6 kDa, 7.7
<i>Solanum tuberosum</i> putative TR	AJ400815	264 AA, 28.7 kDa, 6.9

reducing co-substrate. The enzymes are somewhat permissive for the ketone substrates, but strictly specific for the positioning of the alcohol group to be formed. The stereospecificity is conferred by the selective binding of tropinone by TRI and TRII.<sup>76,88</sup> Both proteins were crystallised, and tropinone binding was studied in detail. In both TRs different charged residues in the substrate binding site conferred different electrostatic environments on the two enzymes. A modelling study indicated that these charged residues control opposite binding orientation of tropinone within the substrate binding site, while NADPH is bound in the same orientation. The reducing hydride then attacks tropinone on the appropriate face to leave the alcohol group in either the equatorial or axial position.<sup>89-92</sup>

*Solanum* species are known to accumulate steroid alkaloids, but were thought not to express the tropane alkaloid pathway. Tropinone reductases are labile enzymes, and isolation of an active tropinone reducing enzyme from *Solanum tuberosum* tissues failed, probably due to high phenolic contents.<sup>93</sup> PCR-based cDNA cloning with primers obtained from consensus sequences of TRs and SDRs yielded several TR-like sequences from potato. The degree of identity in amino acid sequence to tropinone reductases from tropane alkaloid plants is high (91–95%). One of the potato TR sequences after expression in *E. coli* displayed pseudotropine formation and similar kinetic characteristics to the TRII enzymes from *Hyoscyamus*, *Datura* and *Atropa* species. The TRII enzyme is considered specific for pseudotropine formation and devoted to calystegine biosynthesis in potato.<sup>94</sup> Accounting for the tropine appearance after tropinone feeding to potato tissues, TRI-like sequences were also isolated from potato. A *Solanum tuberosum* reductase specific for tropine formation from tropinone was also expressed in *E. coli* (Kaiser, Keiner and Dräger, unpublished). This sequence shows 84–87% identity to other TRI proteins. Both the cDNA and the amino acids sequences are available in the EMBL gene bank as are all other TR sequences as well (Table 2).

A TRII was purified and characterised from *Calystegia sepium* root cultures. The enzyme prefers tropinone as substrate and forms pseudotropine exclusively. PCR-based cloning of TRs from *C. sepium* yielded TR-like sequences (Table 2). One of the cloned TRs from *C. sepium* after *E. coli* expression reduces tropinone to tropine only and was named TRI, indicating that in *C. sepium* again, both TRI and TRII are expressed. The TR-like amino acid sequences from the Convolvulaceae plant show typical length and SDR motifs, but have lower degrees of identity (55–66%) than within the TRs from Solanaceae (Sichhart, Meier and Dräger, unpublished).

### 3.2.3 Putrescine methyltransferase

The reduction of tropinone requires the preceding formation of the bicyclic tropane skeleton, which has been established to start by methylation of the ubiquitous polyamine putrescine **33** (Fig. 6). This reaction is common to both tropane and nicotine biosynthesis, and the enzyme putrescine *N*-methyltransferase

(PMT, EC 2.1.1.53) has been purified and its activity measured from tobacco plant roots<sup>95-98</sup> and callus cultures.<sup>99</sup> Root cultures of *Datura stramonium*,<sup>97,100,101</sup> *Hyoscyamus albus*<sup>102,103</sup> and *H. niger*<sup>104</sup> contain PMT with similar properties to the enzyme from tobacco. The cDNA of PMT was cloned from tobacco (EMBL Accession No. D28506)<sup>105</sup> and from *Nicotiana sylvestris* (EMBL Accession Nos. AB004322, AB004323, AB004324).<sup>106</sup> The gene for PMT was shown to be exclusively expressed in the root pericycle of *Atropa belladonna*<sup>64</sup> and in the endodermis, xylem and outer cortex cells of *Nicotiana sylvestris*.<sup>106</sup> In tobacco, PMT is stress-responsive and inducible by methyl jasmonate<sup>106-109</sup> but, in contrast to the tobacco PMT promoter,<sup>110</sup> in *Atropa belladonna* no jasmonate responsive element was identified in the promoter region.<sup>64</sup> Correspondingly, hyoscyamine was not enhanced and calystegines decreased upon elicitation in root cultures of *A. belladonna*.<sup>55</sup> In all investigations published so far, calystegine concentrations vary considerably among plants and within one plant, but appear not to be inducible by stress, but strictly developmentally regulated.

