

## Review Article

## Amaryllidaceae Isocarbostryril Alkaloids and Their Derivatives as Promising Antitumor Agents

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### Abstract

This review covers the isolation, total synthesis, biologic activity, and more particularly the *in vitro* and *in vivo* antitumor activities of naturally occurring isocarbostryril alkaloids from the Amaryllidaceae family. Starting from these natural products, new derivatives have been synthesized to explore structure–activity relationships within the chemical class and to obtain potential candidates for preclinical development. This approach appears to be capable of providing novel promising anticancer agents.

*Translational Oncology* (2008) 1, 1–13

### Introduction

Natural products have played a highly significant role in the discovery and development of new drugs for the treatment of human diseases [1]. This is particularly evident in the cancer field, where more than 60% of drugs are of natural origin [2]. Plants from the Amaryllidaceae family have long been known for their medicinal and toxic properties. Indeed even in ancient Greece, the oil from *Narcissus* species was already being used successfully for the treatment of cancer [3]. Consequently, efforts have been made to isolate the active ingredients responsible for this antitumor activity. Some 48 alkaloids bearing a variety of carbon skeletons have been isolated from *Narcissus* species [4]. One small group of these alkaloids does not contain basic nitrogen atoms and is represented by hydroxylated benzophenanthridone [5] or isoquinolinone types of structure. The most widely known compounds [5] of this group are narciclasine **1**, lycoricidine **2**, and pancratiastatin **3** (Figure 1), and the most frequently used term in the literature to describe this group of alkaloids is the isocarbostryrils [6,7].

All these natural products have demonstrated potent *in vitro* cytotoxicity against cancer cell lines [7] and potent *in vivo* antitumor activity against mouse M-5076 sarcoma and P388 leukemia [7]. Therefore, this family as a whole seems of interest as a potential source of new lead structures for the development of a future generation of anticancer drugs.

This review focuses on the isocarbostryril alkaloid family, with a special emphasis on the isolation and synthesis of these natural products and their hemi- and fully synthetic derivatives and their *in vitro*

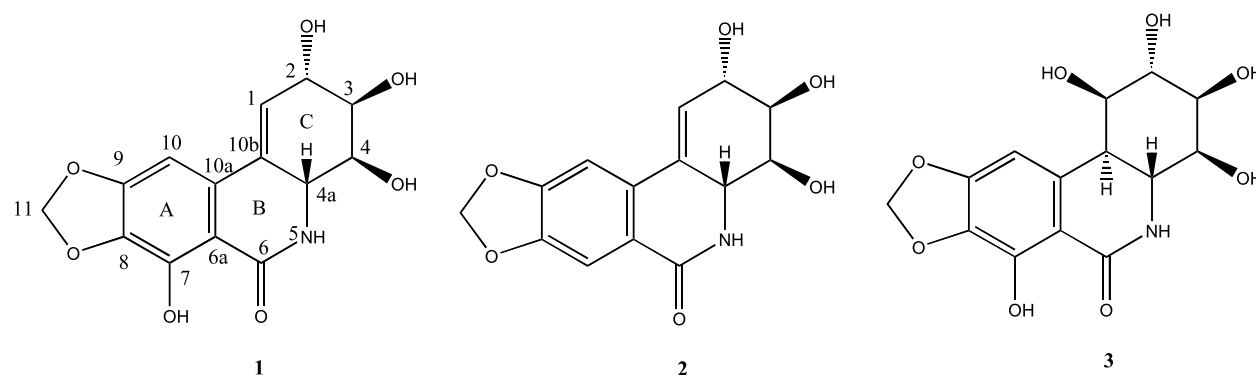
and *in vivo* antitumor activity. Finally, recent advances in understanding the mechanism of action of these antitumoral molecules as well as their original role in the control of the plant growth will be discussed.

### Isolation of Amaryllidaceae Isocarbostryril Alkaloids

#### *Narciclasine and Natural Derivatives*

Narciclasine **1**, also known as lycoricidinol given it is the 7-hydroxy derivative of lycoricidine **2** (Figure 1), was the first alkaloid of the class isolated in 1967 from *Narcissus* (Amaryllidaceae species) bulbs based on its inhibition of the growth of wheat grain radicles [8]. The typical procedure for the isolation of this compound involves ethanolic extraction of fresh daffodil bulbs. The alcohol is then evaporated to leave an aqueous residue, which is itself extracted with dichloromethane to remove nonpolar contaminants. Narciclasine is then extracted from the residual aqueous phase with ethyl acetate. After evaporation of the solvent, the residue is dissolved in ethanol, and after that, solid deposit was further purified by column chromatography [8,9]. Narciclasine is

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Received 29 January 2008; Revised 29 January 2008; Accepted 29 January 2008



**Figure 1.** Chemical structures of narciclasine, lycoricidine and pancratistatin.

obtained as a pure product after alcohol or acetic acid crystallization (as light yellowish needles) of the eluted fraction containing the compound. The structure of narciclasine was elucidated in 1970 by  $^1\text{H}$  nuclear magnetic resonance (NMR) and  $^{13}\text{C}$  NMR, mass spectral, infrared, and elemental analyses and was further characterized with respect to optical rotation [9,10].

One of the most widely reported sources of narciclasine is the bulbs of *Narcissus* species [4,8,11]. Thirty-two species and varieties of this genus have been examined for narciclasine content, which varies from 30 to 200 mg/kg fresh bulbs. The typical content in this genus is 100 to 120 mg/kg (Table 1) and may vary during the year. For example, a content of 200 mg/kg was exceptionally found in bulbs of *Narcissus incomparabilis* Mill. Var. Helios at the flowering stage in March (Table 1). The occurrence of narciclasine was also investigated in others species of Amaryllidaceae, including *Lycoris radiata* [12,13], *Haemanthus* [6,11], *Hymenocallis* [7,11,14,15], *Brachystola magna* [16], *Galanthus* [11], *Leucojum* [11], *Pancreatum maritimum* [11], *Sprekelia formosissima* [11], *Sternbergia lutea* [11], and *Vallota speciosa* [11]. The content of narciclasine in these species does not exceed 50 mg/kg fresh bulbs (Table 1). Narciclasine was exceptionally found in high concentrations (~135 mg/kg) in *Zephyranthes flava*, a tropical and subtropical plant cultivated in India (Table 1) [6].

However, in general and based on analyses to date, *Narcissus* bulbs are the best and easiest to obtain source of narciclasine, given that these plants are relatively common and can grow in many countries with different climates.

**Table 1.** Occurrence of Narciclasine **1**, Lycoricidine **2**, and Pancratistatin **3** (in mg/kg of Bulbs or Seeds) in Some Amaryllidaceae Species.

Amaryllidaceae Species	Compounds		
	<b>1</b>	<b>2</b>	<b>3</b>
<i>N. incomparabilis</i>	200 in March 100-120 in November	NI	NI
<i>Narcissus</i> "Carlton"	100-120	NI	NI
<i>H. kalbreyeri</i>	45	17	157
<i>Z. flava</i>	135	NI	165
<i>P. maritimum</i>	50	NI	6
<i>L. radiata</i>	4.2	3.2	NI
<i>Ismene</i> × "Sulfur Queen"	NI	32	NI
<i>H. littoralis</i>	5	118	100-150 from Hawaii 22 from Arizona
<i>B. magna</i>	1.5	NI	2.5
<i>Z. carinata</i>	NI	NI	7.5

NI indicates not isolated.

