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SYNTHESIS OF 4-AZAPODOPHYLLOTOXINS WITH ANTICANCER ACTIVITY BY MULTICOMPONENT REACTIONS (REVIEW)

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In the last 13 years, considerable advances have been made in the development of 4-azapodophyllotoxins as anticancer agents. The current review covers this research with an emphasis on the synthesis of less stereochemically complex podophyllotoxin analogs by efficient multicomponent reactions. After a short introduction to the 4-azapodophyllotoxins prepared by such reactions, the debate concerning their exact mechanisms is highlighted. This is followed by a description of 4-azapodophyllotoxin libraries and a discussion of their documented anticancer activities. The review concludes with an analysis of the proposed mode of action and possible future directions in this fascinating area of research.

Keywords: 4-azapodophyllotoxins, podophyllotoxin, antiproliferative activity, multicomponent reaction.

In 2011, Alegria and Malhotra published a general review on the syntheses and applications of azapodophyllotoxins, providing a broad overview of the area [1]. However, because the multicomponent reaction (MCR) approach appears to be the most promising and direct method of generating 4-azapodophyllotoxins with single-digit nanomolar activities, it was felt that a focused discussion specifically emphasizing the synthesis of such analogs by way of the MCRs would be helpful to the scientific community. In addition, the current review includes recent mechanistic investigations that could be useful in a further development of this approach to expand its scope and permit access to new azapodophyllotoxins. Finally, the antiproliferative activity of some of the more potent analogs that have been synthesized since 2011 will be discussed as well. It should be noted that the development of the MCR-based approach to access new 4-azapodophyllotoxins in this area of research parallels the recognition of these powerful MCR strategies in medicinal chemistry as a whole [2].

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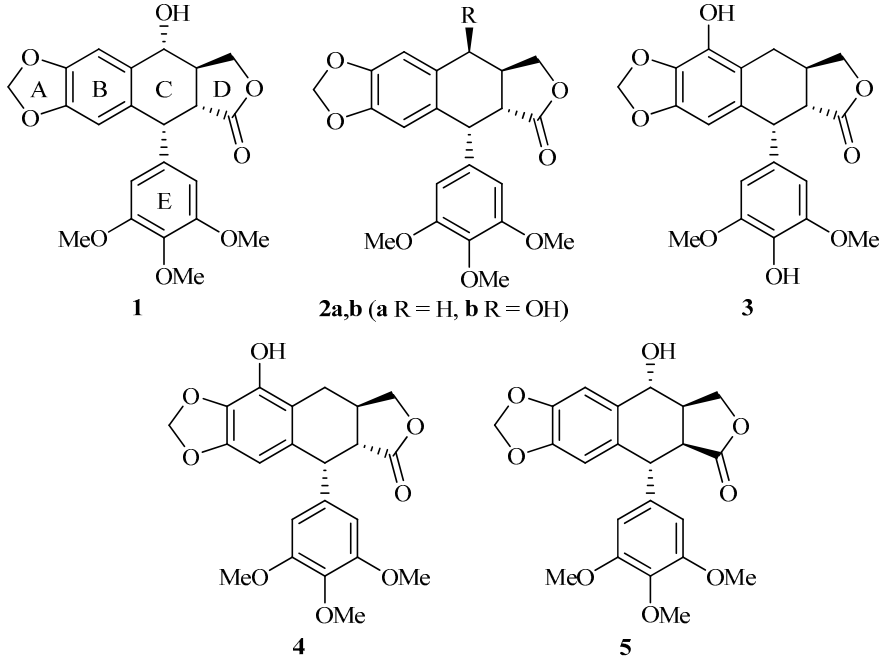
A significant proportion of the cancer drugs currently on the market are either natural products or are based on a natural product lead compound [3]. This is due to the intrinsic bio-relevance of natural molecules, and this insight has led to widespread screening of natural compounds to identify new structural leads [4]. A study by Newman and Cragg revealed that 48.6% of the compounds used for the treatment of cancer between 1981 and 2010 were either natural products, derived from a natural product by semisynthesis, or were based on a natural pharmacophore and made by total synthesis [3].

