J. of Korean Oil Chemists' Soc.,
Vol. 29, No. 2. June, 2012. 248~256

Analytical Characterization of Aza-Indole Alkaloids in the Biosynthesis of *Catharanthus Roseus*

Hyang-Yeol Lee†

†*Department of Biotechnology, Korea National University of Transportation, 380-702, Korea*
(Received May 23, 2012 ; Revised June 14, 2012 ; Accepted June 21, 2012)

Abstract : Aza-indoles are important pharmacophores that have similar size and biological properties of indole. Here we have synthesized 4- and 7-azaindole tryptamines and showed that they are successfully incorporated in the biosynthesis of monoterepene indole alkaloids (MIAs) to form novel azaindole alkaloids by enzymatic reactions of strictosidine synthase(STR) and strictosidine glucosidase(SDG) monitored by UPLC/MS. By using HPLC equipped with a HPLC photo diode array(PDA) detector, each of the UV spectra of azaindole alkaloids was obtained and characterized. When hydrophilicity of azaindole alkaloids was compared, 4-azaindole alkaloids were more hydrophilic than 7-azaindole alkaloids.

Keywords : Catharanthus roseus, Pictet-Spengler reaction, azaindole alkaloids, b-carbolines, deglucosylated azaindole strictosidine.

1. Introduction

Indole moiety can be found in many organic molecules such as tryptophan-containing proteins, in alkaloids and in dyes. Indole as a side chain of tryptophan has been used extensively as an intrinsic fluorescent probe to investigate protein dynamics and the local environment of the tryptophan residue. Several nitrogen-containing tryptophan analogs such as 4~7-Aza-trptophans have been investigated to incorporate into the proteins due to the advantages of stronger fluorescence intensity and red-shifted fluorescence[1]. Tryptamine is a monoamine alkaloid structurally related with an amino acid residue, tryptophan. Tryptamine analogs are biologically active compounds. The most well-known tryptamine analogs among many naturally found in nature are a neurotransmitter, serotonin, and a hormone, melatonin. Tryptamines are also known as hallucinogens. For examples, N,N-dimethyltryptamine(DMT) is a naturally occurring potent psychedelic drug and 4-and 5-substituted tryptamines such as 4-HO-DMT and 5-MeO-DMT are hallucinogenicagents. Tryptamine derived compounds have also been explored as potential therapeutic agents in treating alcoholism, opioid addiction, cluster headaches, stress disorder, obsessive-compulsive disorder, post-traumaticstress disorder[2].

Azaindoles are of great interest as isopharmacores of indole due to their structural similarity and biological properties[1]. They are promising building blocks with potential applications in the field

†Corresponding author (E-mail : hyl@cjnu.ac.kr)

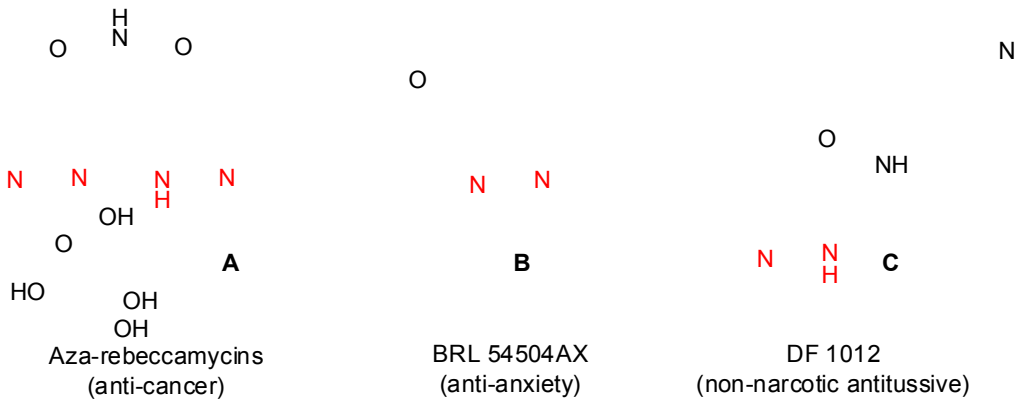


Fig. 1. The Aza-indole moiety in synthetic compounds with pharmaceutical activities.

