JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Design, Synthesis, and Anti-tobacco Mosaic Virus (TMV) Activity of Phenanthroindolizidines and Their Analogues

Ziwen Wang, Peng Wei, Lizhong Wang, and Qingmin Wang*

State Key Laboratory of Elemento-Organic Chemistry, Research Institute of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, People's Republic of China

Supporting Information

ABSTRACT: On the basis of our previous structure−activity relationship (SAR) and antiviral mechanism studies, a series of phenanthroindolizidines and their analogues **3**−**20** were designed, targeting tobacco mosaic virus (TMV) RNA, synthesized, and systematically evaluated for their antiviral activity against TMV. The bioassay results showed that most of these compounds displayed good anti-TMV activity, and some of them exhibited higher antiviral activity than that of commercial Ningnanmycin (perhaps the most successful registered antiplant viral agent). Especially, (*S*)-deoxytylophorinine (**5**) with excellent anti-TMV activity (inactivation activity, 59.8%/500 μg mL^{-1} and 40.3%/100 μg mL^{-1}; curative activity, 65.1%/500 μg mL^{-1} and 43.7%/100 μg mL^{-1}; and protection activity, 70.2%/500 μg mL^{-1} and 51.3%/100 μg mL^{-1}) emerged as a potential inhibitor of the plant virus. Compound **20** exhibited a strong *in vivo* protection effect against TMV at 100 μg mL^{-1}, which indicated that phenanthroindolizidine analogues with a seven-membered D ring have a new and interesting structural scaffold and have great potential for further development as tobacco protection agents.

KEYWORDS: *Phenanthroindolizidines, diazepines, antiviral activity, tobacco mosaic virus, structure−activity relationship, TMV, SAR*

INTRODUCTION

Plant viruses cause a variety of detrimental effects on agriculture and horticulture.[1] Tobacco mosaic virus (TMV), one of the most well-studied plant viruses, is known to infect more than 400 plant species belonging to 36 families, such as tobacco, tomato, potato, and cucumber. It is found that in certain fields 90−100% of the plants show mosic or leaf necrosis by harvesting time. Therefore, this plant virus has the name "plant cancer" and is difficult to control.[2−4]

Ningnanmycin (Figure 1), perhaps the most successful registered anti-plant viral agent, displayed 56.0% *in vivo* curative effect at 500 μg mL^{-1}. Ribavirin (Figure 1) is another widely used plant viral inhibitor. Its inhibitory effects are also less than 50% at 500 μg mL^{-1}. In fact, there are no super chemical treatments that can absolutely inhibit TMV once it has infected the plants. Because of the unsatisfactory cure rate (30−60%) by common antiviral agents (e.g., Ningnanmycin, Ribavirin, and virus A) and economic loss of tobacco, much effort has been directed toward the development of novel, potent, and structurally concise antiviral agents. Some chemicals, such as pyrazole derivatives,[5,6] nucleotides,[7] α-aminophosphonate derivatives,[8,9] 3-acetonyl-3-hydroxyoxindole,[10] triazolyl compounds,[11] oxidized polyamines,[12] thiadiazoles,[13] substituted phenylureas,[14] and some natural products,[15−17] have been found to possess antiviral activity. However, because there are only a few reports on economically viable antiviral chemicals available for application in agriculture,[18] there is great potential for further research in this field.

Natural-product-based antiviral agents offer advantages in that they can sometimes be specific to a target species and often have a unique mode of action with low mammalian toxicity. Another benefit is their ability to decompose rapidly, thereby reducing their risk to the environment.[19,20] An additional advantage is that natural products can be candidates that possess the desirable biological activities.

Natural phenanthroindolizidine alkaloid (*R*)-tylophorine (**2**) and its analogues [e.g., (*R*)-antofine (**4**) and (*R*)-deoxytylophorinine (**6**)] have been isolated primarily from the genera *Cynanchum*, *Pergularia*, and *Tylophora* in the Asclepiadaceae family.[21] These compounds, commonly called tylophora alkaloids, have been targets of synthesis and modification for their significant cytotoxic activities.[22]

In a program aimed at screening of plants for biologically active natural products as alternatives to conventional synthetic antiviral agents, we first found that the alcohol extract of *Cynanchum komarovii* displayed moderate antiviral activity against TMV. Using a bioassay-directed fractionation approach, the main active substances in *C. komarovii* were determined as tylophorine alkaloids, in which (*R*)-antofine (**4**) presents a high level.[23] Moreover, another four alkaloids (Figure 2), 6-hydroxyl-2,3-dimethoxyphenanthroindolizidine (**I**), 7-demethoxytylophorine *N*-oxide (**II**), 14-hydroxyantofine *N*-oxide (**III**), and 2,3-dimethoxy-6-(3-oxobutyl)-7,9,10,11,11a,12-hexahydrobenzo[*f*]pyrrolo[1,2-b]isoquinoline (**IV**), were obtained at lower levels. Bioassay results showed that (*R*)-antofine (**4**) and 6-hydroxyl-2,3-dimethoxyphenanthroindolizidine (**I**) displayed excellent antiviral activity.[24] For example, the commercial antiviral agents 2,4-dioxohexahydro-1,3,5-triazine (DHT) and 1,5-diacetyl-2,4-dioxohexahydro-1,3,5-triazine (DADHT) and moroxydine hydroxychloride copper acetate (virus A) showed 50% inhibition at 500 μg mL^{-1}, whereas 6-

Received: August 15, 2012
Revised: September 22, 2012
Accepted: September 23, 2012
Published: October 4, 2012