Podophyllotoxin (**1**) (Fig. 1) is a naturally-occurring cyclolignan that has been studied extensively in the last few decades as one such anticancer lead, owing to its disruption of cell mitosis through the destabilization of microtubules [5]. Podophyllotoxin was isolated from podophyllin – a resin produced following the alcohol extraction of members of the genus *Podophyllum* [4, 5]. The pharmacological value of the podophyllotoxins has been known for centuries. These compounds were used as cathartic and anthelmintic agents in ancient times, and podophyllotoxin-related lignans were used as topical treatments of the type I herpes simplex virus and measles [6]. The antiviral efficacy of podophyllotoxin (**1**) has also been demonstrated through its successful use for the treatment of venereal and perianal warts [4, 7]. Among its diverse pharmacological properties the antitumor activities have generated the most interest. It has been an effective agent in the treatment of various oncological conditions, such as Wilms' tumors, genital tumors, non-Hodgkin lymphomas, and lung cancer [4]. Podophyllotoxin (**1**) and structurally related compounds, deoxypodophyllotoxin **2a** (Fig. 1), α- and β-peltatins (**3**), (**4**), picropodophyllotoxin (**5**), among others, all inhibit cell proliferation through the destabilization of microtubules during the metaphase of the mitotic cycle. Notwithstanding highly potent antiproliferative action and the inhibition of tumor cell growth, podophyllotoxin failed clinical trials due to severe gastrointestinal side effects [3-5]. Fortunately, its failure in this clinical context led to the development of semisynthetic derivatives that have found widespread use as late stage cancer treatments in the clinic.



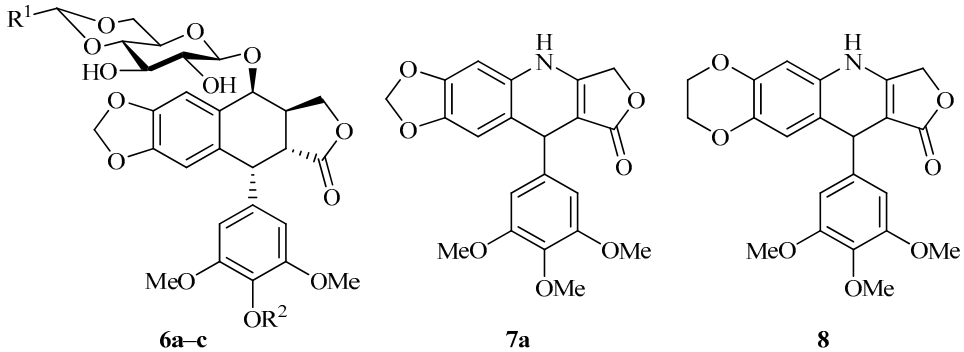
1 **2a,b** (**a** R = H, **b** R = OH) **3**

4 **5**

Fig. 1. Podophyllotoxin and related cyclolignans.

Among the latter, there are two glycoside-substituted epimers, etoposide (**6a**) and teniposide (**6b**), and the water-soluble prodrug Etopophos (**6c**) (Fig. 2). It is interesting to note that these derivatives operate through a different mechanism of action compared to that of the parent compound. Whereas podophyllotoxin destabilizes the assembly of microtubules, the semisynthetic derivatives **6a-c** inhibit tumor growth by stabilizing a cleavable complex with DNA and topoisomerase II [4].

Although effective, these drugs are still only available through semisynthetic methods, as total syntheses of the podophyllotoxin scaffold are still long and inefficient due to the presence of the four contiguous stereocenters on the C ring. For example, Norman and co-workers synthesized an analog of podophyllotoxin with a phenyl E ring in 19 steps [8]. The most recent formal synthesis of podophyllotoxin (**1**) was reported by Ishikawa and co-workers in 2013, to produce podophyllotoxin (**1**) in 10 steps [9], opening up the possibility to reengineer the natural compound with other groups in the A, B, and E rings [10]. (For another formal synthesis of podophyllotoxin (**1**), see [11].) This structural complexity, and the associated tedious synthetic approaches, has led to investigations into the necessity of the chiral centers for the biological activity, and these efforts led to the discovery of the so-called 4-azapodophyllotoxins.