of pharmaceuticals, natural product synthesis and a variety of synthetic intermediates on the purpose to find novel therapeutic agents[3~5]. It has been reported that 4- and 7-azaindoles are not noticeably toxic in comparisons to indole along with pharmacological properties based on the *in vivo* study in mice. This can be observed in mice as the LD_{50} for 4-azaindole is 260 mg/kg and for 7-azaindole is 490 mg/kg compared to 316 mg/kg for indole. A few natural products with azaindole moieties have been found in nature. For examples, Didemnolines and Variolin B were isolated from an ascidian of the genus *Didemnum* and antatic sponge *Kirkpatricki* respectively[6]. Synthetic products have been broadly synthesized based on azaindole scaffolds in natural product analogues and in drugs, most of synthetic products shown in fig. 1 are under the clinical trials. After the synthesis of the two azaindole tryptamines 4 and 5, it was demonstrated that two of these compounds were successfully converted to azaindole alkaloids by enzymatic reactions. Moreover, masses and UV spectra of those new azaindole alkaloids were measured by UPLC/MS and HPLC.

2. Materials and Method

2.1. Materials

4-Trimethylsilyl-3-butyn-1-ol was purchased from Wako Co.. 2-Amino-3-iodopyridine, 3-amino-4-iodopyridine and 4-amino-3-iodopyridine were purchased from Alfa Acer. 3-Amino-2-iodopyridine was prepared from 3-amino-2-bromopyridine that was purchased from Alfa Acer Co. according to the method described in the literature. Tryptamine, $Pd(dppf)Cl_2$. CH_2Cl_2 and trans-N,N'-dimethylcyclohexane were purchased from Aldrich Co.

2.2. UPLC/MS analysis

Ultra performance LC analysis was performed on an Acquity Ultra Performance BEH C18 column with a 1.7 mm particle size, 2.1 x 100 mm dimension, and a flow rate of 0.6 ml min^{-1} in tandem with a Micromass LCT Premier TOF Mass Spectrometer with an ESI source (Waters Corporation). The capillary and sample cone voltages were 3000 V and 30 V, respectively. The desolvation and source temperatures were 300 ℃ and 100 ℃, respectively. Analysis was performed with MassLynx 4.1.

2.3. HPLC analysis of enzyme reactions with azaindole tryptamine 5 or 6

Strictosidine synthase(*C. roseus*) was expressed in *E. coli* and purified as

previously described[7,8]. Reactivity was measured using an high-performance liquid chromatography (HPLC) assay following previously reported procedures[7]. Secologanin(2.5 mM), 1-naphathalene acetic acid as an internal standard(60 mM), and stryptamine analogs **5** or **6** (500 mM) in PBS buffer(50 mM, pH 7.0) were incubated at 30 ℃. Enzymatic reactions were quenched by addition of 2.0 M aqueous sodium hydroxide. Quenched aliquots of the reaction were directly injected onto an analytical HPLC using a solvent gradient of 10 % to 70 % acetonitrile in 0.1 % aqueous trifluoroacetic acid. The UV absorbance of azaindole tryptamine alkaloids were obtained by HPLC photo diode array(PDA) detector.

2.4. UPLC/MS analysis of enzyme reactions with azaindole tryptamine 5 and 6

4-Azaindole tryptamine **5** or 7-azaindole tryptamine **6** (500 mM), secologanin(2.5 mM), strictosidine synthase(STR), strictosidine glucosidase (SDG) were incubated at 30 ℃ in PBS buffer(50 mM, pH 7.0). Enzymatic reactions were quenched by addition of 2.0 M aqueous sodium hydroxide. Quenched aliquots of the reaction were directly injected onto UPLC/MS after 24 hours to monitor reaction progress.