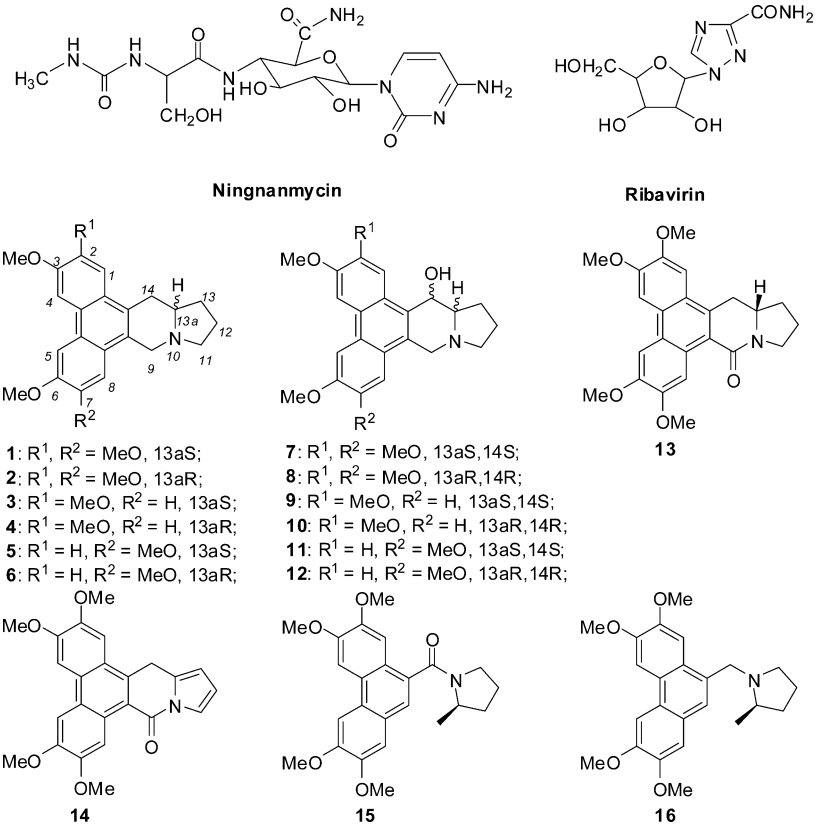


Figure 1. Chemical structures of Ningnanmycin, Ribavirin, and compounds **1**−**16**.

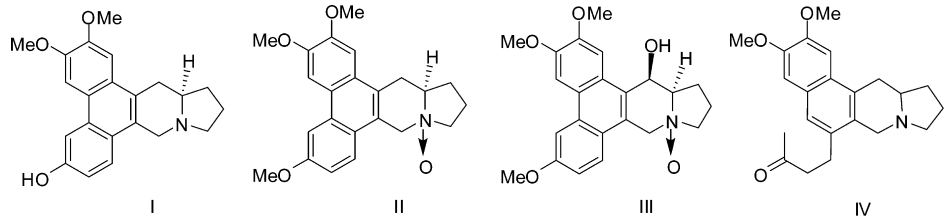


Figure 2. Chemical structures of compounds **I**−**IV**.

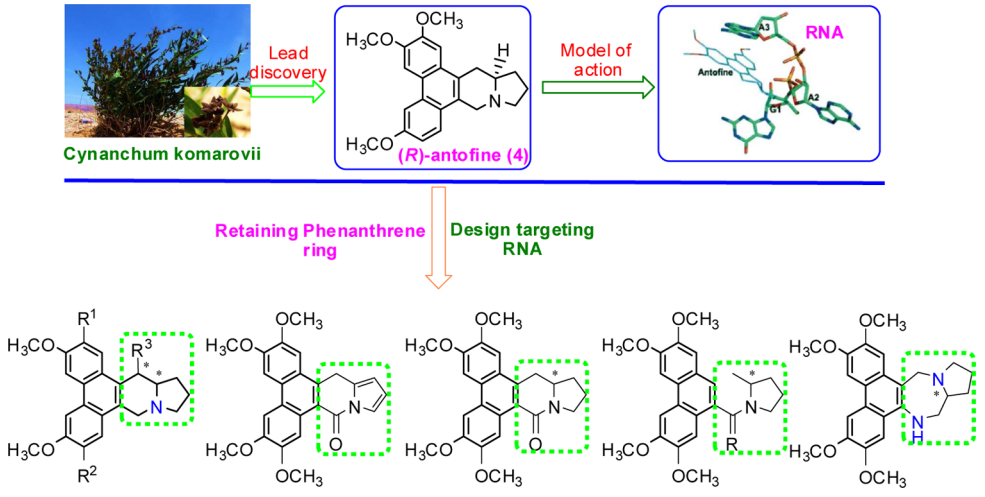


Figure 3. Design of phenanthroindolizidines and their analogues.

hydroxyl-2,3-dimethoxyphenanthroindolizidine (**I**) has 70% inhibitory activity, even at the concentration of 1.0 μg mL^{-1}, which was 10−100 times more active than any reported plant virus inhibitor.[24,25] Moreover, the structure−activity relationship studies showed that most compounds of the antofine-based library with structural diversity exhibited inhibitory

dx.doi.org/10.1021/jf303550a | J. Agric. Food Chem. 2012, 60, 10212−10219

activity against TMV higher than that of commercial antiviral agents[26,27] and the presence of free nitrogen in tertiary amine and phenanthrene ring was essential for high antiviral activity.[28]

Further antiviral mechanism studies revealed that antofine-based alkaloids have a favorable interaction with the origin of TMV RNA (oriRNA), likely exerting its virus inhibition by binding to oriRNA and interfering with virus assembly initiation.[29]

On the basis of the above findings, a series of phenanthroindolizidines and their analogues (**3**−**20**) were designed, targeting TMV RNA (Figure 3), synthesized, and systematically evaluated for their antiviral activity against TMV.

MATERIALS AND METHODS

Synthetic Procedures. The melting points were determined using an X-4 binocular microscope melting point apparatus (Beijing Tech Instruments Co., Beijing, China), and the thermometer was uncorrected. Mass spectra were obtained on a VG ZAB-HS instrument spectrometer and a LCQ Advantage instrument spectrometer using the electron ionization (EI) or fast atom bombardment (FAB) method and electrospray ionization (ESI) method, respectively. High-resolution mass spectrometry (HRMS) was obtained on Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) (Ionspec, 7.0 T). The enantiomeric excess (ee) of (*S*)-tylophorine (**1**) and (±)-tylophorine was determined by high-performance liquid chromatography (HPLC) on a chiralcel AD-H column using Agilent 1100 series, and the ee of compounds **17**−**20** was determined by HPLC on a chiralcel AD-H column using Rheodyne 3725i-038 series. Optical rotations were recorded on a Perkin-Elmer 341 MC polarimeter. Chromatographic separations were carried out under pressure on silica gel using flash column techniques. Reagents were purchased from commercial sources and used as received. All anhydrous solvent were dried and purified by standard techniques just before use. Reaction progress was monitored by thin-layer chromatography on silica gel GF_{254} with ultraviolet (UV) detection. ^1H and ^{13}C nuclear magnetic resonance (NMR) and infrared (IR) data of compounds **1**, **7**, **17**, **19**, and **22**−**24** were given in the Supporting Information.