PMT expression is also responsive to auxin. In nicotine and in tropane alkaloid producing plants, PMT is suppressed upon auxin application.<sup>109,111,112</sup> PMT resembles spermidine synthase (SPDS, EC 2.5.1.16), and this ubiquitous enzyme is considered the evolutionary ancestor of PMT.<sup>113</sup> *Solanum tuberosum* as well as calystegine-forming Convolvulaceae must contain PMT activity to build up the tropane alkaloids skeleton. However, it could be hypothesised that PMT is not required for calystegine formation and the bicyclic nortropane skeleton arises directly from putrescine without methylation *via* oxidation and addition of three extra carbons. In fact one proposed pathway does not contain either a TR or PMT.<sup>8</sup> As TRs were isolated from *Solanum tuberosum* and *Calystegia sepium*, a cloning of PMTs from these plants was undertaken and yielded PMT-typical sequences which are currently being expressed (Stenzel, Teuber and Dräger, unpublished).

### 3.2.4 Further metabolic steps

**Demethylation.** A characteristic feature of most calystegines is the secondary amino group in the ring, *i.e.* the loss of the *N*-methyl group. There are clues for the assumption that *N*-demethylation will occur as the next metabolic step after formation of pseudotropine: norpseudotropine was isolated from *Morus albus* fruits.<sup>12</sup> In *C. sepium* root cultures nortropinone was detectable upon tropinone application (Sichhart, Meier and Dräger, unpublished). In Solanaceae, demethylation of the nitrogen in the tropane skeleton is not unusual. Several esterified nortropane alkaloids have been reported.<sup>18</sup> Nortropane alkaloids have also been isolated from *Erythroxylum* species.<sup>9,10,114,115</sup> Ideas on the mechanism of *N*-demethylation exist from the formation of nornicotine from nicotine. Oxidative hydrolysis can be concluded from degradation of *N*-<sup>13</sup>C-methyl-labelled nicotine, after which the label was retrieved in carbon dioxide and in the amino acids serine and

methionine. Folate is consequently assumed to assist in the transfer of the C<sub>1</sub>-group.<sup>116</sup> Demethylation of pseudotropine may even be a rate-limiting step for calystegine biosynthesis. Tropinone reductase activity in most tissues is higher than required for tropane alkaloid biosynthesis,<sup>74</sup> and reduction products are esterified with acetic acid or tiglic acid if they accumulate in excess upon tropinone application.<sup>117</sup> Norpseudotropine, however, is found in calystegine-forming tissues in traces only, if at all.

**Hydroxylation and dehydroxylation.** The subsequent hydroxylating steps are not known at all. It may be speculated whether every hydroxyl position on every calystegine is introduced by a specialised enzyme. Some of the plentitude of calystegine structures may be imagined to arise by isomerisation through the monocyclic aminoheptanone **38** (Fig. 7), the reduced form of which was also isolated from plant tissue.<sup>118</sup> Dihydroxynortropans lacking a hydroxyl group on carbon 3 pose a problem for the current model of calystegines biosynthesis, because it must be postulated that they are dehydroxylated. Incorporation of labelled <sup>15</sup>N-tropinone into 2,7-dihydroxynortropane *ent*-**28** reveals that these metabolites also derive from tropinone in *C. sepium*, by whichever mechanism.<sup>35,51</sup> The same feeding experiments unequivocally proved descent of calystegines from labelled tropinone *via* labelled pseudotropine, and enabled an estimate of the flux of tropane skeletons into calystegine biosynthesis.<sup>35,51</sup>

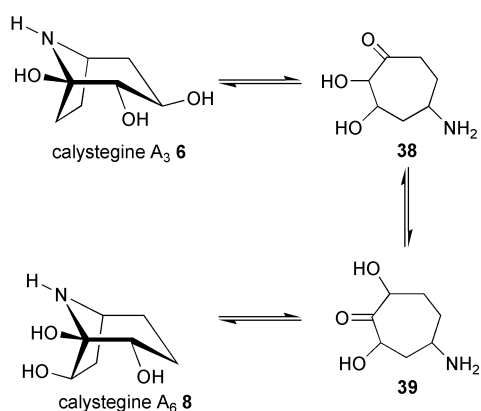


Fig. 7 Possible origin of calystegines by isomerisation.