2.5. Synthesis of 4-azaindole tryptamine 5 and 7-azaindole tryptamine 6

The title compounds were prepared following a general procedure reported by Pullagurla et al as shown in Fig. 2[2, 9~12]. A solution of 4-trimethylsilylbut-3-yn-1-ol (1.0 g, 7.03 mmol) and dry CH_2Cl_2 was cooled to 0 °C and Et_3N(1.08 ml, 7.73 mmol) was added in a dropwise manner. Methane sulfonyl chloride(600 ml, 7.73 mmol) in CH_2Cl_2(5 ml) was added to the reaction mixture. It was allowed to stir at room temperature for 1h and then the reaction mixture was extracted with brine and CH_2Cl_2 and dried under the vacuum. The solid was dissolved in THF(5 ml) and liquid NH_3 was added at -78 °C to the reaction mixture and stirred 3 days at room temperature in a sealed tube. Free amine product was isolated by column chromatography with 5 % dichloromethane in MeOH and starting material, 4-trimethylsilylbut-3-yn-1-ol, was recovered for further reaction. A solution of 4-trimethylsilylbut-3-yn-1-amine(272 mg, 1.91 mmol), Et_3N (293 ml, 2.10 mmol) in dry CH_2Cl_2(2 ml) and THF(2 ml) under Ar atmosphere was cooled to 0 °C and acetic anhydride (199 ml, 2.10 mmol) was added in a dropwise manner to the reaction mixture. The reaction mixture was allowed to stir at room temperature for 2h and then it was extracted with brine and CH_2Cl_2 and evaporated to dryness to give brown solid **5** or **6**.

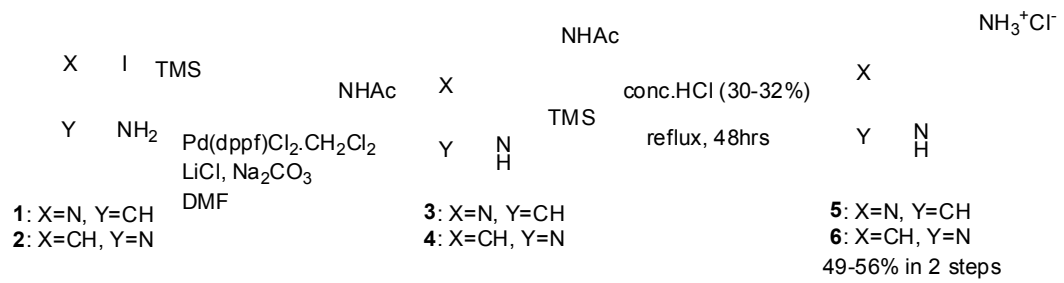
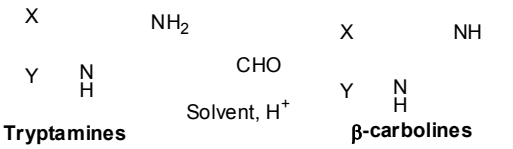


Fig. 2. Synthesis of aza-indole tryptamines **5** and **6**.

3. Results and Discussion

3.1. Chemical reactivity of 5, 6 and 7.

To determine the relative chemical reactivity of tryptamine and 4- or 7-azaindole tryptamines **5** and **6** respectively, reactions were carried out in the same condition, which used 5 times excess of propionaldehyde and an equivalent of acetic acid as an acid catalyst. After chromatographic separation, the b-carboline product, 1-ethyl-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole **10**, was isolated in 44 % yield from tryptamine and propionaldehyde mixture as shown in Table 1; however only trace amount of azaindole carboline products from azaindole tryptamines **5** and **6** and an excess of propionaldehyde were detected by LC/Mass. NMR data of **10** was identical with the known product **10**. Even attempts to use an acid catalyst, trifluoroacetic acid, and Lewis acid catalysts, $Zn(OTf)_3$ or $Sc(OTf)_3$, to facilitate the reaction, were unsuccessful.



Tryptamines **β-carbolines**

5: X=N, Y=CH
6: X=CH, Y=N
7: X=CH, Y=CH

8: X=N, Y=CH
9: X=CH, Y=N
10: X=CH, Y=CH

Table 1. Chemical Reactivity Comparison of Tryptamine **7** and Azaindole Tryptamines **5**, **6** with Propionaldehyde

Compound	solvent	Yield
7	MC	39%[a]
7	DMF	44%[a]
5	DMF	trace
6	DMF	trace

[a] isolated yield of b-carbolines. Reaction time: overnight.