*(S)-Methyl-1-[(9-bromo-2,3,6,7-tetramethoxyphenanthren-10-yl)methyl]pyrrolidine-2-carboxylate (**22**).* A mixture of bromide **21** (5.00 g, 11 mmol), methyl L-prolinate hydrochloride (2.64 g, 16 mmol), and potassium carbonate (4.40 g, 32 mmol) in *N*,*N*-dimethylformamide (DMF) (150 mL) was refluxed for 8 h. After cooling, the reaction mixture was concentrated *in vacuo*. The residue was taken up in ethyl acetate (250 mL) and washed with brine (2 × 100 mL). The organic phase was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (3:1 PE/EA) to give carboxylate **22** (5.12 g, 93%) as a white powder. Melting point (mp): 227−229 °C (dec). $[\alpha]_D^{20}$ −9.5 (*c* 2.0 in $CHCl_3$). HRMS (ESI) calcd for $C_{25}H_{29}BrNO_6$ $(M + H)^+$ 518.1173, found 518.1166.

*(S)-1-[(9-Bromo-2,3,6,7-tetramethoxyphenanthren-10-yl)-methyl]pyrrolidine-2-carboxylic acid (**23**).* To a solution of carboxylate **22** (2.00 g, 4 mmol) in methanol (80 mL) was added a 2 N solution of potassium hydroxide (20 mL). The reaction mixture was refluxed for 6 h and then made acidic with concentrated hydrochloric acid. The product was extracted with CH_2Cl_2 (150 mL) and crystallized from methanol to yield acid **23** (1.36 g, 84%) as a slight yellow powder. mp: 238−240 °C (dec). $[\alpha]_D^{20}$ 17 (*c* 1.0 in $CHCl_3$). HRMS (ESI) calcd for $C_{24}H_{27}BrNO_6$ $(M + H)^+$ 504.1016, found 504.1016.

*(13aS,14S)-14-Hydroxytylophorine (**7**).* To a solution of acid **23** (1.00 g, 2 mmol) and tetramethylethylenediamine (TMEDA) (0.76 g, 7 mmol) in dry tetrahydrofuran (THF) (150 mL), *n*-BuLi (4.23 mL, 6 mmol) was added at −78 °C, and the resulting mixture was stirred at this temperature for 4 h under a nitrogen atmosphere. Then, methanol (30 mL) and sodium borohydride (0.75 g, 20 mmol) were added to the mixture. After stirring at −40 °C for 1 h and room temperature for 8 h, the reaction was quenched with water (100 mL). The products were extracted with methylene dichloride (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (50:1 CH_2Cl_2/CH_3OH) to give alcohol **7** (0.45 g, 56%) as a slight yellow powder. mp: 267−269 °C (dec) (literature[30] mp: 270 °C). $[\alpha]_D^{20}$ 148.5 (*c* 2.0 in $CHCl_3$). HRMS (ESI) calcd for $C_{24}H_{28}NO_5$ $(M + H)^+$ 410.1962, found 410.1970.

*(S)-Tylophorine (**1**).* To a solution of **7** (1 g, 2 mmol) in trifluoroacetic acid (30 mL) was added triethylsilane (0.64 g, 6 mmol), and the resulting mixture was stirred at room temperature for 10 h in the dark. The solvent was evaperated *in vacuo*, and the residue was made basic with 10% aqueous solution of sodium carbonate. The product was extracted with methylene dichloride (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give the pure compound **1** (0.90 g, 94%) as a slight yellow powder. mp: 282−284 °C (dec). $[\alpha]_D^{20}$ 102 (*c* 1.0 in $CHCl_3$) [literature[31] mp: 284−286 °C; $[\alpha]_D^{20}$ 73 (*c* 0.7 in $CHCl_3$)]. ee: 97% [flow rate of 1.0 mL/min for 75:25 *n*-hexane/*i*-propanol and 0.1% triethylamine, at 41 bar and a 254 nm UV detector, with t_R (major) = 10.38 min and t_R (minor) = 13.32 min]. HRMS (ESI) calcd for $C_{24}H_{28}NO_4$ $(M + H)^+$ 394.2013, found 394.2009.

*(S)-1-[(9-Bromo-2,3,6,7-tetramethoxyphenanthren-10-yl)-methyl]pyrrolidine-2-carboxamide (**24**).* A solution of bromide **21** (5.00 g, 11 mmol), L-prolinamide (1.46 g, 13 mmol), and potassium carbonate (2.20 g, 16 mmol) in DMF (150 mL) was refluxed for 8 h. After cooling, the reaction mixture was concentrated *in vacuo*. The residue was washed with brine to give carboxamide **24** (4.92 g, 92%) as a white powder. mp: 252−253 °C (dec). $[\alpha]_D^{20}$ −14 (*c* 1.0 in $CHCl_3$). HRMS (ESI) calcd for $C_{24}H_{28}BrN_2O_5$ $(M + H)^+$ 503.1176, found 503.1167.

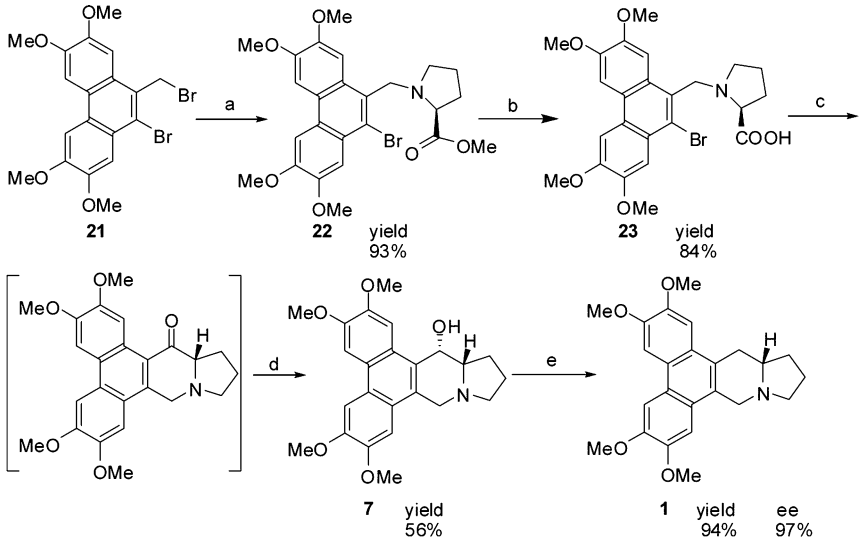
*(R)-1-[(9-Bromo-2,3,6,7-tetramethoxyphenanthren-10-yl)-methyl]pyrrolidine-2-carboxamide (**25**).* The analogue procedure for the preparation of compound **24** was used. The bromide **21** (5.00 g, 11 mmol), D-prolinamide (1.46 g, 13 mmol), and potassium carbonate (2.20 g, 16 mmol) gave compound **25** (4.87 g, 91%) as a white powder. mp: 246−248 °C. $[\alpha]_D^{20}$ 13 (*c* 1.0 in $CHCl_3$). Other data are the same as those of compound **24**.