6a–c **7a** **8**

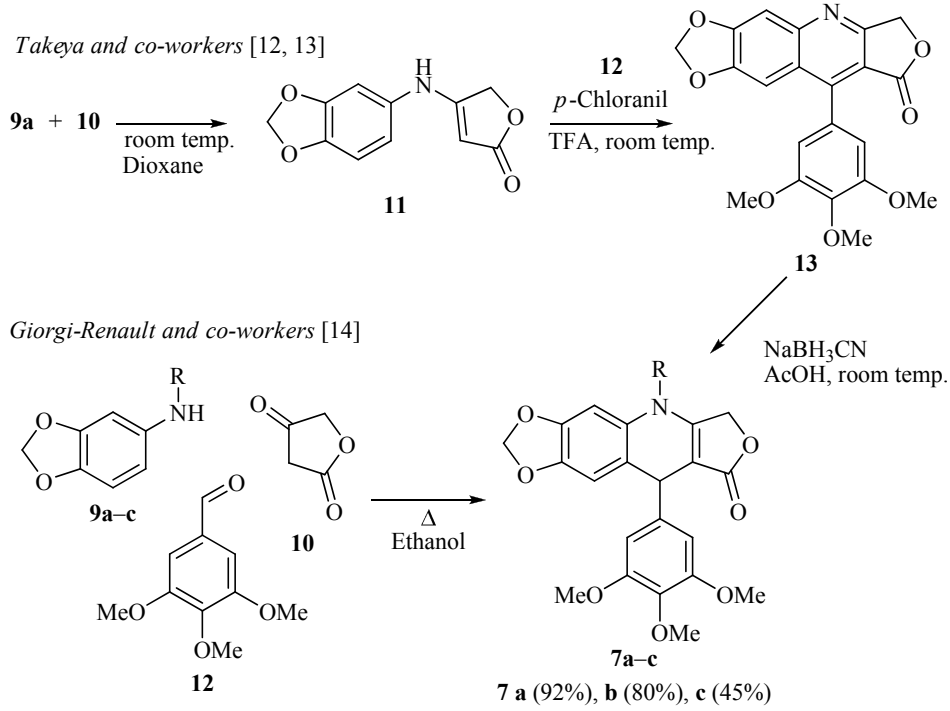
6 a R^1 = Me, R^2 = H; **b** R^1 = thiophen-2-yl, R^2 = H; **c** R^1 = Me, $R^2 = PO_3^-$

Fig. 2. Semisynthetic podophyllotoxin derivatives **6a-c**, and 4-azapodophyllotoxins **7a** and **8**.

In 2000, Takeya and co-workers reported the synthesis of two 4-azapodophyllotoxin analogs **7a** and **8** (Scheme 1, Fig. 2) which had an activity of more than twice that of the naturally occurring cyclolignan **1**, even as racemic mixtures. In addition, a number of other synthesized compounds from this group had activities comparable to podophyllotoxin (**1**) [12]. A substantial advantage of these compounds was that they were prepared in only three steps and in high yields [12, 13]. Even though these analogs were still as toxic as podophyllotoxin, the relative ease with which they could be synthesized provided an opportunity to explore different ring systems by changes in the A, B, and E rings of the podophyllotoxin scaffold (see Fig. 1 for the ring labelling of podophyllotoxins). The variation of the AB ring system was limited by the anilines that could be used in this method [14]. The first step in the synthesis involved the reaction of aniline **9a** with tetronic acid (**10**) leading to anilinolactone **11**, followed by a condensation with an appropriate aldehyde **12** in trifluoroacetic acid and with the addition of *p*-chloranil to afford the intermediate quinoline **13**. This limited the scope of this method to non-*N*-substituted anilines and required reduction with a weak reducing agent, such as sodium cyanoborohydride, to form the intended product **7a** [12, 13].

A much more straightforward synthesis of these analogs was then reported by Giorgi-Renault and co-workers in 2002 who described a one-pot MCR for the synthesis of 4-aza-2,3-didehydropodophyllotoxin analogs **7a-c** in good to excellent yields (Scheme 1) [14]. Also the reaction conditions were very simple: the corresponding aniline **9a-c**, benzaldehyde **12**, and tetronic acid (**10**) were stirred at reflux in ethanol as solvent. The MCR introduced by Giorgi-Renault and co-workers relies on the innate nucleophilicity and electrophilicity of tetronic acid (**10**) to presumably first form a Knoevenagel adduct between the benzaldehyde and tetronic acid, which then undergoes nucleophilic attack by the aniline **9a-c** to form the 4-aza-2,3-didehydro-podophyllotoxin scaffold **7** (see the debate concerning the exact mechanism later in this review). This means that, without the quinoline intermediate involved, *N*-substituted anilines can be used, and the position 4 is open for further derivatization [14]. The only limitation was that, for the success of the MCR, the aniline **9** had to have an electron-donating group in the *meta* position.