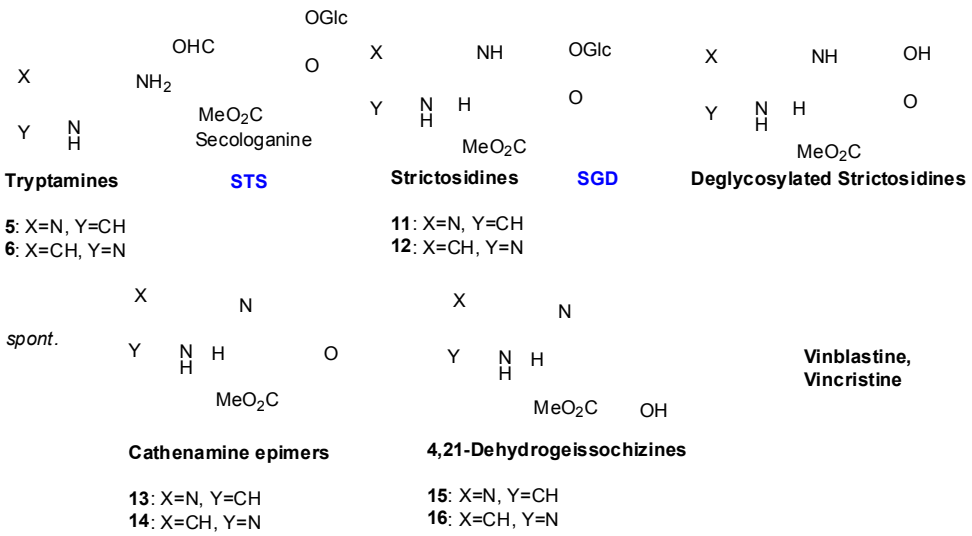


Fig. 3. Reaction of tryptamines in monoterpene indole alkaloid biosynthesis.

The result of this experiment indicates that tryptamine is very reactive by chemical reaction to form b-carboline whereas azaindole tryptamines **5** and **6** are not. The reason that azaindole tryptamines are less reactive than tryptamine is because azaindoles are protonated at neutral pH which results in reducing electron density and chemical reactivity for the Pictet-Spengler reaction.

3.2. UPLC/MS analysis of azaindole alkaloids by enzymatic reaction.

Strictosidine synthase catalyzes the first step in monoterpene indole alkaloid biosynthesis(MIAs)[13,14]. Tryptamine and secologanin are condensed by strictosidine synthase(STS) to form strictosidine, the common precusor to all TIAs. This step involves an asymmetric Pictet-Spengler reaction between tryptamine and secologanin. In the second step of this alkaloid pathway, strictosidine is deglucosylated by strictosidine b-D-glucosidase(SGD), that has been isolated from *Catharanthus roseus*. Deglucosylated structosidine is converted several unstable intermediates to cathenamine and 4,21-dehydrogeissoschizine[17]. Using synthetic tryptamines monoterpene indole alkaloid (MIA) biosynthesis, the medicinal plant *Catharanthus rosues* has a surprisingly broad tolerance for stereochemical perturbations *in vitro*[15~17].

When strictosidine synthase(STS) is incubated with 4- or 7-azaindole tryptamines **5** and **6** with secologain, 4- or 7-azastrictosidine **11** and **12** respectively are successfully formed. 4- Or 7-azastrictosidine **11** and **12** is then deglucosylated by strictosidine b-D-glucosidase(SGD) to form deglucosylated azastrictosidines which are spontaneously turned to aza-cathenamine epimers **13**, **14** and aza-4,21-dehydrogeissochizene **15**, **16** as shown in Fig. 3.

When strictosidine synthase(STS) was incubated with 4-azaindole tryptamine and secologanin, the peak of 4-aza-strictosidine(**11**, m+1/z 532) was shown on LC/MS in Fig. 4. Then strictosidine glucosidase(SGD) was incubated with 4-aza-strictosidine and the peak of deglucosylated strictosidine(m+1/z 352) appeared in Fig. 5 (1). The peak at 3.6 min in Fig. 5 (1) could be the 4-aza-4,21-dehydrogeissoschizine intermediate based on retention time. 7-Aza-strictosidine was deglucosylated by SGD to form deglucosylated strictosidine varients such as 7-aza-cathenamine epimers **14** and 7-aza-