**Location of biosynthesis and possible transport.** Tropane and nicotine alkaloid biosyntheses in Solanaceae are believed to take place in the roots, and the alkaloids are transported into aerial parts of the plant by the xylem. This knowledge arose from early grafting experiments<sup>119,120</sup> and analysis of xylem sap.<sup>121</sup> Tropane alkaloids can also be transported by the phloem sap.<sup>122</sup> Calystegines were isolated from aerial parts and roots of several plants, but biosynthetic enzymes, *i.e.* tropinone reductases were isolated from roots only. Isolated potato tubers, which are stem organs, produce high amounts of calystegines during sprouting, proving calystegine biosynthesis in non-rooted stem tissues that normally grow underground. The TRII transcript is expressed in potato plants, especially in the sprouts, as expected from calystegine accumulation, but also in roots and aerial parts. The PMT transcript is localised in sprouting tuber eyes as well (Stenzel and Dräger, unpublished). The observation that the distribution of the TRII transcript differs from the tissues of highest calystegine accumulation suggests transport of calystegines or their precursors between potato organs.<sup>94</sup>

### 3.3 Biological activity

#### 3.3.1 Biochemical activities

Calystegines are glycosidase inhibitors with selective activity for certain glycosidase. Their inhibitory activity is strong in most

cases;  $K_i$ -values of less than 1  $\mu\text{M}$  were reported, *e.g.*  $K_i$  0.86  $\mu\text{M}$  for calystegine B<sub>2</sub> towards coffee bean  $\alpha$ -galactosidase and  $K_i$  0.45  $\mu\text{M}$  for calystegine C<sub>1</sub> towards almond  $\beta$ -glycosidase.<sup>8</sup> The inhibitory potency is comparable to other alkaloidal glycosidase inhibitors like swainsonine with a  $K_i$  of 2  $\mu\text{M}$  against human  $\alpha$ -mannosidase.<sup>123</sup> The therapeutically applied deoxynojirimycin derivative miglitol **5** exhibits an inhibition of  $K_i$  0.11  $\mu\text{M}$  against rat sucrase.<sup>124</sup>

Calystegines, like most other glycosidase inhibitors, compete with the substrate for the binding in the active site, as concluded from their behaviour in kinetic interaction measurements.<sup>8,54</sup> There are two exceptions: calystegine N<sub>1</sub><sup>54</sup> and the 3-O- $\beta$ -D-glucoside of calystegine B<sub>1</sub> are non-competitive inhibitors.<sup>125</sup> Most glycosidases perform an acid-assisted hydrolysis with the aid of a glutamic acid residue in the active cleft.<sup>126</sup> Calystegines may mimic the transition state of the enzymatic hydrolysis reaction, a semiplanar glycosyl cation. Alternatively, they may resemble the reaction product, a monosaccharide, but they do not leave the active centre due to their positively charged nitrogen atom bound to the glutamate residue. The calystegine inhibitory mechanism is not known yet. The models of action derive from the knowledge of glycosidase structures for which lysozyme is a protagonist<sup>127</sup> and from the inhibitory mechanism of other glycosidase inhibitors that have been studied in detail.

The glycosidase inhibitory activity of most calystegines has been reviewed.<sup>43,44,128</sup> The authors either gave qualitative indications or grouped the inhibitory potency into three categories: weak, moderate, and potent, and they refrained from giving exact data. Indeed much of the data in the literature is given as IC<sub>50</sub> values for calystegine inhibition of a particular glycosidase. The IC<sub>50</sub> value is meant to indicate the concentration of the inhibitor, which leads to a 50% inhibition of the reaction velocity. With competitive inhibition, this value varies of course with the substrate concentration. As many of the references do not give the substrate concentration used for inhibition experiments, those IC<sub>50</sub> data are only valid for comparison within one series of measurements. Thus, an estimate as *e.g.* potent or moderate inhibitor is mostly the appropriate summary of the data.  $K_i$  values are more precise and preferable, because they are valid independently from individual assay mixtures. However, they require more measurements, which sometimes may have been prevented by the shortage of individual calystegines.