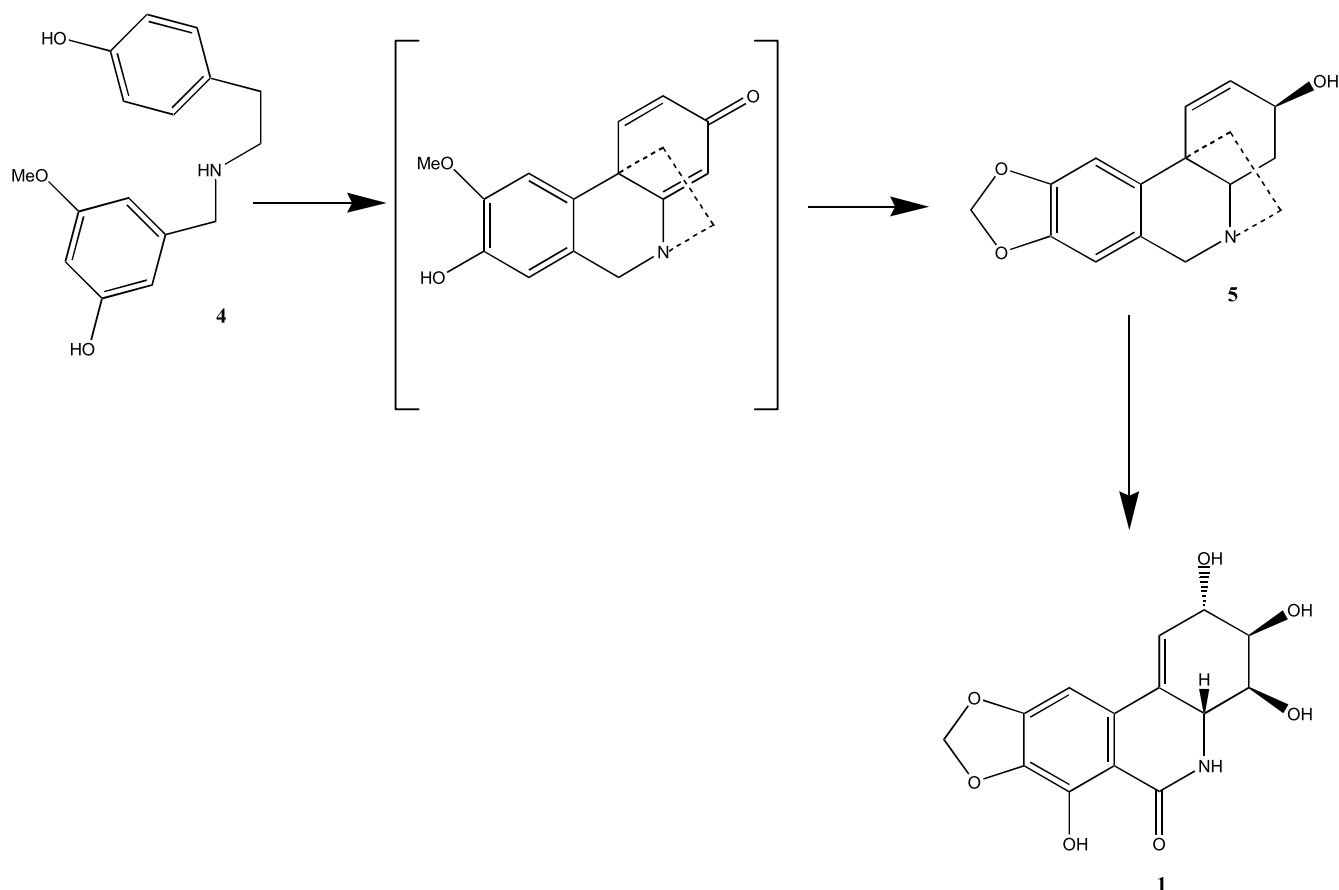
The biosynthesis of these Amaryllidaceae alkaloids in plants was deciphered in 1971 [17]. Narciclasine is synthesized from *O*-methyl-norbelladine **4** by *para-para* phenol coupling to obtain vittatine **5** as an intermediate (Scheme 1) [17,18]. Subsequent elimination of two carbon atoms and hydroxylations of compound **5** then leads to narciclasine (Scheme 1).

In 1985, the first biosynthetic derivative of narciclasine, compound **6** (Figure 2), was isolated in good quantities from *Haemanthus kalbreyeri* [19] and *Z. flava* [20] (concentrations of 78 and 165 mg/kg, respectively), two tropical and subtropical Amaryllidaceae species. So this natural product was named kalbreclasin, and it is the 2-*O*- $\beta$ -D-glucopyranoside derivative of narciclasine. This compound, whose structure was fully elucidated by spectroscopic and chemical methods, provoked a significant activation of splenic lymphocytes *in vitro*, a feature characteristic of immunostimulants [19]. Another  $\beta$ -D-glucoside derivative of narciclasine **7** (Figure 2) was isolated in 1991 in trace amounts from *P. maritimum* (~3.5 mg/kg) [21]. Currently, the last known natural derivative of narciclasine (Figure 2) isolated is *trans*-dihydranarciclasine **8**, which is extracted at low levels (11 mg/kg) from *Zephyranthes candida* [22].

### Lycoricidine and Natural Derivatives

7-Deoxynarciclasine **2** (Figure 1), more generally known as lycoricidine, was first discovered in *L. radiata* in 1968 [12]. The product was isolated from methanol extracts of the plant's bulbs by a similar procedure to that used for narciclasine. Lycoricidine, which was obtained only at low levels (~3 mg/kg; Table 1), was found similar to narciclasine to be a plant growth inhibitor [12]. The structure of lycoricidine was fully established particularly by  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR and by chemical reactions [12,23]. Lycoricidine and narciclasine were shown in 1983 to have a potent insecticidal action, in addition to reducing feeding activity in the larvae of certain butterfly species [13].

Lycoricidine was also isolated in low quantities (~17 mg/kg) from *H. kalbreyeri* [6] and in moderate quantities (~32 mg/kg) from *Ismene* × "Sulphur Queen" [15]. The best sources of lycoricidine are bulbs of *Hymenocallis littoralis* collected from the wild in Hawaii as these yield in the order of 118 mg/kg [14,24]. However, if this rare tropical plant is cultivated in Arizona, the content of lycoricidine is much reduced, varies during the year, and only a maximum of 15 mg/kg can be isolated even in the peak month of October [14]. This difference in the content to the native Hawaiian bulb could be explained by an Arizona climate that is much hotter, drier, and sunnier [14].



**Scheme 1.** The biosynthesis of narciclasine.

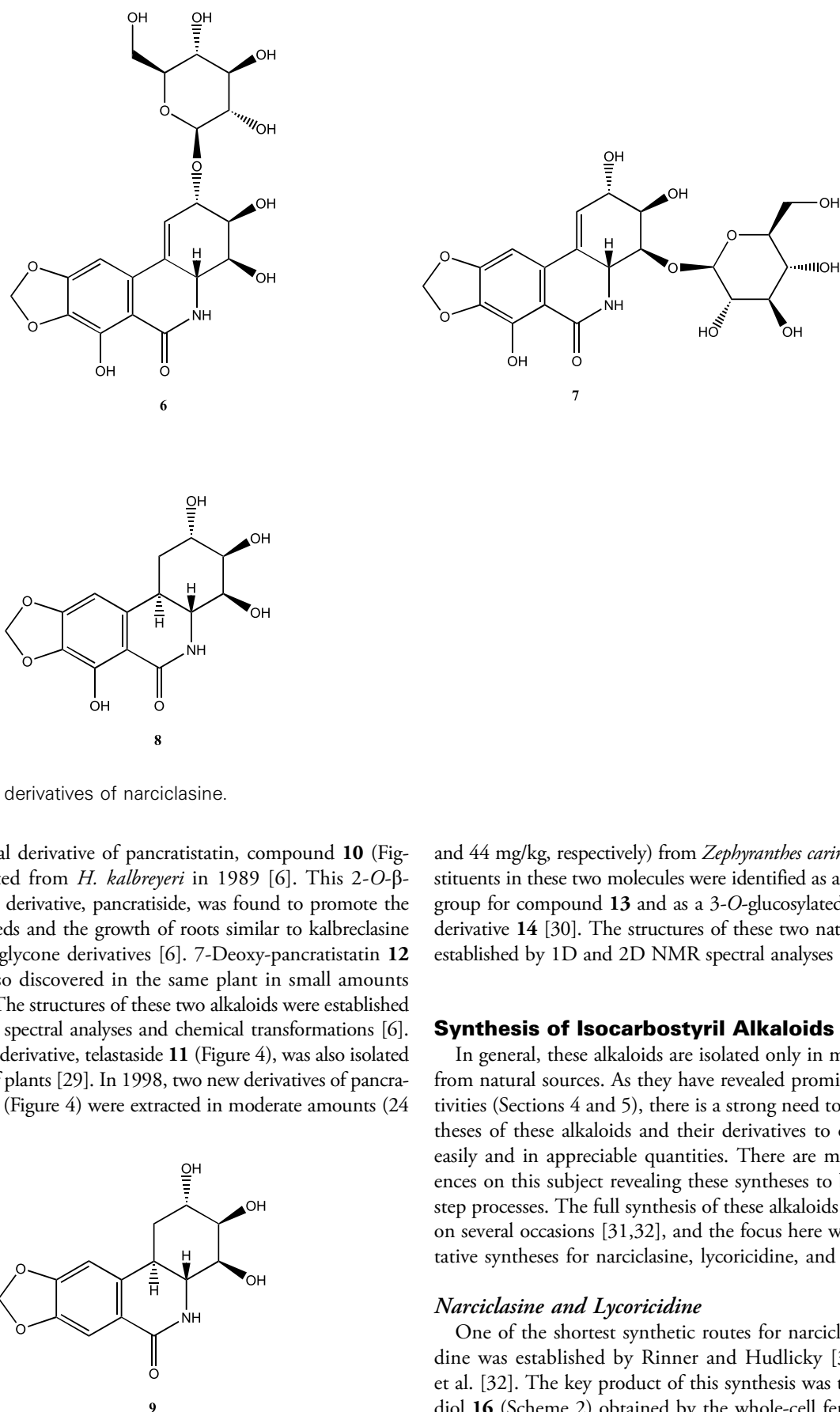
To date, only one natural derivative of lycoricidine **9** (Figure 3) has been isolated from *H. littoralis* [14,23,25] (the tropical spider lily) and *Hymenocallis caribaea* [7]. Compound **9** (7-deoxy-*trans*-dihydronarciclasine or alternatively *trans*-dihydro lycoricidine) is a *trans*-dihydro derivative of lycoricidine in which the two ring cycles are *trans* linked. *Hymenocallis littoralis* contains a maximum of 16 mg/kg of **9** when sampled in October [14], whereas *H. caribaea* contains a maximum of only 3.8 mg/kg. The structure has been fully elucidated by spectroscopic and chemical methods [7,25].