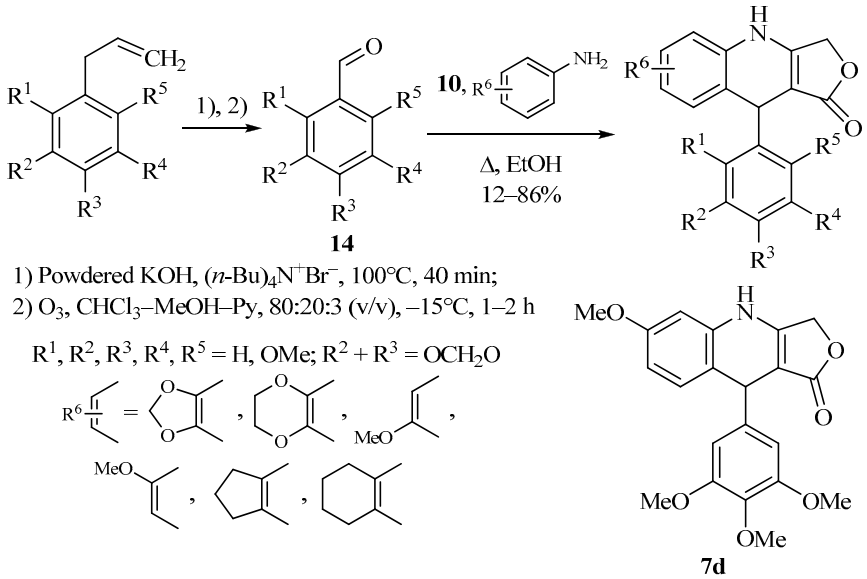
Scheme 1



7, **9 a** R = H, **b** R = Me, **c** R = $(CH_2)_3NMe_2 \cdot HCl$

A comparison of MCR [14] and stepwise synthesis [12, 13] of 4-azapodophyllotoxins

Scheme 2



Synthesis of polyalkoxy 4-azapodophyllotoxins by Semenova and co-workers [15]

Since this first report of a MCR being used to generate the 4-azapodophyllotoxin scaffold, there has been a veritable explosion in the field of synthetic podophyllotoxin analogs and an exploration of a wide variety of different substituents in the A, B, and E rings of the scaffold (see the references below). Both of the above approaches have been utilized by Semenova and co-workers who have prepared various polyalkoxy analogs using similar protocols and employing allylpolyalkoxybenzenes, isolated from parsley oil, that were

subsequently converted into the corresponding benzaldehydes **14** (Scheme 2) [15]. These aldehydes were then utilized in the MCR strategy, resulting in a set of 4-azapodophyllotoxins with significant variation in E ring substitution, as well as the modification of the A and B rings (representative examples are shown in Scheme 2 – for more examples, see [15]). Interestingly, the methylenedioxy moiety in the AB ring system appears to be of no critical importance for anticancer activity, since the 6-methoxy derivative **7d** also showed potent antiproliferative action [15, 16].

Although this review focuses on the anticancer activity of synthetic 4-azapodophyllotoxins, it is interesting to note that, similar to the natural podophyllotoxin (**1**), these scaffolds do have other applications as well [17, 18]. For example, a 2009 study by Frackenpohl and co-workers described an investigation of insecticidal activities of a library of MCR products against the mustard beetle (*Phaedon cochleariae*) and the fall armyworm (*Spodoptera frugiperda*) [17].