*(S)-2,3,6,7-Tetramethoxy-10a,11,12,13-tetrahydro-9H-phenanthro[9,10-e]pyrrolo[1,2-a][1,4]diazepin-10(15H)-one (**17**).* A mixture of carboxamide **24** (3.00 g, 6 mmol), Cs_2CO_3 (3.89 g, 12 mmol), CuI (0.45 g, 2 mmol), and *N*,*N*-dimethylglycine hydrochloride (0.67 g, 5 mmol) in 1,4-dioxane (240 mL) was refluxed for 5 h under a nitrogen atmosphere. After cooling, ethyl acetate (100 mL) was added and the resulting mixture was filtrated through a short silica gel (100−200 mesh) pad. The filtrate was concentrated, and the residue was purified by flash column chromatography on silica gel (1:2 CH_2Cl_2/EA) to give compound **17** (2.09 g, 83%) as a white powder. mp: 261−263 °C (dec). $[\alpha]_D^{20}$ 233 (*c* 1.0 in $CHCl_3$). ee: more than 99% (flow rate of 1.0 mL/min for 85:15 *n*-hexane/*i*-propanol and 0.1% triethylamine, at 570−620 psi and a 254 nm UV detector, with t_R = 16.88 min). HRMS (ESI) calcd for $C_{24}H_{27}N_2O_5$ $(M + H)^+$ 423.1914, found 423.1904.

*(R)-2,3,6,7-Tetramethoxy-10a,11,12,13-tetrahydro-9H-phenanthro[9,10-e]pyrrolo[1,2-a][1,4]diazepin-10(15H)-one (**18**).* The analogue procedure for the preparation of compound **17** was used. The carboxamide **25** (3.00 g, 6 mmol) gave compound **18** (2.11 g, 84%) as a white powder. mp: 254−256 °C. $[\alpha]_D^{20}$ −243 (*c* 1.0 in $CHCl_3$). ee: more than 99% (flow rate of 1.0 mL/min for 85:15 *n*-hexane/*i*-propanol and 0.1% triethylamine, at 570−620 psi and a 254 nm UV detector, with t_R = 13.96 min). Other data are the same as those of compound **17**.

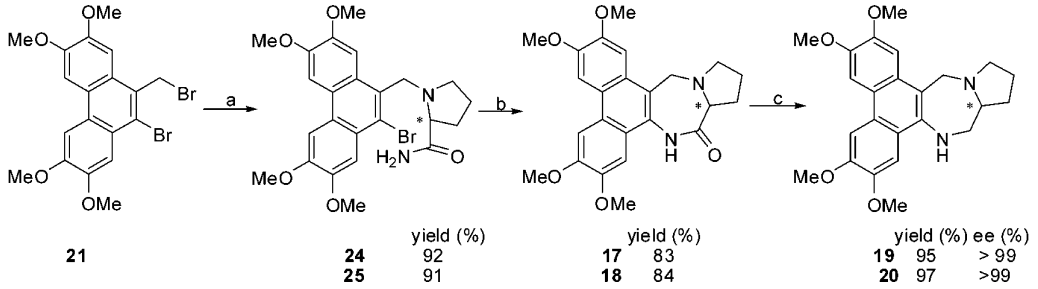
*(S)-2,3,6,7-Tetramethoxy-10,10a,11,12,13,15-hexahydro-9H-phenanthro[9,10-e]pyrrolo[1,2-a][1,4]diazepine (**19**).* A mixture of compound **17** (0.50 g, 1 mmol) and $LiAlH_4$ (0.14 g, 4 mmol) in THF (150 mL) was refluxed for 4 h under a nitrogen atmosphere. After cooling to 0 °C, water (3 mL) was added. The precipitated inorganic salts were removed by filtration through Celite and washed with chloroform (50 mL). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to give compound **19** (0.46 g, 95%) as a slight

Scheme 1. Synthesis of the Target Compounds 1 and 7[a]



[a]Reagents and conditions: (a) methyl L-prolinate hydrochloride, K_2CO_3, DMF, reflux; (b) KOH aqueous, MeOH, reflux; (c) nBuLi, THF, TMEDA, −78 °C; (d) MeOH, $NaBH_4$, −40 °C to room temperature; and (e) Et_3SiH, CF_3COOH, room temperature.

Scheme 2. Synthesis of Compounds 17−20[a]



[a]Reagents and conditions: (a) L- or D-prolinamide, K_2CO_3, DMF, reflux; (b) CuI, DMGC, Cs_2CO_3, 1,4-dioxane, reflux; and (c) $LiAlH_4$, THF, reflux.

yellow powder. mp: 167−169 °C. $[\alpha]_D^{20}$ −35 (*c* 1.0 in $CHCl_3$). ee: more than 99% (flow rate of 1.0 mL/min for 85:15 *n*-hexane/*i*-propanol and 0.1% triethylamine, at 570−620 psi and a 254 nm UV detector, with t_R = 39.12 min). HRMS (ESI) calcd for $C_{24}H_{29}N_2O_4$ $(M + H)^+$ 409.2122, found 409.2115.

*(R)-2,3,6,7-Tetramethoxy-10,10a,11,12,13,15-hexahydro-9H-phenanthro[9,10-e]pyrrolo[1,2-a][1,4]diazepine (**20**).* The analogue procedure for the preparation of compound **19** was used. The compound **18** (0.50 g, 1 mmol) gave compound **20** (0.47 g, 97%) as a slight yellow powder. mp: 179−181 °C. $[\alpha]_D^{20}$ 47 (*c* 1.0 in $CHCl_3$). ee: more than 99% (flow rate of 1.0 mL/min for 85:15 *n*-hexane/*i*-propanol and 0.1% triethylamine, at 570−620 psi and a 254 nm UV detector, with t_R = 27.70 min). Other data are the same as those of compound **19**.