#### Pancreatistatin and Natural Derivatives

In 1984, pancreatistatin **3** (Figure 1) was first discovered in a Hawaiian species originally designated as *Pancretium littorale*, but later reclassified as *H. littoralis* [26]. The bulb section of this native Hawaiian plant was extracted with dichloromethane–methanol–water and the extract was concentrated to obtain an aqueous phase. Organic compounds were extracted with *n*-butanol and purification of the resulting crude product was undertaken using gel permeation chromatography (Sephadex LH-20) and precipitation/crystallization to give the pure natural product **3** [26]. This experimental procedure is very effective for laboratory-scale isolation of pancreatistatin, but for large-scale production, the cost is too high. Another procedure was then developed to avoid the use of gel permeation, but this approach remains more complicated than the isolation of narciclasine [14]. The structure of pancreatistatin was fully elucidated by  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR, mass spectrometry, infrared, and elemental analyses [24,26] and was corroborated by an X-ray crystal structure determination of the hemisynthesized 7-methoxy derivative [26]. As with

narciclasine and lycoricidine, pancreatistatin has been found to exhibit strong *in vitro* RNA antiviral activity [27]. *In vivo*, pancreatistatin increased survival by 100% when it was used to treat mice infected with Japanese encephalitis [27]. Furthermore, this product showed activity against two other RNA-containing flaviviruses and bunyaviruses, namely Punta Toro and Rift Valley fever [27]. Pancreatistatin and lycoricidine also showed an *in vitro* antiparasitic effect against a microsporidium causing infections in humans [28].

The rare tropical bulbs of *H. littoralis* seem to be a good source of pancreatistatin as it can be isolated from these in the order of 100 to 150 mg/kg when bulbs are obtained from the wild in Hawaii (Table 1) [14,24]. However, the compound has to be commercially extracted from field- and greenhouse-grown bulbs or from tissue cultures cultivated, for example, in Arizona, which generate lower levels of pancreatistatin (a maximum of 22 mg/kg) even in the peak month of October (Table 1) [14]. After October, when the bulb becomes dormant, levels of pancreatistatin drop to only 4 mg/kg by May [14]. Field-grown bulbs, which show monthly changes in pancreatistatin content, generate somewhat smaller amounts (2–5 mg/kg) compared to those grown in greenhouse cultivated over the same period [14]. The occurrence of pancreatistatin has also been investigated in other Amaryllidaceae, including several species of *Hymenocallis* [15], *B. magna* [16], and *P. maritimum* [15]. The pancreatistatin content of these species does not exceed 29 mg/kg of fresh bulbs. Pancreatistatin was exceptionally found in good quantity in *Z. flava* [20] and *H. kalbreyeri* [6] (Table 1), two tropical and subtropical plants cultivated in India. Consequently and to date, the best sources of pancreatistatin are rare, tropical, and subtropical bulbs of *Z. flava*, *H. kalbreyeri*, and *H. littoralis* when these plants are cultivated in their native region.



**Figure 2.** Natural derivatives of narciclasine.

The first natural derivative of pancratistatin, compound **10** (Figure 4), was isolated from *H. kalbreyeri* in 1989 [6]. This 2-*O*- $\beta$ -D-glucopyranoside derivative, pancratistide, was found to promote the germination of seeds and the growth of roots similar to kalbreclisine **6** but unlike its aglycone derivatives [6]. 7-Deoxy-pancratistatin **12** (Figure 4) was also discovered in the same plant in small amounts (~30 mg/kg) [6]. The structures of these two alkaloids were established by comprehensive spectral analyses and chemical transformations [6]. Another glycoside derivative, telastaside **11** (Figure 4), was also isolated from this family of plants [29]. In 1998, two new derivatives of pancratistatin **13** and **14** (Figure 4) were extracted in moderate amounts (24

and 44 mg/kg, respectively) from *Zephyranthes carinata* [30]. Key substituents in these two molecules were identified as a 3-hydroxy-butyryl group for compound **13** and as a 3-*O*-glucosylated butyryl group for derivative **14** [30]. The structures of these two natural products were established by 1D and 2D NMR spectral analyses [30].

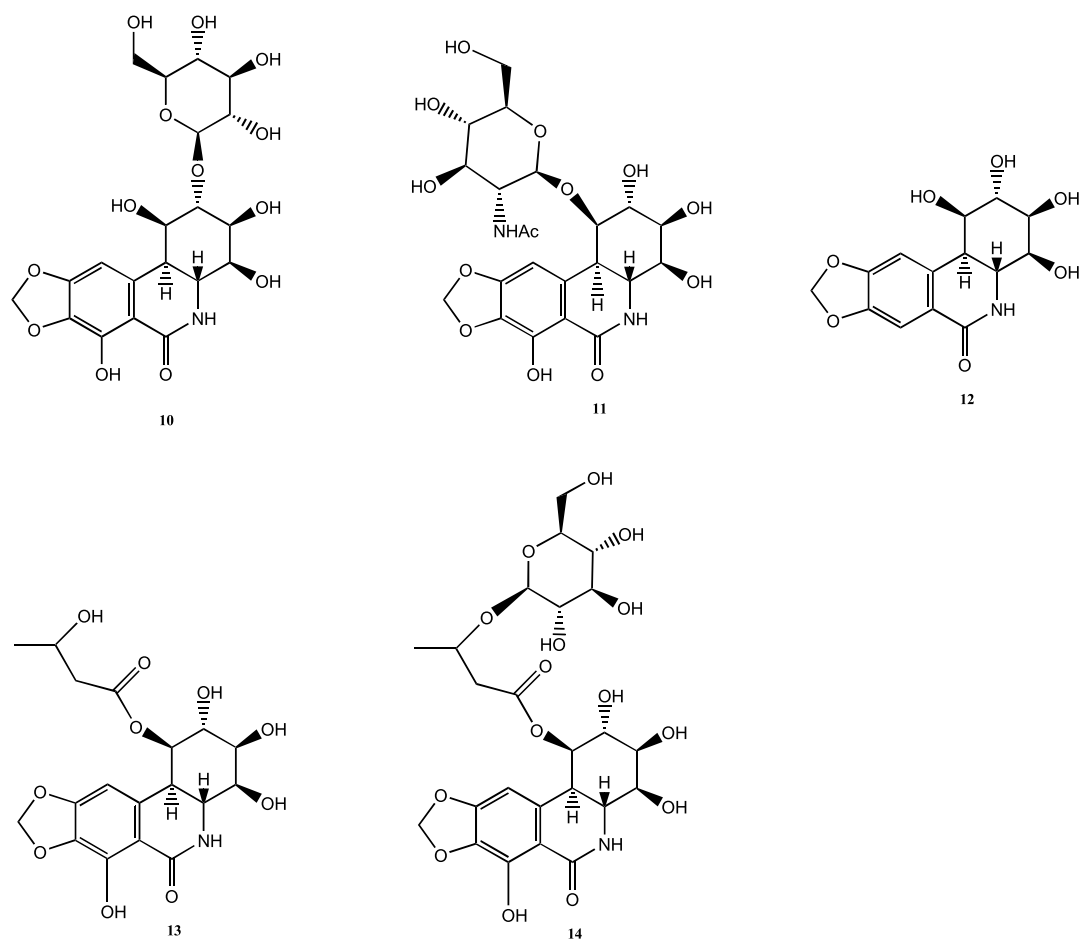
### Synthesis of Isocarbostryl Alkaloids

In general, these alkaloids are isolated only in moderate quantities from natural sources. As they have revealed promising antitumor activities (Sections 4 and 5), there is a strong need to develop total syntheses of these alkaloids and their derivatives to obtain them more easily and in appreciable quantities. There are more than 25 references on this subject revealing these syntheses to be complex multi-step processes. The full synthesis of these alkaloids has been reviewed on several occasions [31,32], and the focus here will be on representative syntheses for narciclasine, lycoricidine, and pancratistatin.