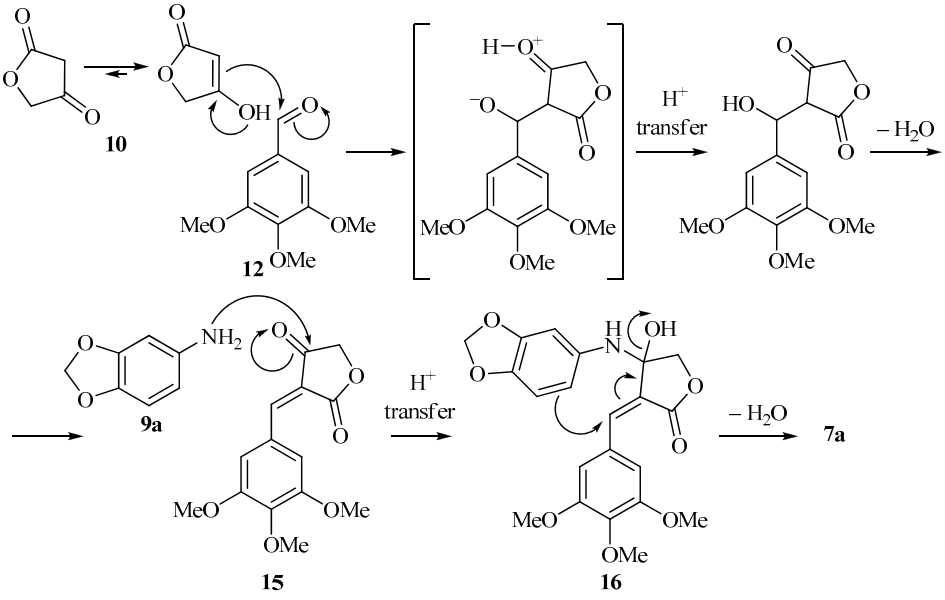
MECHANISTIC INVESTIGATIONS

In their initial report, Giorgi-Renault and co-workers proposed a mechanism for the MCR resulting in compound **7a** containing a dihydropyridine scaffold [14]. As previously mentioned, the first step proposed was the condensation of a substituted benzaldehyde **12** with tetronic acid (**10**) to form the Knoevenagel adduct **15** (Scheme 3), followed by a nucleophilic attack of the aniline **9a** on the ketone functionality of the adduct **15** to form the proposed intermediate **16**. Through the loss of a water molecule, the product **7a** is then formed.

This proposed sequence was investigated by first doing a two-step reaction, i.e., by only adding aniline **9** after formation of the Knoevenagel adduct **15**. With the use of this two-step process, the 4-azapodophyllotoxin analog **7a** was readily obtained in 85% yield. Following the success of this approach, the authors added all three components in one pot, which gave compound **7a** in a yield of 92%. It is interesting to observe that even *N*-substituted anilines were tolerated; for example, the MCR, when using *N*-methylaniline, gave a yield of 80%.

Another group, Tu and co-workers, proposed an alternative mechanism in which aniline first reacts with aromatic aldehyde. The resulting imine **17** then reacts with tetronic acid (**10**) to form the Knoevenagel adduct which, in turn, eliminates aniline (Scheme 4) [19]. Aniline then adds to the elimination product **18**, and the amino group attacks the ketone functionality in compound **19**. After a proton transfer, a cyclic intermediate is formed. The loss of water then leads to the formation of a 4-azapodophyllotoxin derivative.

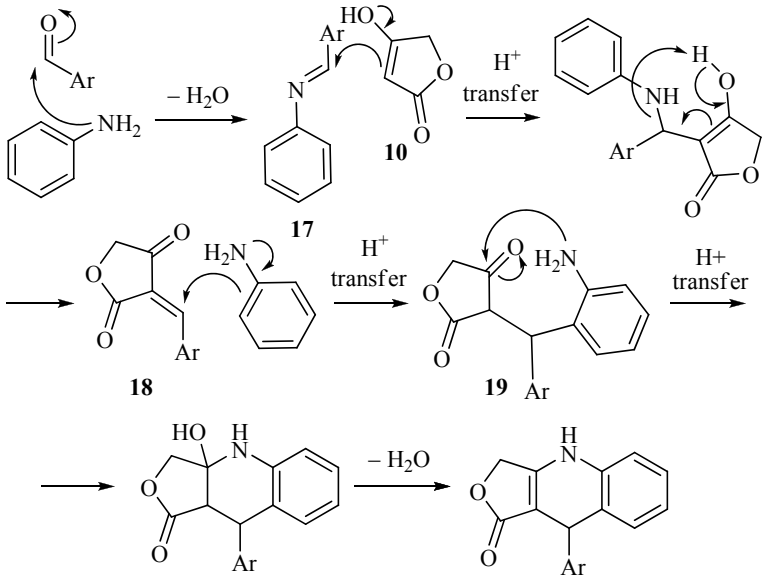
Scheme 3



Mechanism suggested by Giorgi-Renault and co-workers [14]