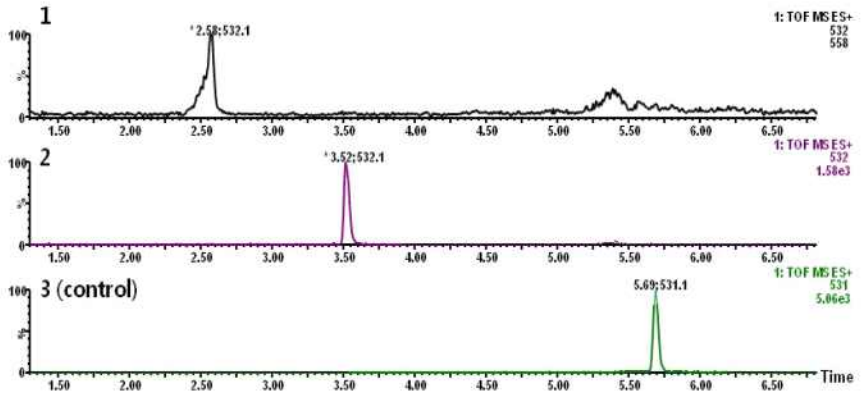


Fig. 4. (1) LC/MS spectra showing production of 4-azaindole strictosidine **11**, (2) 7-azaindole strictosidine **12**.

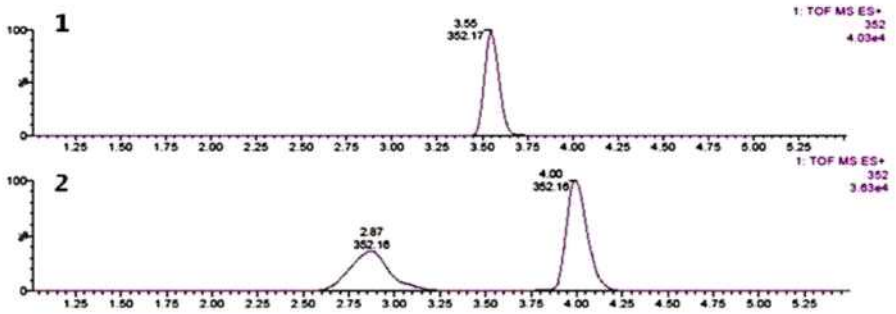


Fig. 5. (1) LC/MS spectra showing production of 4-aza-deglucosylated strictosidine isomers. (2) 7-aza-deglucosylated strictosidine isomers.

4,21-dehydrogeissoschizine **16**. The peaks appeared at 2.9 min and at 4.0 min seem to be **16** and **14**, respectively in Fig. 5 (2).

3.3. HPLC analysis of 4-azaindole alkaloids by enzymatic reaction.

Reactivity of 4-azaindole tryptamine **5** was measured using an high-performance liquid chromatography(HPLC) assay[16]. Secologanin (2.5 mM) and azaindole tryptamine **5**(500 mM) with STS and DGS in PBS buffer(50 mM, pH 7.0) were incubated at 30 ℃. Enzymatic reactions were quenched by an addition of 2.0 M aqueous sodium hydroxide. Quenched aliquots of the reaction were directly injected onto an analytical HPLC using a solvent gradient of 0 % to 70 % acetonitrile in 0.1 % aqueous trifluoroacetic acid. The UV absorbance of azaindole tryptamine alkaloids were obtained by a HPLC photo diode array(PDA) detector.

Peak A at 9.8 min and peak B at 10.4 min are starting materials, 4-azaindole tryptamine **5** and secologanin. Peak C at 11.6 min represents 4-azaindole strictosidine **11** which then gradually converts to peak D and E at 13.8 min & 14 min, respectively. Those peaks correspond to 4-azaindole-cathenamine epimers **13** and 4-azaindole-4,21-dehydrogeissoschizine **15** which are converted spontaneously from deglucosylated strictosidine.