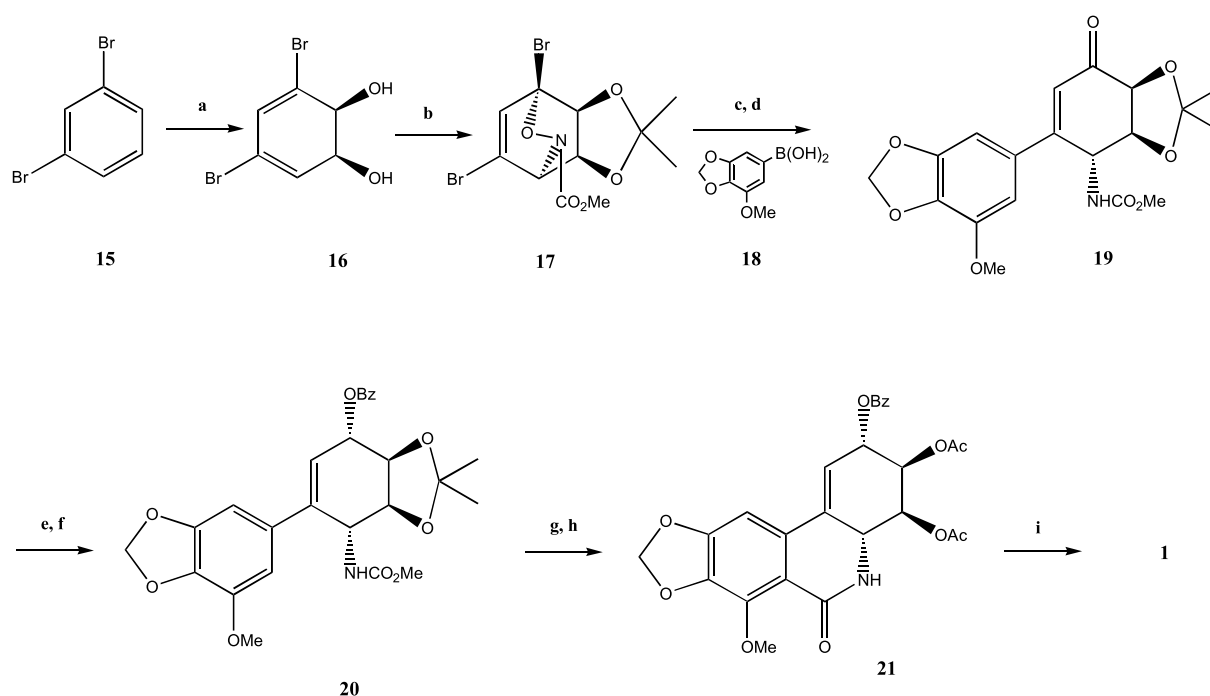
#### *Narciclasine and Lycoricidine*

One of the shortest synthetic routes for narciclasine and lycoricidine was established by Rinner and Hudlicky [31] and Hudlicky et al. [32]. The key product of this synthesis was the cyclohexadiene diol **16** (Scheme 2) obtained by the whole-cell fermentation of 1,3-dibromobenzene **15** with recombinant *Escherichia coli* JM0109 [33]. This diol **16** was then protected by an acetonide group, and the resulting product subject to a hetero-Diels-Alder reaction to generate

**Figure 3.** Chemical structure of the only one isolated natural derivative of lycoricidine.



**Figure 4.** Chemical structures of natural derivatives of pancratistatin.



**Scheme 2.** a: *E. coli* JM109 (pDTG601A), 4 g/L. b: i) DMP, acetone, *p*-TsOH, r.t.; ii) NHCO<sub>2</sub>Me, NaIO<sub>4</sub>, r.t., 70%. c: borate 18, Pd(PPh<sub>3</sub>)<sub>4</sub>, aq Na<sub>2</sub>CO<sub>3</sub>, PhH, reflux, 30%. d: TTMSS, AIBN, PhH, reflux, 80%. e: NaBH<sub>4</sub>, CeCl<sub>3</sub>, MeOH, 0°C, 80%. f: BzOH, Bu<sub>3</sub>P, DEAD, THF, r.t., 65%. g: i) Dowex 50X8-100, MeOH, r.t.; ii) Ac<sub>2</sub>O, pyridine, DMAP, r.t., 70%. h: Tf<sub>2</sub>O, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 0°C, 40%. i: i) Amberlyst A21, MeOH; ii) LiCl, DMF, 120°C, 20%.

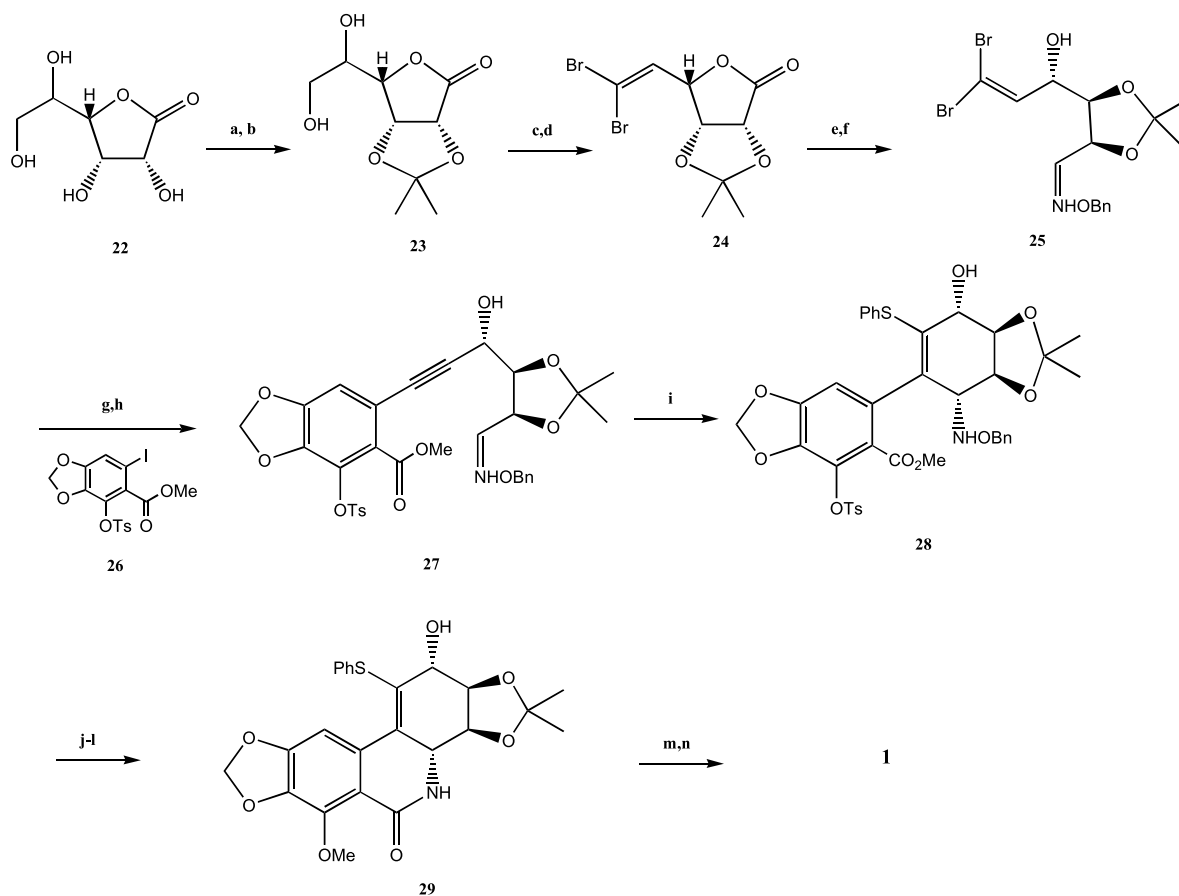
oxazine **17**. A Suzuki coupling of one of the bromines on **17** with the boronic acid derivative **18** afforded the desired coupled product, which was treated with a silylsilane derivative to form the ketone **19**. The reduction of the ketone function was followed by a Mitsunobu reaction to obtain benzoate **20**. After replacement of the acetonide by acetates, a modified Bischler–Napieralski ring-closure gave the protected product **21**. Cleavage of the phenolic methyl function and removal of protecting groups afforded narciclasine **1** in 12 steps with an overall yield of 0.5% from the cyclohexadiene diol **16**. The synthesis of lycoricidine **2** was realized in nine steps (30% overall yield) by the same method using a cyclohexadiene diol such as **16** obtained by biooxidation with the same bacteria *E. coli* [34].

A second method of synthesis used for narciclasine and lycoricidine was established in 1999 by Keck as reviewed in the work of Rinner and Hudlicky [31]. The starting material of these total syntheses is the commercially available D-gulonolactone **22** that fits correctly with three of the four stereocenters of these isocarbostryl products (Scheme 3). After 2,3-acetonide protection, compound **23** was obtained in two steps. Oxidation of **23** followed by a Corey–Fuchs reaction of this latter product gave dibromide **24**. The lactone function of **24** was then converted to the *O*-benzyl amine derivative **25**. A Pd-catalyzed coupling reaction of **25** with compound **26** provided the alkyne **27**. The radical cyclization of this latter product afforded compound **28** with an 88% yield. Cleavage of the tosyl group in