The mechanism discussed above differs from that proposed by Giorgi-Renault and co-workers in how aniline reacts with the Knoevenagel adduct. Instead of a nucleophilic attack by the lone pair of the nitrogen atom of the aniline, a Hantzsch-type addition [20, 21] of the aniline to the Knoevenagel adduct is suggested. This mechanism is again discussed in the later work of Tu and co-workers [22-24], where the nucleophilic attack occurs by way of the *ortho*-carbon atom of the aniline ring. It should be noted that this mechanism has also been proposed by the group of Shi and co-workers [25].

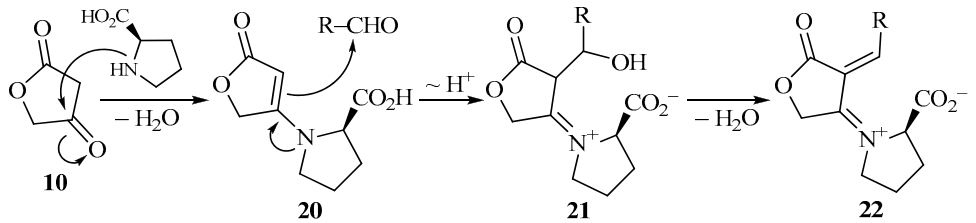
Scheme 4



Mechanism proposed by Tu and co-workers [19, 22-24] and Shi and co-workers [25]

Of interest is that Shi and co-workers have employed a modified MCR by using a catalyst such as L-proline to afford products in greater yields and with less limitation on the substrates [26]. With L-proline acting as catalyst, the mechanism proposed involves the enamine **20** formation through the reaction of tetronic acid (**10**) with L-proline (Scheme 5). Compound **20** then reacts with aldehyde to generate intermediate **21** which, through the loss of water, forms the iminium Knoevenagel adduct **22**. Using catalyst loadings of 10 mol% L-proline, Tu and co-workers [23] showed that the requirement of the presence of an electron donor in the *meta* position of the aniline, initially identified by Giorgi-Renault and co-workers [14], no longer applied. For example, the use of 4-methylaniline in this modified MCR gave a 4-azapodophyllotoxin analog in 93% yield.

Scheme 5



Iminium Knoevenagel adduct formation as suggested by Tu and co-workers [24]

With the successful application of L-proline as catalyst, a more recent study by Shi and co-workers then explored the use of five other organocatalysts (Fig. 3), along with L-proline, for use in similar MCRs to synthesize compound **23** (Fig. 3) [25]. None of these additions, however, proved to be as efficient as L-proline.