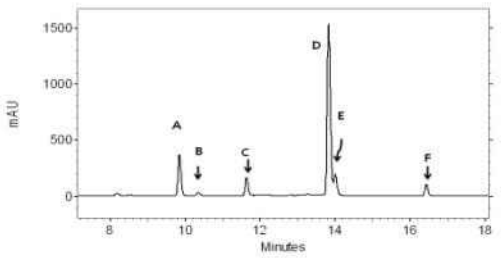
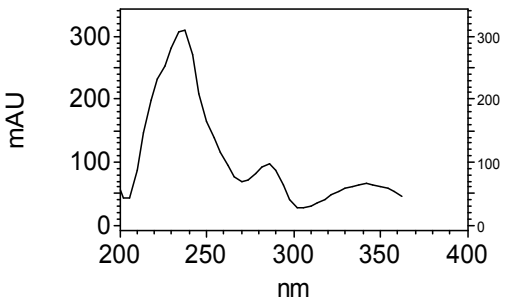
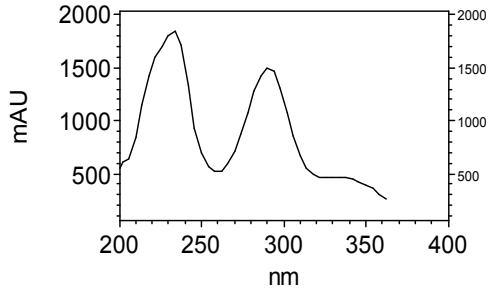


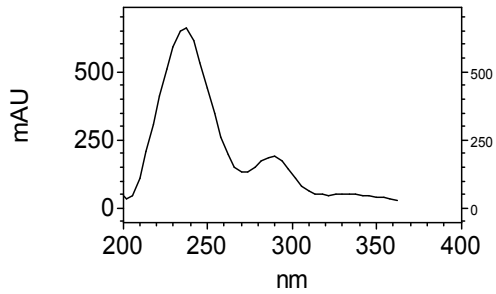
Fig. 6. HPLC chromatogram showing deglucosylation of azastrictosidine derived from **5**. (A) 9.8 min **5**, (B) 10.4 min Secologanin, (C) 11.6min **11**, (D, E) 13.8min & 14min **13** & **15** respectively, (F) 16.4 min naphthalene acetic acid as an internal standard. Gradient, 0-70% acetonitrile in water with 0.1% trifluoroacetic acid over 20min, monitoring at 290nm.



4-Azaindole strictosidine **11**; peak C at 11.6min.



4-azaindole-4,21-dehydrogeissoschizine **15**; peak D at 13.8 min.



4-azaiondole-cathenamine epimers **13**; peak E at 14.0 min.

Fig. 7. UV absorption spectra of 4-azaindole alkaloids were obtained by HPLC photo diode array (PDA) detector.

3.4. HPLC analysis of 7-azaindole alkaloids by enzymatic reaction.

Reactivity of 7-azaindole tryptamine **6** was measured using the same high-performance liquid chromatography (HPLC) assay as described above. Secologanin(2.5 mM) and 7-azaindole tryptamine **6**(500 mM) with STS and DGS in PBS buffer(50 mM, pH 7.0) were incubated at 30 ℃. Quenched aliquots of the reaction were directly injected onto an analytical HPLC using a solvent gradient of 5 % to 70 % acetonitrile in 0.1 % aqueous trifluoroacetic acid.

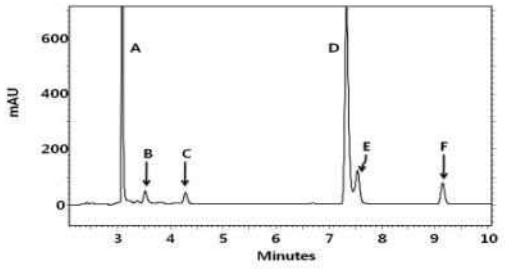
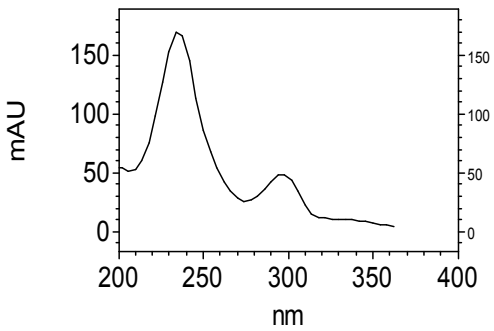
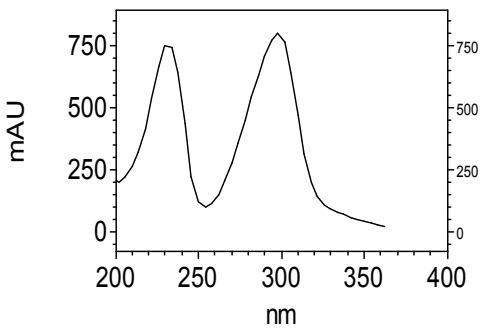