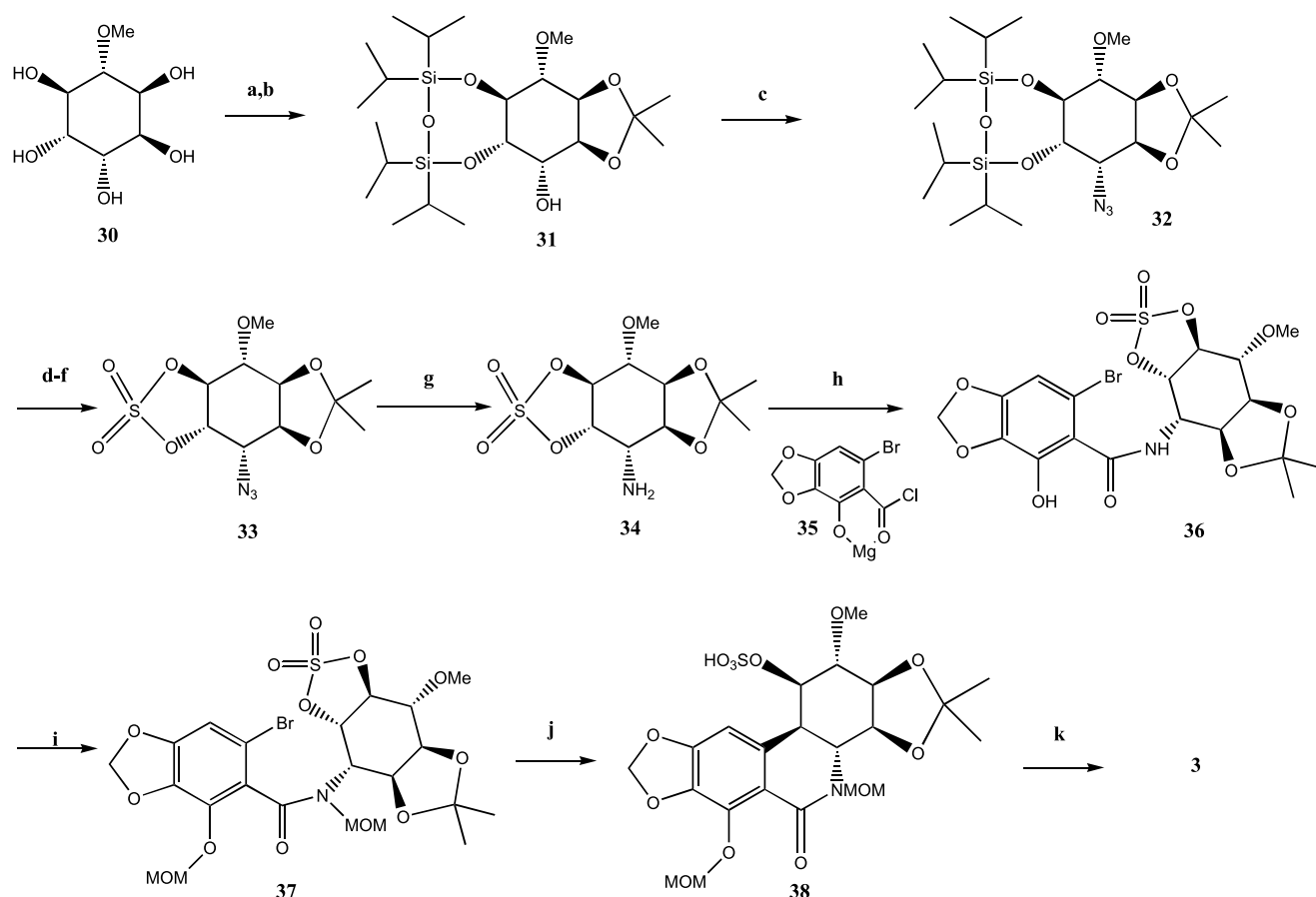
**28** and methylation of the phenolic hydroxyl functionality allowed aluminum-catalyzed cyclization to deliver the protected product **29**. Deprotection of the thioether and removal of acetonide by acid treatment afforded narciclasine **1** in 14 steps from D-gulonolactone with an overall yield of 16%. This method of synthesis was also used for lycoricidine in 11 steps with an overall yield of 27% starting from D-gulonolactone **22** and the appropriate aromatic analog of **26** [31].

### Pancreatistatin

Danishefsky et al., as reviewed in the work of Rinner and Hudlicky [31], reported in 1989 the first total synthesis of racemic pancreatistatin. Thereafter, numerous research groups published the stereoselective synthesis of pancreatistatin. These syntheses were previously reviewed [31] and include those of Tian et al. [35], Doyle et al. [36], Trost and Pulley [37], Rigby et al. [38], Magnus and Sebat [39], and Ko et al. [40]. Another interesting synthesis of pancreatistatin was reported in 2006 by Li et al. [41] and started from pinitol **30** whose stereogenic centers exactly match those of the C-ring of pancreatistatin (Scheme 4). Protection of the diol functions of compound **30** gave compound **31**. The free hydroxyl of this was subsequently substituted by an azide to give **32**. After removal of the silyl function, a cyclic sulfate was installed to obtain product **33**. The Staudinger reaction gave the free amine **34** from azide **33**. The coupling reaction between **34** and **35** gave compound **36** with a moderate yield. Methocymethyl protection of both



**Scheme 3.** a: DMP, *p*-TsOH, DMF, 79%. b: AcOH, H<sub>2</sub>O, THF, 79%. c: NaIO<sub>4</sub>, CH<sub>2</sub>Cl<sub>2</sub>. d: CBr<sub>4</sub>, PPh<sub>3</sub>, Et<sub>3</sub>N, 80% (more than two steps). e: *L*-Selectride, Et<sub>2</sub>O, −78°C. f: HCl·H<sub>2</sub>NOBn, pyridine, 90% (more than two steps). g: *n*-BuLi, Et<sub>2</sub>O, −90°C, 93%. h: Pd(OAc)<sub>2</sub>, PPh<sub>3</sub>, CuI, Et<sub>3</sub>N, **26**, THF, 89%. i: PhSH, hν, toluene, 27°C, 88%. j: Sml<sub>2</sub>, THF, H<sub>2</sub>O, 0°C, 94%. k: MeI, K<sub>2</sub>CO<sub>3</sub>, DMF, 96%. l: Me<sub>3</sub>Al, THF, −15°C to 65°C, 72%. m: Sml<sub>2</sub>, MeOH, THF, 0°C, 87%. n: TFA, 0°C, 89%.



**Scheme 4.** a: TIPDSCl<sub>2</sub>, imidazole, DMAP, DMF, 24%. b: DMP, *p*-TsOH, acetone, 81%. c: PPh<sub>3</sub>, DEAD, CH<sub>3</sub>SO<sub>3</sub>H, CH<sub>2</sub>Cl<sub>2</sub>, 0°C to r.t. then NaN<sub>3</sub>, DMF, 60°C, 72%. d: TBAF, THF, 0°C to r.t., 100%. e: SOCl<sub>2</sub>, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, 0°C. f: NaIO<sub>4</sub>, RuCl<sub>3</sub>, aq CH<sub>3</sub>CN, 87% (more than two steps). g: PPh<sub>3</sub>, aq THF, 0°C to r.t., 94%. h: Et<sub>2</sub>O, **35**, 0°C, 64%. i: K<sub>2</sub>CO<sub>3</sub>, MOMCl, DMF, 84%. j: *t*-BuLi, CeCl<sub>3</sub>, ultrasound, THF, -78°C to r.t., 72%. k: BBr<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, -78°C to 0°C, 1 hour then MeOH, -78°C to 0°C, 2 hours, 52%.

the amide and the free phenol gave compound **37**. Treatment of this latter product with *t*-BuLi followed by addition of cerium chloride gave compound **38**. Full deprotection of **38** by BBr<sub>3</sub> and methanol afforded pancratistatin **3** in 12 steps from commercially available pinitol with an overall yield of 2.3%.

Given the higher availability of narciclasine in extracts of *Narcissus* species (up to 200 mg/kg), Pettit et al. [42,43] have developed and patented a second process to synthesize pancratistatin from Amaryllidaceae-derived narciclasine. The hydroxyl functions of narciclasine are first protected and the olefin function oxidized to give epoxide **39** (Scheme 5). Hydrogenation of this latter product and subsequent saponification yields a mixture of four compounds, one of which is the desired diol **40**. Activation of this diol with thionyl chloride and oxidation of the epimeric sulfites give the cyclic sulfate **41**. Nucleophilic opening of compound **41** and hydrolysis of the sulfate and acetonide functions affords the benzoate product **42**. Deprotection of the benzoyl group at C-1 position gives pancratistatin **3** in 10 steps and an overall yield of 3.6% from narciclasine **1**.