Antiviral Biological Assay. The anti-TMV activity of the synthesized compounds was tested using our previously reported method.[27]

Antiviral Activity of Compounds against TMV in Vitro. Fresh leaf of the 5−6 growth stage of tobacco (*Nicotiana tabacum* var. Xanthi nc) inoculated by the juice-leaf rubbing method (concentration of TMV is 5.88×10^{-2} μg mL^{-1}) was cut into halves along the main vein. The halves were immersed into the solution of 500 μg mL^{-1} of the compounds and double-distilled water for 20 min and then cultured at 25 °C for 72 h. Each compound was replicated at least 3 times.

Protective Effect of Compounds against TMV in Vivo. The compound solution was smeared on the left side, and the solvent served as a control on the right side for growing *N. tabacum* var. Xanthi nc leaves of the same ages. The leaves were then inoculated with the virus after 12 h. A brush was dipped in TMV of 6×10^{-3} mg mL^{-1} to inoculate the leaves, which were previously scattered with silicon carbide. The leaves were then washed with water and rubbed softly along the nervature once or twice. The local lesion numbers appearing 3−4 days after inoculation were counted. There are three replicates for each compound.

Inactivation Effect of Compounds against TMV in Vivo. The virus was inhibited by mixing with the compound solution at the same volume for 30 min. The mixture was then inoculated on the left side of the leaves of *N. tabacum* var. Xanthi nc, whereas the right side of the leaves was inoculated with the mixture of solvent and the virus for control. The local lesion numbers were recorded 3−4 days after inoculation. There are three replicates for each compound.

Curative Effect of Compounds against TMV in Vivo. Growing leaves of *N. tabacum* var. Xanthi nc of the same ages were selected. TMV (concentration of 6.0×10^{-3} mg mL^{-1}) was dipped and inoculated on the whole leaves. Then, the leaves were washed with water and dried. The compound solution was smeared on the left side, and the solvent was smeared on the right side for control. The local lesion numbers were then counted and recorded 3−4 days after inoculation. There are three replicates for each compound.

The *in vitro* and *in vivo* inhibition rates of the compound were then calculated according to the following formula ("av" means average, and controls were not treated with compound):

$$\text{inhibition rate (\%)} = [(\text{av local lesion number of control} - \text{av local lesion number of drug-treated}) / \text{av local lesion number of control}] \times 100\%$$

dx.doi.org/10.1021/jf303550a | *J. Agric. Food Chem.* 2012, 60, 10212−10219

Table 1. *In Vitro* Antiviral Activity against TMV

compound	concentration (μg mL^{-1})	inhibition rate (%)	compound	concentration (μg mL^{-1})	inhibition rate (%)
3	500	60.2	**14**	500	15.3
	100	30.5		100	0.0
4	500	52.8	**15**	500	38.4
	100	27.4		100	15.3
5	500	71.2	**16**	500	29.8
	100	50.0		100	0.0
6	500	56.5	**17**	500	34.8
	100	32.9		100	4.6
7	500	62.4	**18**	500	32.2
	100	34.7		100	20.0
8	500	51.2	**19**	500	50.0
	100	23.0		100	18.5
9	500	30.0	**20**	500	40.4
	100	0.0		100	23.1
10	500	43.5	(*S*)-tylophorine	500	54.1
	100	21.6		100	36.9
11	500	45.2	(*R*)-tylophorine	500	49.7
	100	29.6		100	21.1
12	500	47.7	Ribavirin	500	40.0
	100	33.1		100	12.5
13	500	48.1	Ningnanmycin	500	71.4
	100	30.9		100	30.4

RESULTS AND DISCUSSION

Chemistry. Compounds **2**–**6** and **8**–**16** were prepared using our previously reported methodology.[32,33]

*Synthesis of the Target Compounds **7** and **1**.* The synthetic route of enantiopure (*S*)-tylophorine (**1**) was illustrated in Scheme 1. The dibromide **21**[32] was coupled with methyl L-prolinate hydrochloride[33] to give ester **22** in 93% yield. After hydrolysis, the ester **22** was converted to acid **23** in 84% yield. As a key step, the intramolecular cyclization reaction that employs aryllithiums generated by lithium–halogen exchange, known as the Parham cyclization reaction,[34−36] was successfully used to assemble the indolizidine ring in our procedure, and the unstable ketone intermediate was directly and stereoselectively reduced to the alcohol **7** to avoid decomposition and racemization. The absolute configuration of compound **7** was determined by comparison to authentic sample DCB-3503.[32] The alcohol **7** was reduced using triethylsilane and trifluoroacetic acid to give (*S*)-tylophorine (**1**) in 94% yield and 97% ee.

*Synthesis of the Target Compounds **17**–**20**.* To explore the effect of the D ring of phenanthroindolizidine alkaloids on biological activity, diazepines **19** and **20** were prepared. As shown in Scheme 2, the dibromide **21** was coupled with L- or D-prolinamide[37] to give the amides **24** and **25** in excellent yield. CuI-catalyzed intramolecular C–N cross-coupling, known as Ullmann–Goldberg condensation,[38−40] was successfully used to assemble the diazepine ring as a key step. Diazepinones **17** and **18** upon reduction afforded diazepines **19** and **20** in excellent yields with more than 99% ee.

Antiviral Activity. The anti-TMV activity of compounds **3**–**20** was tested. The commercial plant virucides Ningnanmycin and Ribavirin and our previously reported (*S*)- and (*R*)-tylophorine[27] were used as the controls.

Phytotoxic Activity. The phenanthroindolizidines and their analogues **3**–**20** were first tested for their phytotoxic activity against the test plant, and the results indicated that these natural-product-based compounds showed no phytotoxic activity at 500 μg mL^{-1}.

In Vitro Anti-TMV Activity. The *in vitro* antiviral assay results of all of the synthesized compounds were listed in Table 1. As the control, Ribavirin exhibited a 40.0% inhibitory effect at 500 μg mL^{-1}, whereas most of the synthesized compounds exhibited higher antiviral activity than that of Ribavirin. The commercial plant virucide Ningnanmycin, perhaps the most successful registered antiplant viral agent, exhibited 71.4% inhibitory effect at 500 μg mL^{-1} and 30.4% inhibitory effect at 100 μg mL^{-1}. The optimum compound **5** exhibited nearly equal inhibitory effect to Ningnanmycin at 500 μg mL^{-1}. When the concentration was lowered to 100 μg mL^{-1}, compounds **3**–**7** and **11**–**13** displayed nearly equal or higher inhibitory effect than that of Ningnanmycin, especially for compound **5** with 50% inhibition rate emerging as a new lead. In comparison to our previously reported (*S*)- and (*R*)-tylophorine (**1** and **2**), the (*S*)- and (*R*)-antofine (**3** and **4**) displayed nearly equal inhibitory effect, which indicated that the removal of the methoxyl at the 7 position is tolerant. However, the removal of methoxyl at the 2 position significantly increased the activity (inhibition rate: **5** > **1**; **6** > **2**). The bioassay results also showed that the 13a*S* configuration is the preferred antiviral configuration for phenanthroindolizidine alkaloids (inhibition rate: **1** > **2**; **3** > **4**; **5** > **6**).