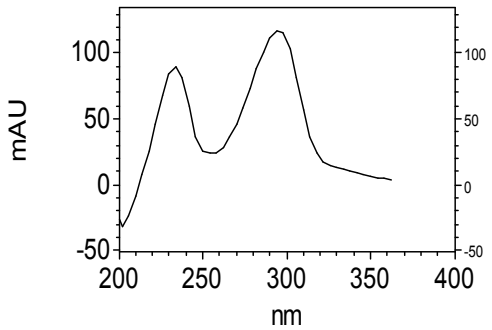
Fig. 8. HPLC chromatogram showing deglucosylation of azastrictosidine derived from **6**. (A) 3.1 min **6**, (B) 3.5min Secologanin, (C) 4.3 min **12**, (D, E) 7.3 min & 7.5 min **14** & **16**, respectively (F) 9.1 min NAA(naphthalene acetic acid) as an internal standard. Gradient, 5-70 % acetonitrile over 15 min, monitoring at 290 nm.



7-Azaindole strictosidine **12**; peak C at 4.3 min.



7-Azaindole-4,21-dehydrogeissoschizine **16**; peak D at 7.3 min.



7-Azaiondole-cathenamine epimers **14**; peak E at 7.5 min.

Fig. 9. UV absorption spectra of 7-azaindole alkaloids were obtained by HPLC photo diode array (PDA) detector.

Azaindole alkaloids were UV-detected and characterized from their specific UV absorbance spectra. 4-Azaindole strictosidine **11** shows maximum UV-absorbance at 238 due to the p-p* excitation. Other maximum UV-absorbance of **11** due to the n-p* excitation are at 285 and 342. Interestingly **11** shows two UV-absorbance for n-p* excitation whereas other azaindole alkaloids have one. Moreover, the peak of 7-azaindole alkaloids are red shift for n-p* transition with blue shift for p-p* transition compared to 4-azaindole alkaloids.

Hydrophilicity of azaindole alkaloids are also compared on HPLC. 4-Azaindole alkaloids eluted much earlier than 7-azaindole alkaloids which implies that 4-azaindole alkaloids are more hydrophilic substances.

4. Conclusions

Tryptamine is very sensitive to chemical reactions to form b-carboline; however azaindole tryptamines **5** and **6** show very poor chemical reactivity due to the reduced electrons on indole ring. 4- or 7-Azaindole tryptamines **5** and **6** with secologain formed 4- or 7-azastrictosidine **11** and **12**, respectively by strictosidine synthase(STS). 4- Or 7-azastrictosidine **11** and **12** is then deglucosylated by strictosidine b-D-glucosidase(SGD) to form deglucosylated aza-strictosidines which are spontaneously turned to aza-cathenamine epimers **13**, **14** and aza-4,21-dehydrogeissochizene **15**, **16** respectively. The peak of 7-azaindole alkaloids are red shift for n-p* transition with blue shift for p-p* transition compared to 4-azaindole alkaloids. When hydrophilicity of azaindole alkaloids are compared on HPLC, 4-azaindole alkaloids are more hydrophilic than 7-azaindole alkaloids.

Table 2. Specific Properties of Terepene Indole Alkaloids for HPLC Detection

Alkaloids	l_{max} (nm)	Hydrophilicity	ref.
Tryptamine	218, 278	-	[16]
Secologanin	238	-	[16]
Strictosidine	228, 239, 271, 280	0	[14, 16]
11	238, 285, 342	+++	
15	234, 290	+	
13	238, 290	+	
12	234, 296	++	
16	230, 298	+	
14	230, 295	+	

* Hydrophilicity of azaindole alkaloids are compared with strictosidine as a standard.

Acknowledgement

This research was supported by a grant from the Academic Research Programs of Korea National University of Transportation in 2012.

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