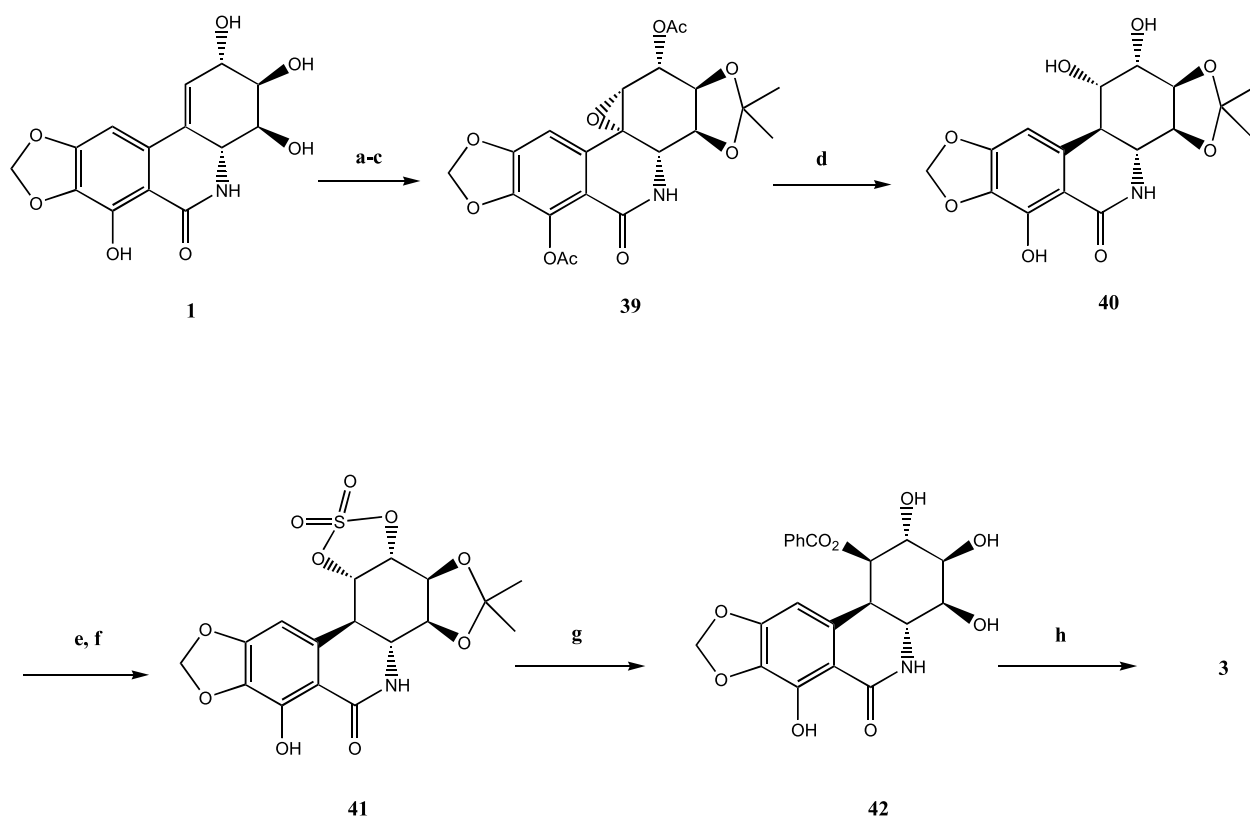
### In Vitro Antitumor Activity

#### Narciclasine, Lycoricidine, Pancratistatin, and Natural Derivatives

The cytotoxicity of these isocarbostryl alkaloids was first demonstrated in basic assays some 30 years ago [12,44] and later confirmed

in standard MTT colorimetric assays on diverse human tumor cell lines by the National Cancer Institute (NCI) [7,23,45,46].

Narciclasine **1** exhibits *in vitro* potent cytotoxicity against human cancer cell lines and murine P388 leukemia cells (Table 2) [45,46]. The cytotoxicity of this product was evaluated in 60 cancer cell lines by the NCI, and the mean IC<sub>50</sub> value (the concentration which inhibits overall cell growth in culture by 50%) was 0.046 μM [45]. With respect to narciclasine, lycoricidine **2** demonstrates cytotoxic activity against these 60 cancer cell lines, which is ~10 times weaker (mean IC<sub>50</sub> = 0.33 μM), and pancratistatin **3** an activity ~5 times weaker (IC<sub>50</sub> = 0.26 μM) [46]. The observed cytotoxicity of these three isocarbostryls against murine P388 leukemia cell line is similar but results against human cancer cell lines (Table 2) confirm those found by the NCI for lycoricidine but not for pancratistatin [23,45]. Against the murine P388 leukemia cell line, dehydronarciclasine **8** showed more potent cytotoxicity than narciclasine (Table 2), but in the 60 cancer cell line panel, the cytotoxicity was of the same order of magnitude with a mean IC<sub>50</sub> of 0.053 μM [23,45,46]. Dehydrolycoricidine **9** revealed the same level of cytotoxicity as lycoricidine (Table 2) [23]. These results with compounds **8** and **9** revealed that the double bond between the C-1 and C-10b is not essential for the cytotoxicity of these isocarbostryl alkaloids. For glycoside derivatives **6**, **7**, **10**, and **11** (Figures 2 and 4), there are no data in the literature on the cytotoxicity measured by MTT assays against cancer cells. However, for glucoside derivative **7**, a potato disc cytotoxicity assay has revealed an activity very similar to



**Scheme 5.** a: DMF, DMP, *p*-TsOH, 97%. b: Ac<sub>2</sub>O, pyridine, 81%. c: *m*-CPBA, phosphate buffer, CH<sub>2</sub>Cl<sub>2</sub>, H<sub>2</sub>O, 52%. d: i) H<sub>2</sub>, Pd/C 10%; ii) K<sub>2</sub>CO<sub>3</sub>, MeOH, H<sub>2</sub>O, 28%. e: SOCl<sub>2</sub>, Et<sub>3</sub>N, THF; f: RuCl<sub>3</sub>·3H<sub>2</sub>O, NaIO<sub>4</sub>, MeCN, CCl<sub>4</sub>, H<sub>2</sub>O, 47% (more than two steps). g: i) PhCO<sub>2</sub>H, CsCO<sub>3</sub>, DMF; ii) THF, H<sub>2</sub>O, H<sub>2</sub>SO<sub>4</sub>, 74%. h: K<sub>2</sub>CO<sub>3</sub>, MeOH, 75%.

narciclasine [21]. For 7-deoxy pancratistatin **12**, only two cancer cell lines have been tested and this product demonstrated a 10- to 20-fold lower cytotoxic activity than pancratistatin (Table 2) [47]. It can be concluded that the phenolic hydroxyl group of compounds **1** and **3** contributes to the greater ( $\geq 10$ -fold) activity of these products compared to that of their congeners **12** and **2**.

The hydroxybutyryl derivative of pancratistatin **13** gave two-fold better cytotoxicity than pancratistatin against P388 leukemia cells [30]. This better activity was then confirmed by the NCI in the 60-cancer cell line panel with a mean IC<sub>50</sub> of 0.037  $\mu$ M [46]. The glucoside derivative **14** revealed the same order of cytotoxicity as pancratistatin against P388 leukemia cells [30].

### Fully and Hemisynthesized Derivatives

A series of derivatives and analogs have been synthesized by full or hemisynthesis to obtain more active and/or soluble products (Figure 5)

that could be potentially selected for preclinical development as anti-cancer agents.

Only one compound that has been synthesized, a derivative of narciclasine (Scheme 5) [42,43], has been found to be more active than pancratistatin. This 1-benzoate pancratistatin derivative **42**, phenpanstatin, has revealed a cytotoxicity 10 times higher than pancratistatin **3** against a panel of human cancer cell lines (Tables 2 and 3) [45].

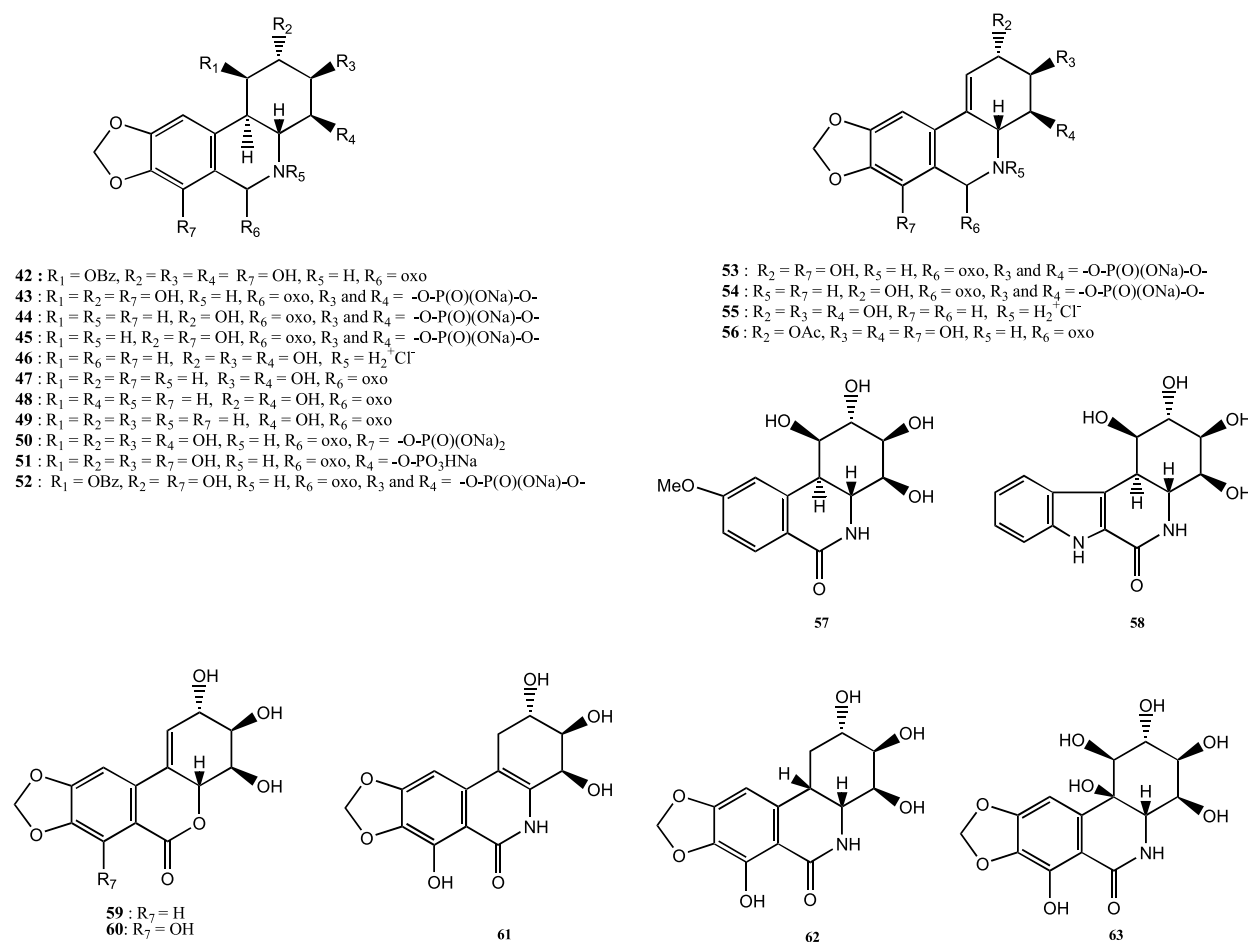
Naturally occurring isocarbostryl alkaloids such as pancratistatin have low aqueous solubility ( $\sim 50$   $\mu$ g/ml) [45]. This has limited their clinical evaluation in intravenous formulations. To increase aqueous solubility, phosphate prodrugs, which release the active drug through the action of cellular phosphatases, have been synthesized [23,45,48]. Several positions (4-OH, 7-OH, and 3,4-OH) on the natural isocarbostryl alkaloids were used to obtain 3,4-cyclic phosphate derivatives **43**, **44**, **45**, **50** (7-*O*-phosphate pancratistatin), **51** (4-*O*-phosphate pancratistatin), **52**, **53** (narcistatin), and **54** (Figure 5) [23,45,48]. These