Introduction of a hydroxyl at the 14 position of phenanthroindolizidines preserved or decreased the *in vitro* antiviral activity (inhibition rate: **7** ≈ **1**; **8** ≈ **2**; **9** < **3**; **10** < **4**; **11** < **5**; **12** ≈ **6**). Introduction of a carbonyl group at the 9 position of (*S*)-tylophorine slightly decreased the antiviral activity (inhibition rate: **13** < **1**). A rigid indolizidine ring led to a dramatic decrease of the inhibition rate (inhibition rate: **14** < **13**). Opening the D ring or inserting an amino group into the D ring decreased the antiviral activity (inhibition rate: **15**–**17** and **19** < **1**; **18** and **20** < **2**).

As shown in Table 1, most of the compounds displayed good *in vitro* antiviral activity against TMV. Therefore, these

compounds were bioassayed further to investigate their antiviral activity *in vivo*.

In Vivo Anti-TMV Activity. As shown in Table 2, most of the compounds also displayed higher *in vivo* activity than that of the control Ribavirin. (*S*)-Deoxytylophorinine (**5**) showed the best *in vivo* anti-TMV activity (inactivation activity, 59.8%/500 μg mL^{-1} and 40.3%/100 μg mL^{-1}; curative activity, 65.1%/500 μg mL^{-1} and 43.7%/100 μg mL^{-1}; and protection activity, 70.2%/500 μg mL^{-1} and 51.3%/100 μg mL^{-1}), which is significantly higher than that of Ningnanmycin (inactivation activity, 68.5%/500 μg mL^{-1} and 37.7%/100 μg mL^{-1}; curative activity, 56%/500 μg mL^{-1} and 18.9%/100 μg mL^{-1}; and protection activity, 66.6%/500 μg mL^{-1} and 25.2%/100 μg mL^{-1}).

Table 2. *In Vivo* Antiviral Activity against TMV

compound	concentration (μg mL^{-1})	inactivation effect (%)	curative effect (%)	protection effect (%)
3	500	51.1	55.5	56.4
	100	37.2	26.7	28.4
4	500	42.7	46.0	45.5
	100	15.3	19.4	20.4
5	500	59.8	65.1	70.2
	100	40.3	43.7	51.3
6	500	48.9	44.7	60.5
	100	23.6	24.5	31.7
7	500	57.8	61.9	63.7
	100	32.9	30.6	20.0
8	500	49.2	43.4	53.9
	100	26.7	32.8	26.5
9	500	24.8	19.3	21.2
	100	0.0	0.0	0.0
10	500	38.5	34.9	39.6
	100	14.2	10.2	7.3
11	500	32.4	40.0	41.3
	100	0.0	17.2	24.5
12	500	51.2	56.2	54.4
	100	33.3	27.8	30.2
13	500	37.0	30.1	43.5
	100	20.5	14.8	23.4
14	500	22.6	17.5	21.5
	100	8.5	0.0	5.3
15	500	31.3	30.1	43.3
	100	9.6	6.2	18.9
16	500	25.2	25.8	39.6
	100	0.0	0.0	13.1
17	500	32.9	31.5	29.7
	100	22.2	0.0	7.6
18	500	34.9	35.7	36.4
	100	21.2	18.9	19.7
19	500	42.5	44.6	53.6
	100	20.3	22.7	28.5
20	500	31.5	44.6	46.5
	100	12.6	28.7	33.7
(*S*)-tylophorine	500	50.5	53.8	56.8
	100	34.2	37.0	28.6
(*R*)-tylophorine	500	39	44.2	47.6
	100	15.1	26.4	22.5
Ribavirin	500	33.8	36.7	38.9
	100	9.8	8.2	7.5
Ningnanmycin	500	68.5	56.0	66.6
	100	37.7	18.9	25.2

Just as the antiviral activity *in vitro*, (*S*)-phenanthroindolizidine alkaloids **3** and **5** showed enhancement *in vivo* anti-TMV activity compared to their corresponding enantiomers **4** and **6**, which indicated that the 13a*S* configuration is the preferred antiviral configuration for phenanthroindolizidine alkaloids. The phenanthrene substitution pattern greatly affects the *in vivo* anti-TMV activity of the phenanthroindolizidines. Removal of the methoxyl at the 2 position significantly increased the activity (*in vivo* activity: **5** > **1**; **6** > **2**).

Introduction of a hydroxy group at the 14 position of phenanthroindolizidines greatly affects the *in vivo* anti-TMV activity. The anti-TMV activity of compound **7** was increased in comparison to (*S*)-tylophorine (**1**) at 500 μg mL^{-1} and slightly decreased at 100 μg mL^{-1}. The activity of compound **8** was significantly increased in comparison to (*R*)-tylophorine (**2**). The activity of compounds **10** and **12** were preserved in comparison to compounds **4** and **6**, respectively. The introduction of a hydroxy group at the 14 position dramatically decreased the activity levels for compounds **3** and **5** (*in vivo* activity: **3** > **9**; **5** > **11**). It ought to be mentioned that the (13a*S*,14*S*) configuration is the preferred antiviral configuration for 14-hydroxytylophorine (*in vivo* activity: **7** > **8**). In contrast, the (13a*R*,14*R*) configuration is found to be the preferred antiviral configuration for 14-hydroxyantofine (*in vivo* activity: **10** > **9**) and 14-hydroxydeoxytylophorinine (*in vivo* activity: **12** > **11**).

The rigid indolizidine ring and the introduction of a carbonyl group at the 9 position both dramatically decreased the antiviral activity (*in vivo* activity: **14** < **13** < **1**). The D-ring-opened analogues **15** and **16** also exhibited good anti-TMV activity at 500 μg mL^{-1}, although the activity is lower than that of (*S*)-tylophorine (**1**).