Calystegine effects on proteins other than glycosidases have not yet been reported, but may be expected. Other polyhydroxy alkaloids possess additional biochemical effects, *e.g.* inhibition of glycogen phosphorylase by isofagomine<sup>129</sup> or of eukaryotic DNA polymerases by the pyrrolidine alkaloid 1,4-dideoxy-1,4-imino-D-ribitol.<sup>130</sup>

#### 3.3.2 Toxicity

While castanospermine and fagomine were isolated from *Castanospermum australe*<sup>131</sup> and from *Fagopyrum esculentum*<sup>132</sup> respectively in the course of a phytochemical screening, swainsonine was found as toxic agent in the Australian Fabaceae *Swainsona canescens* that causes toxicosis in cattle.<sup>133</sup> The symptoms of swainsonine intoxication are similar to mannosidosis, a genetic lysosomal storage disease sometimes found in Angus cattle, because swainsonine is a strong  $\alpha$ -mannosidase inhibitor.<sup>134</sup> Swainsonine was also detected in North American range plants that were known to cause intoxication in cattle. Those plants were summarised as locoweeds, and the symptoms of toxicity were called locoism. Many of the locoweeds are Fabaceae, and swainsonine was identified in *Astragalus lentiginosus*,<sup>135</sup> and further *Astragalus* and *Oxytropis* species.<sup>136</sup> Castanospermine in contrast appears to cause gastrointestinal symptoms like diarrhoea mainly by inhibition of carbohydrate digesting  $\alpha$ -glycosidases, sucrase in particular.<sup>137-139</sup> When the compound is injected it displays toxic effects by inhibition of

cellular  $\alpha$ -glucosidases and alteration of glycogen distribution in experimental animals.<sup>140</sup> While swainsonine is readily absorbed from the intestine, castanospermine absorption will be low due to its lower octanol-water partition coefficient (calculated log *P* values  $-1.6$  vs.  $-0.8$ ). Toxic effects resulting from oral ingestion are therefore mainly described for swainsonine; the literature has been reviewed.<sup>25,45,68,128,141</sup>

Calystegines were suspected to contribute to toxicity of Convolvulaceae as range plants, but this remains to be proved. *Calystegia sepium* and *Convolvulus arvensis* were suspected to cause intestinal fibrosis and weight loss in horses, but calystegines were not found in those plants.<sup>82</sup> Swainsonine was identified in toxic Weir vine from Queensland, Australia, *Ipomoea* sp. Q6 [aff. *calobra*], together with calystegines of the B-group in approximately equal quantities (0.048–0.058% dm). The symptoms of intoxication in sheep resembled mannosidosis caused by swainsonine. Additional epilepsy-like seizures cannot clearly be attributed to calystegines.<sup>142</sup> De Balogh and co-workers described *Ipomoea carnea* as causative for poisoning of goats. The plants were analysed positive for swainsonine and the symptoms of intoxication were entirely like those of swainsonine.<sup>143</sup> In a quantitative evaluation of alkaloid content of *I. carnea*, the highest concentration of calystegine B<sub>2</sub> (0.021% fm) in seeds was comparable to that of swainsonine, and the question was raised whether calystegines by their  $\beta$ -glucosidase inhibition can cause different lysosomal storage diseases.<sup>52</sup> Another *Ipomoea* species, *I. asarifolia*, that causes tremors and lack of coordination in goats, was analysed and found to contain no calystegines and swainsonine only in traces. The authors concluded that the toxicity must be due to other phytotoxins or tremorgenic mycotoxins.<sup>144</sup> In summary, a lot of suspicion has been raised about calystegines causing intoxication due to their potent glycosidase inhibitory activity, but the proof of action remains to be given. Such proof would need to include proof of absorption from the intestine after oral ingestion.

### 3.3.3 Medicinal applications

Alkaloidal glycosidase inhibitors are applied as pharmaceutical compounds for diabetic patients. Miglitol (Fig. 1) and acarbose, a nitrogen-containing pseudotetrasaccharide, prevent a sudden rise of blood glucose after a carbohydrate meal, because they delay carbohydrate absorption in the gut.<sup>145</sup> Miglustat, another deoxynojirimycin derivative (*N*-butyl-deoxynojirimycin), was made available in 2003 for the treatment of mild to moderate type I Gaucher's disease in patients for whom enzyme replacement therapy is unsuitable.<sup>146,147</sup> The same compound proved a promising candidate for a male contraceptive, interfering with the production of glucosphingolipids and thereby inhibiting sperm development without hormone effect.<sup>148</sup>

Other glycosidase inhibitors show promising activities as anti-cancer or anti-viral compounds including activity against the AIDS virus, and they are being intensively studied in preclinical and clinical tests. Activity as a chemical chaperone was shown for deoxygalactonojirimycin. The current state of knowledge has been summarised without any mention of calystegines.<sup>149,150</sup> There are no clinical tests published with these compounds, although they have original and very promising activities in *in-vitro* assays. The major obstacle to research on calystegine action and biological role is their deficient availability. Recently efficient synthetic procedures were made available, and this will signify a major step forward in calystegine research.

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