**Table 2.** *In Vitro* Cytotoxic-Related Antitumor Effects (IC<sub>50</sub> Values in  $\mu$ M)\* of Certain Naturally Occurring Isocarbostryl Alkaloids [23,44].

Compounds	Cancer Cell Lines						
	Leukemia P388	Pancreas BXPC-3	Breast MCF-7	CNS SF268	Lung-NSC NCI-H460	Colon KM20L2	Prostate Du-145
<b>1</b>	0.042	0.011	0.010	0.010	0.027	0.011	0.011
<b>2</b>	0.065	0.24	0.16	0.41	0.18	0.29	0.17
<b>3</b>	0.052	0.061	0.071	0.043	0.098	0.077	0.046
<b>8</b>	0.0078	0.039	0.017	0.065	0.030	0.049	0.021
<b>9</b>	0.099	0.16	0.12	0.20	0.15	0.17	0.14
<b>12</b>	1.42	NT	NT	NT	NT	0.71	NT

\*Data are initially given in  $\mu$ g/ml [50% effective dose (ED<sub>50</sub>) or 50% growth inhibition (GI<sub>50</sub>)]. NT indicates not tested.





**Figure 5.** Chemical structures of fully and hemisynthesized isocarbostryril derivatives.

phosphate derivatives had considerably improved aqueous solubility, notably the sodium salt of narcistatin **53** (60 mg/ml) and have been duly patented [49–52]. All phosphate derivatives have markedly lower cytotoxicity *in vitro* compared to their active parent compound (Tables 2

and 3) [23,45,48]. However, as indicated above, hydrolysis of the phosphate groups to release the active isocarbostryril is expected to be very effective *in vivo*. *In vivo* antitumor evaluations will be undertaken shortly to validate this concept of water-soluble phosphate prodrugs [23].

**Table 3.** *In Vitro* Cytotoxic-Related Antitumor Effects (IC<sub>50</sub> Values in μM)\* of Fully and Hemisynthetic Isocarbostryril Alkaloid Derivatives.

Compounds	Cancer Cell Lines						
	Leukemia P388	Pancreas BXPC-3	Breast MCF-7	CNS SF268	Lung-NSC NCI-H460	Colon KM20L2	Prostate Du-145
42	0.0037	0.0044	0.00072	0.0013	0.00023	0.00086	0.00049
43	8.1	8.1	7.1	7.1	9.3	9	5.6
44	4.2	> 26.5	19.1	> 26.5	> 26.5	> 26.5	> 26.5
45	2.2	14.2	11.7	22.4	19.8	24.4	13.2
46	10.5	4.4	5.4	5.1	2.5	5.4	2.2
47	5	> 36.1	> 36.1	> 36.1	> 36.1	> 36.1	> 36.1
48	1.6	NT	NT	NT	NT	NT	NT
49	153.6	NT	NT	NT	NT	NT	NT
50	0.53	0.44	0.44	0.18	0.42	0.38	0.058
51	0.042	0.42	0.42	0.28	0.89	0.56	2.2
52	0.12	0.49	0.08	0.33	0.056	0.25	0.25
53	0.031	0.18	0.15	0.12	0.15	0.15	0.08
54	4.5	14.1	10.7	16.8	12.5	14.9	10.4
55	22.6	14	10.8	9.2	10.8	> 32	> 10.2
56	< 0.03	0.78	0.72	0.57	0.96	0.54	0.78
57	14.6	16.6	14.9	11.2	9.5	12.2	8.8
58	60.2	> 32.9	> 32.9	NT	NT	> 32.9	NT

\*Data are initially given in μg/ml (ED<sub>50</sub> or GI<sub>50</sub>).

NT indicates not tested.

To explore the structure–activity relationship (Figure 5 and Tables 2 and 3) of these isocarbostryril alkaloids, several other key positions in the molecule were modified. One modification involved the reduction of the lactam carbonyl group. For this purpose, 2-acetate lycoricidine **56** (Figure 5) was synthesized as an intermediate, but this modification was found to have a low impact on cytotoxicity. In contrast, compounds **46** and **55** (Figure 5) were found to be inactive against the panel of human cancer cell lines (Table 3) [25]. Fully synthetic compounds **59** and **60** (Figure 5) in which the lactam carbonyl was also replaced by a lactone function, which is considered an analog [5], showed no significant cytotoxic activity against L1210 murine leukemia cells [5]. All these modulations appear to indicate that the lactam carbonyl function is essential for cytotoxic activity.

Three deoxy-analogs of these isocarbostryril alkaloids **47**, **48**, and **49** (Figure 5) were synthesized to determine the minimum structural pharmacophore [53,54]. These three analogs showed weak antitumor activity (Table 3) and so revealed that the C-2, C-3, and C-4 hydroxyl groups were essential for the cytotoxic activity and can be considered as a minimum pharmacophore [53,54].

Another modulation was the B/C ring junction stereochemistry (Figure 1) and was evaluated through the synthesis of derivatives **61**, **62**, and **63** (Figure 5) [7,55]. For the isonarciclasine **61**, the B/C ring junction is planar because a double bond connects the two rings. A *cis*-B/C ring junction was obtained in products **62** and **63** after hydrogenation or oxidation of the double bond of narciclasine [7,22]. All these products showed weaker cytotoxicity than the parent natural compounds. Compound **61** gave an IC<sub>50</sub> in the order of 12 μM and **62** an IC<sub>50</sub> of ~4 μM [7]. The cytotoxicity of compound **63** against P388 murine leukemia and several human cancer cell lines was found to be about 1000 times less potent than pancratistatin [55]. All these results appear to confirm the need for a *trans*-B/C ring junction in these compounds to maintain potent cytotoxicity.

Modulation of the benzodioxole moiety of pancratistatin was investigated through the full synthesis of analogs **57** and **58** (Figure 5) [47,54]. The methoxy analog **57** and the β-carboline-1-one derivative **58** were revealed to be 100-fold less cytotoxic than the corresponding natural product [47,54]. These results show that an intact methylenedioxyphenyl or benzodioxole functionality is essential for significant cancer cell cytotoxicity in this class of natural compounds.

### In Vivo Antitumor Activity

Pancratistatin and narciclasine have proven to be effective in murine P388 lymphocytic leukemia and M-5076 ovary sarcoma models *in vivo* [24]. Table 4 (A and B) shows the *in vivo* survival values from the NCI database [46] for the antitumor activities of pancratistatin (A) and narciclasine (B) in different mouse models. The *T/C* index represents the percentage survival ratio of drug-treated (*T*) compared to untreated (*C*) mice. A *T/C* value >125% indicates that drug treatment significantly enhances the survival of the tumor-bearing mice.

The promising antineoplastic activity of pancratistatin has led to its preclinical development and selection as an important synthetic starting point for a growing number of research groups. Pancratistatin is particularly attractive for clinical development because of its potent activity against experimental melanoma and ovary carcinoma. Unfortunately, pancratistatin exhibits very low water solubility (53 μg/ml) and this property has complicated its formulation for intravenous administration. Although the solubility of this isocarbostryril can be increased in organic solvents, such as dimethylformamide

**Table 4.** National Cancer Institute Pancratistatin (A) and Narciclasine (B) *In Vivo* Antitumor Activities in a Range of Different Mouse Tumor Models.