The new structural diazepine analogues **17**−**20** also exhibited good anti-TMV activity, which is equal to or significantly higher than that of Ribavirin, although the activity is lower than that of (*S*)-tylophorine (**1**), except for the *in vivo* protection effect (100 μg mL^{-1}) of compound **20**. The activity of compounds **19** and **20** is higher than that of compounds **17** and **18**, which indicated that the D ring of phenanthroindolizidine and the unshared electron pair on nitrogen of diazepines **19** and **20** are important for maintaining high anti-TMV activity. The *in vivo* curative and protection effects (100 μg mL^{-1}) of diazepine **20** are significantly higher than those of Ningnanmycin, which indicated that phenanthroindolizidine derivatives with a seven-membered D ring have a new structural scaffold and could have great potential for further development as selective tobacco protection agents.

In summary, a series of phenanthroindolizidines and their analogues were prepared and systematically evaluated for their antiviral activity against TMV. The bioassay results showed that most of these compounds displayed good anti-TMV activity and some of them exhibited higher antiviral activity than that of commercial Ningnanmycin. The optimal compound (*S*)-deoxytylophorinine (**5**) emerged as a potential inhibitor of the plant virus. The 13a*S* configuration was found to be the preferred antiviral configuration for these compounds. The phenanthrene substitution pattern and the introduction of a hydroxy group at the 14 position greatly affect the anti-TMV activity. *R*-Diazepine **20** exhibited a strong *in vivo* protection effect against TMV at 100 μg mL^{-1}, which indicated that the phenanthroindolizidine analogues with a seven-membered D ring have a new structural scaffold for further development as tobacco protection agents. Further studies on structural

optimization and mode of action are currently underway in our laboratories.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR, IR, and HRMS spectra of compounds **1**, **7**, **17**, **19**, and **22**−**24**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone/Fax: 0086-22-23503952. E-mail: wangqm@nankai.edu.cn.

Funding

We gratefully acknowledge assistance from the National Key Project for Basic Research (2010CB126100), the National Natural Science Foundation of China (21132003 and 21121002), the China Postdoctoral Science Foundation (2011M500519 and 2012T50207), the National Key Technology Research and Development Program (2011BAE06B02-17), and the Tianjin Natural Science Foundation (11JCZDJC20500), and we also thank China Agricultural University for supplying some of the chemical reagents and the National Key Technology Research and Development Program (2012BAK25B03-3).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Bos, L. Crop losses caused by viruses. *Crop. Prot.* **1982**, *1*, 263−282.

(2) Craeger, A. N.; Scholthof, K. B.; Citovsky, V.; Scholthof, H. B. Tobacco mosaic virus: Pioneering research for a century. *Plant Cell* **1999**, *11*, 301−308.

(3) Ritzenthaler, C. Resistance to plant viruses: Old issue, new answer. *Curr. Opin. Biotechnol.* **2005**, *16*, 118−122.

(4) Liu, L. R. *The Control of Disease and Pests of Tobacco*; Science Press: Beijing, China, 1998; p 31.

(5) Ouyang, G. P.; Cai, X. J.; Chen, Z.; Song, B. A.; Bhadury, P. S.; Yang, S.; Jin, L. H.; Xue, W.; Hu, D. Y.; Zeng, S. Synthesis and antiviral activities of pyrazole derivatives containing an oxime moiety. *J. Agric. Food Chem.* **2008**, *56*, 10160−10167.

(6) Ouyang, G. P.; Chen, Z.; Cai, X. J.; Song, B. A.; Bhadury, P. S.; Yang, S.; Jin, L. H.; Xue, W.; Hu, D. Y.; Zeng, S. Synthesis and antiviral activity of novel pyrazole derivatives containing oxime esters group. *Bioorg. Med. Chem.* **2008**, *16*, 9699−9707.

(7) Reichman, M.; Devash, Y.; Suhadolnik, R. J.; Sela, I. Human leukocyte interferon and the antiviral factor (AVF) from virus-infected plants stimulate plant tissues to produce nucleotides with antiviral activity. *Virology* **1983**, *128*, 240−244.

(8) Chen, M. H.; Chen, Z.; Song, B. A.; Bhadury, P. S.; Yang, S.; Cai, X. J.; Hu, D. Y.; Xue, W.; Zeng, S. Synthesis and antiviral activities of chiral thiourea derivatives containing an α-aminophosphonate moiety. *J. Agric. Food Chem.* **2009**, *57*, 1383−1388.

(9) Zhou, J.; Fan, H. T.; Song, B. A.; Jin, L. H.; Bhadury, P. S.; Hu, D. Y.; Yang, S. Synthesis and antiviral activities of α-aminophosphonate derivatives containing a pyridazine moiety. *Phosphorus Sulfur Silicon Relat. Elem.* **2011**, *186* (1), 81−87.

(10) Li, Y. M.; Zhang, Z. K.; Jia, Y. T.; Shen, Y. M.; He, H. P.; Fang, R. X.; Chen, X. Y.; Hao, X. J. 3-Acetonyl-3-hydroxyoxindole: A new inducer of systemic acquired resistance in plants. *Plant Biotechnol. J.* **2008**, *6*, 301−308.

(11) Sidwell, R. W.; Huffman, J. H.; Khare, G. P.; Allen, L. B.; Witkowski, J. T.; Robins, R. K. Broad-spectrum antiviral activity of virazole: 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* **1972**, *177*, 705−706.

(12) Bachrach, U. Antiviral activity of oxidized polyamines. *Amino Acids* **2007**, *33*, 267−272.

(13) Xue, W.; Song, B. A.; Wang, H.; He, W.; Yang, S.; Jin, L. H.; Hu, D. Y.; Liu, G.; Lu, P. Synthesis and anti-TMV activity of 2-[5-(3,4,5-trimethoxyphenyl)-1,3,4-thiadiazole-2-ylthiomethyl]-1-(2,3,4-trimethoxy)phenyl ketoxime ether derivatives. *Chin. J. Org. Chem.* **2006**, *26*, 702−706.

(14) Yuan, D. K.; Zhang, D. Q.; Li, R. X.; Wang, D. Q.; Yang, X. L. Synthesis and anti-TMV activity of novel *N*-(pyrimidin-5-yl)-*N*′-phenylureas. *Chin. Chem. Lett.* **2011**, *22*, 18−20.

(15) Wu, Z. J.; Ouyang, M. A.; Wang, C. Z.; Zhang, Z. K.; Shen, J. G. Anti-tobacco mosaic virus (TMV) triterpenoid saponins from the leaves of *IIex oblonga*. *J. Agric. Food Chem.* **2007**, *55*, 1712−1717.

(16) Ouyang, M. A.; Wein, Y. S.; Zhang, Z. K.; Kuo, Y. H. Inhibitory activity against tobacco mosaic virus (TMV) replication of pinoresinol and syringaresinol lignans and their glycosides from the root of *Rhus javanica* var. *roxburghiana*. *J. Agric. Food Chem.* **2007**, *55*, 6460−6465.