(A) Pancratistatin, mg/kg					
<i>T/C</i> Survival	B16 Melanoma	L1210 Leukemia	P388 Leukemia	M5076 Sarcoma	M5076 Sarcoma
Tumor Site Inoculation	i.p.	i.p.	i.p.	i.p.	s.c.
0.18	NT	NT	113%	153%	101%
0.25	NT	NT	NT	NT	106%
0.37	NT	NT	122%	NT	NT
0.38	NT	120%	119%	NT	NT
0.5	NT	NT	NT	NT	112%
0.75	NT	122%	139%	159%	106%
0.78	NT	NT	133%	132%	NT
1	NT	NT	NT	NT	109%
1.5	100%	121%	142%	166%	111%
1.56	108%	131%	144%	129%	NT
2	NT	NT	NT	NT	112%
3	103%	133%	161%	184%	125%
3.12	129%	131%	151%	143%	NT
4	NT	NT	NT	NT	113%
6	114%	NT	165%	177%	126%
6.25	110%	NT	173%	152%	NT
8	NT	NT	NT	NT	121%
12	NT	NT	156%	144%	NT
12.5	NT	NT	203%	NT	NT
24	NT	NT	NT	Toxic	NT
25	Toxic	Toxic	Toxic	NT	NT

(B) Narciclasine, mg/kg					
<i>T/C</i> Survival	B16 Melanoma	L1210 Leukemia	P388 Leukemia	M5076 Sarcoma	Lewis Lung Carcinoma
Site of Inoculation	i.p.	i.p.	i.p.	i.p.	i.v.
0.62	115%	110%	110%	NT	93%
1.25	111%	108%	129%	NT	109%
2.5	109%	113%	162%	NT	117%
3	NT	NT	NT	133%	NT
5	123%	115%	100%	NT	NT
6	NT	NT	NT	160%	NT
10	NT	NT	87%	NT	NT
12.5	NT	NT	NT	NT	NT

All compounds were administrated intraperitoneally.

*i.p.*, intraperitoneal; *i.v.*, intravenous; *NT*, not tested; *s.c.*, subcutaneous.

and dimethylsulfoxide, and lower boiling aliphatic alcohols, their use as formulation components is not desirable.

### Mechanism of Action

Little is known regarding the anticancer mechanism of action of this family of compounds. Narciclasine and lycoricidine were first identified as plant growth inhibitors [8,12]. In plants, there is clear evidence that narciclasine inhibits the synthesis of proteins and the development of chloroplasts. Additionally, the compound isolated from the mucilage of *Narcissus* bulbs showed inhibitory effects on the growth and plastid development in excised radish cotyledons [56]. Narciclasine (0.1 μM) starts to show inhibitory effects on plant isocitrate lyase and hydroxypyruvate reductase activities after 24 hours of incubation in light. When the concentration is increased to 10 μM, the activities of both enzymes are completely inhibited [56]. From ultrastructure studies, 1 μM narciclasine markedly prevents the degradation of protein bodies and lipid bodies, as well as totally preventing chloroplast formation in excised radish cotyledons [56]. Visible symptoms of tepal senescence in cut *Iris* × “Hollandica” (cv. Blue Magic) flowers are delayed by placing one cut daffodil flower (*Narcissus pseudonarcissus*, cv. “Carlton”) in the same vase

[57]. Addition of mucilage, exuded by daffodil stems to the vase water has the same effect as the flowering daffodil stem. The active compound in the mucilage was identified as narciclasine. This delay of senescence by narciclasine correlates with a delayed increase in protease activity and marked reduction in maximum protease activity [57]. Narciclasine, although not affecting *in vitro* protease activity, is known to inhibit protein synthesis at the ribosomal level. Narciclasine was also originally described as an antimitotic substance displaying colchicine-like effects [8]. It was also found to be an inhibitor of peptide bond formation in eukaryotic ribosomes given its ability to bind to the 60-S ribosomal subunit and more precisely to the peptidyl-transferase center [58,59]. Furthermore, unlike many other anticancer drugs, narciclasine has been found not to interact or form a complex with DNA [60]. More recently, McLachlan et al. [61] demonstrated that pancratistatin, whose chemical structure is very close to that of narciclasine, induced rapid apoptosis in neuroblastoma cells accompanied by disruption of the mitochondrial membrane potential. Additionally, a decrease in ATP synthesis and an increase in the production of reactive oxygen species, indicative of a dysfunction of the mitochondrial respiratory chain, were observed in intact mitochondria incubated with the molecule [61]. These changes were not observed in normal fibroblasts [61,62] and normal endothelial cells [63]. Caspase-3 activation and exposure of phosphatidyl serine on the outer leaflet of the plasma membrane were earlier events than the generation of reactive oxygen species and DNA fragmentation observed following pancratistatin treatment of cancer cells [64]. By investigating isolated mitochondria, McLachlan et al. [61] demonstrated that pancratistatin acted directly on mitochondria and confirmed that this effect was selective for mitochondria isolated from cancer cells. Quite recently, Griffin et al. [62] undertook a synthetic structure–activity relationship evaluation, which indicated that the minimum cytotoxic pharmacophore of pancratistatin comprises the trans-fused B/C ring system containing the 2,3,4-triol unit in the C-ring. The results of their study further indicated that the phenanthridone skeleton in natural Amaryllidaceae alkaloids may be a significant common element for selectivity against cancer cells [62]. McNulty et al. [65] have previously demonstrated that a 2,3,4-triol–functionalized ring-C is required as the minimum pharmacophoric element for potent anticancer activity of pancratistatin.

We have recently reported that narciclasine shows potent *in vitro* cytotoxic activity against six different human cancer cell lines. In contrast, normal human lung fibroblasts appear to be markedly less sensitive to the molecule with a mean cytotoxic  $IC_{50}$  value (the concentration that reduces by 50% the number of viable cells after 3 days of treatment) of 7.5  $\mu$ M compared to 0.03  $\mu$ M for the six human cancer cell lines [66]. The cytotoxic activity of narciclasine was similar in the six cancer cell lines investigated, a feature consistent with NCI data that revealed a mean  $IC_{50}$  value of 0.046  $\mu$ M for the compound across a panel of 60 cancer cell lines, including doxorubicin-resistant cancer cells [46]. Additionally, based on comparative evaluations assessing morphologic changes, after internucleosomal DNA fragmentation or the externalization of phosphatidyl serine in normal fibroblasts and in human cancer cells, the proapoptotic effects of narciclasine at concentrations up to 1  $\mu$ M were confined to cancer cells [66]. These results collectively emphasize the marked selectivity of narciclasine for cancer cells, as also demonstrated by Griffin et al. [62] for pancratistatin. We have shown that the narciclasine-induced apoptosis-mediated cytotoxic effects in human cancer cells not evident in normal fibroblasts is paralleled by the triggering of the activation of the initiator caspases of the death re-

ceptor pathway (caspase-8 and -10), at least in human MCF-7 breast and PC-3 prostate carcinoma cells. This is a feature not observed in normal human fibroblasts [66]. Indeed, formation of the Fas and DR4 death-inducing signaling complex was clearly evidenced in MCF-7 and PC-3 cancer cells [66]. Additionally, in these two cancer cell lines, caspase-8 was found to interact with the Fas and DR4 receptors on narciclasine treatment. However, after this initial step, narciclasine-induced downstream apoptotic pathways in MCF-7 cells diverged from those found in PC-3 cells, where caspase-8 directly activated effector caspases such as caspase-3 in the absence of any further release of mitochondrial proapoptotic effectors [66]. In contrast, in MCF-7 cells, the apoptotic process was found to require an amplification step that is mitochondria-dependent with Bid processing, release of cytochrome *c* and caspase-9 activation [66].

We have postulated that the high selectivity of narciclasine for cancer cells might be linked at least in part to this activation of the death receptor pathway. Normal human fibroblasts appear approximately 250-fold less sensitive to the cytotoxic effects of narciclasine, which does not induce apoptosis in these normal fibroblasts probably due to the absence of death receptor pathway activation [67–69]. Moreover, it has been suggested that Amaryllidaceae alkaloids, such as narciclasine, have a modulating activity on inflammatory reactions by inhibiting tumor necrosis factor- $\alpha$  production in stimulated macrophages [70]. Inflammation has been implicated in the development of many human epithelial cancers, including those of the stomach, lung, colon, and prostate, and tumor necrosis factor- $\alpha$  is a potent pleiotropic, proinflammatory cytokine produced by many cells in response to injury and inflammation [71].

## Conclusions

Since the discovery of narciclasine, the first member of isocarbostryl class of alkaloids in 1967, the search for new natural compounds from the Amaryllidaceae plant family has resulted in the isolation of other isocarbostryls such as lycoricidine and pancratistatin.

To date, for the synthesis of novel isocarbostryl derivatives for the potential discovery and development of new anticancer drug candidates, the best starting material for these products is narciclasine, which can be isolated from *Narcissus* species with a good yield. The total synthesis of these products is still very complex and requires too many steps to be developed commercially for the synthesis and anticancer testing of novel isocarbostryls.

The biologic activities of isocarbostryl alkaloids include excellent *in vitro* and *in vivo* cytotoxicity against many tumor cell lines and high selectivity for cancer cells *versus* normal cells. This has encouraged several research groups to identify new derivatives or analogs of these isocarbostryl alkaloids for preclinical development. It is to be hoped that continuing interest in structure modulation of these isocarbostryl alkaloids will provide novel promising anticancer agents.

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