(17) Ge, Y. H.; Liu, K. X.; Zhang, J. X.; Mu, S. Z.; Hao, X. J. The limonoids and their antitobacco mosaic virus (TMV) activities from *Munronia unifoliolata* Oliv. *J. Agric. Food Chem.* **2012**, *60*, 4289−4295.

(18) Hansen, A. J. Antiviral chemicals for plant disease control. *Crit. Rev. Plant Sci.* **1989**, *8*, 45−88.

(19) Qian, X. H.; Lee, P. W.; Cao, S. China: Forward to the green pesticides via a basic research program. *J. Agric. Food Chem.* **2010**, *58*, 2613−2623.

(20) Seiber, J. N. Sustainability and agricultural and food chemistry. *J. Agric. Food Chem.* **2011**, *59*, 1−21.

(21) Gellert, E. Structure and synthesis of phenanthroindolizidine alkaloids and some related compounds. In *The Alkaloids: Chemical and Biological Perspectives*; Pelletier, S. W., Ed.; Academic Press: New York, 1987; pp 55−132.

(22) Suffness, M.; Cordell, G. A. In *The Alkaloids, Chemistry and Pharmacology*; Brossi, A., Ed.; Academic Press: New York, 1985; Vol. *25*, pp 3−355.

(23) An, T. Y.; Huang, R. Q.; Yang, Z.; Zhang, D. K.; Li, G. R.; Yao, Y. C.; Gao, J. Alkaloids from *Cyanachum komarovii* with inhibitory activity against the tobacco mosaic virus. *Phytochemistry* **2001**, *58*, 1267−1269.

(24) Huang, Z. Q.; Liu, Y. X.; Fan, Z. J.; Wang, Q. M.; Li, G. R.; Yao, Y. C.; Yu, X. S.; Huang, R. Q. Antiviral activity of alkaloids from *Cynanchum komarovii*. *Fine Chem. Intermed.* **2007**, *37* (20−24), 46.

(25) Wang, Q. M.; Yao, Y. C.; Huang, R. Q.; Fan, Z. J.; Li, G. R.; Yu, X. S. Antiviral activity of antofine from *Cynanchum komarovii*. *Agrochemicals* **2007**, *46*, 425−427.

(26) Wang, Q. M.; Wang, K. L.; Huang, Z. Q.; Liu, Y. X.; Li, H.; Hu, T. S.; Jin, Z.; Fan, Z. J.; Huang, R. Q. Derivatives and salts of phenanthro indolizidine and phenanthro quinolizidine, and their application as agricultural virucides. CN 101189968A, 20080604, CAN 149:97630, 2008.

(27) Wang, K. L.; Su, B.; Wang, Z. W.; Wu, M.; Li, Z.; Hu, Y. N.; Fan, Z. J.; Mi, N.; Wang, Q. M. Synthesis and antiviral activities of phenanthroindolizidine alkaloids and their derivatives. *J. Agric. Food. Chem.* **2010**, *58* (5), 2703−2709.

(28) Cui, M. B.; Wang, K. L.; Wang, Q. M.; Huang, R. Q. Concise synthesis of benzoindolizidine derivatives and bioactivity evaluation. *Lett. Org. Chem.* **2008**, *5*, 98−102.

(29) Gao, S.; Zhang, R. Y.; Yu, Z. H.; Xi, Z. Antofine analogues can inhibit tobacco mosaic virus assembly through small-molecule−RNA interactions. *ChemBioChem* **2012**, *13*, 1622−1627.

(30) Buckley, T. F.; Rapoport, H. Amino acids as chiral educts for asymmetric products. Chirally specific syntheses of tylophorine and cryptopleurine. *J. Org. Chem.* **1983**, *48*, 4222−4232.

(31) Nordlander, J. E.; Njoroge, F. G. A short synthesis of (*S*)-(+)-tylophorine. *J. Org. Chem.* **1987**, *52*, 1627−1630.

(32) Wang, Z. W.; Li, Z.; Wang, K. L.; Wang, Q. M. Efficient and chirally specific synthesis of phenanthroindolizidine alkaloids by Parham-type cycloacylation. *Eur. J. Org. Chem.* **2010**, *2*, 292−299.

(33) Wang, Z. W.; Wang, K. L.; Cui, M. B.; Wang, Q. M. Synthesis of (*S*)-(+)-tylophorine and its seco-analogues using free radical reaction. *Sci. China, Ser. B: Chem.* **2009**, *52*, 1288−1299.
(34) Bradsher, C. K.; Hunt, D. A. Schiff bases as external and internal electrophiles in reactions of functionalized aryllithium reagents. A new route to isoindoline derivatives and 1,2,3,4-tetrahydroisoquinolines. *J. Org. Chem.* **1981**, *46*, 327−330.
(35) Collado, M. I.; Sotomayor, N.; Villa, M. J.; Lete, E. ChemInform abstract: Parham-type cyclization and nucleophilic addition-*N*-acyliminium ion cyclization sequences for the construction of the isoquinoline nucleus. *Tetrahedron Lett.* **1996**, *37*, 6193−6196.
(36) Collado, M. I.; Manteca, I.; Sotomayor, N.; Villa, M. J.; Lete, E. Ready access to isoquinoline derivatives via *N*-acyliminium ions and Parham-type cyclizations. *J. Org. Chem.* **1997**, *62*, 2080−2092.
(37) Tang, Z.; Jiang, F.; Cui, X.; Gong, L. Z.; Mi, A. Q.; Jiang, Y. Z.; Wu, Y. D. Enantioselective direct aldol reactions catalyzed by l-prolinamide derivatives. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *16*, 5755−5760.
(38) Ley, S. V.; Thomas, A. W. Modern synthetic methods for copper-mediated caryl−O caryl−N and caryl−S bond formation. *Angew. Chem., Int. Ed.* **2003**, *42*, 5400−5449.
(39) Cristau, H. J.; Cellier, P. P.; Spindler, J. F.; Taillefer, M. Highly efficient and mild copper-catalyzed *N*- and *C*-arylations with aryl bromides and iodides. *Chem.—Eur. J.* **2004**, *10*, 5607−5622.
(40) Monnier, F.; Taillefer, M. Catalytic C−C, C−N, and C−O Ullmann-type coupling reactions. *Angew. Chem., Int. Ed.* **2009**, *48*, 6954−6971.