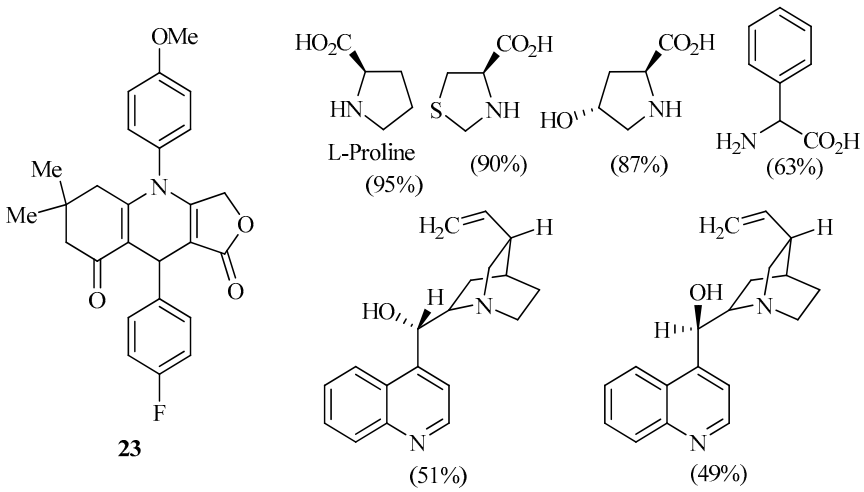
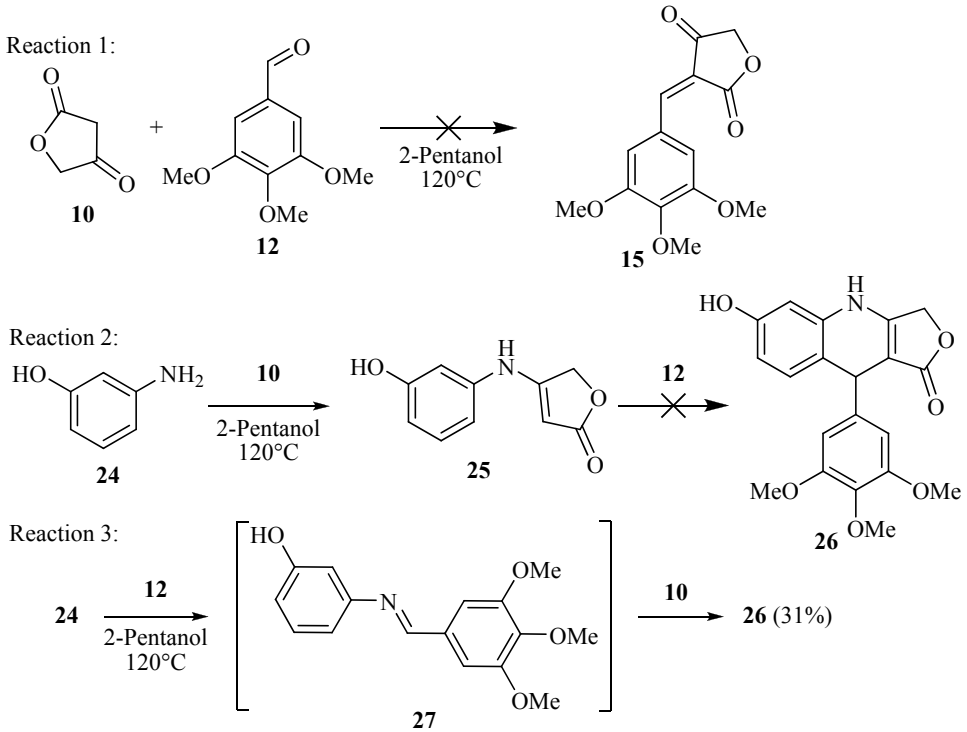


Fig. 3. Organocatalysts used in the synthesis of compound **23** by Shi and co-workers with the corresponding yields of the MCRs indicated [25].

However, these mechanisms were still just conjecture, and the research groups focused more on the activity of the compounds produced than on investigations into the reaction mechanism. Therefore, after a report by Bayer CropScience [17], in which the high yields for MCRs reported by Giorgi-Renault and co-workers [14] were not achieved, Roche and co-workers investigated the mechanism of the MCR by performing stepwise reactions between the different components [27]. In this work, three reaction sequences were carried out, all in 2-pentanol at 120°C. The study began with the investigation of the reaction of tetronic acid (**10**) and 3,4,5-trimethoxybenzaldehyde **12** (Reaction 1, Scheme 6). However, no Knoevenagel adduct **15** formed after an hour. This was followed by using the same reaction order as originally published by Takeya and co-workers

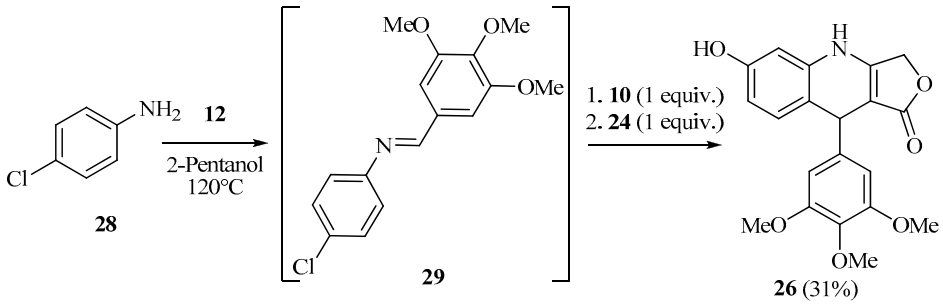
Scheme 6



Studies on the mechanism of MCRs by Roche and co-workers [27]

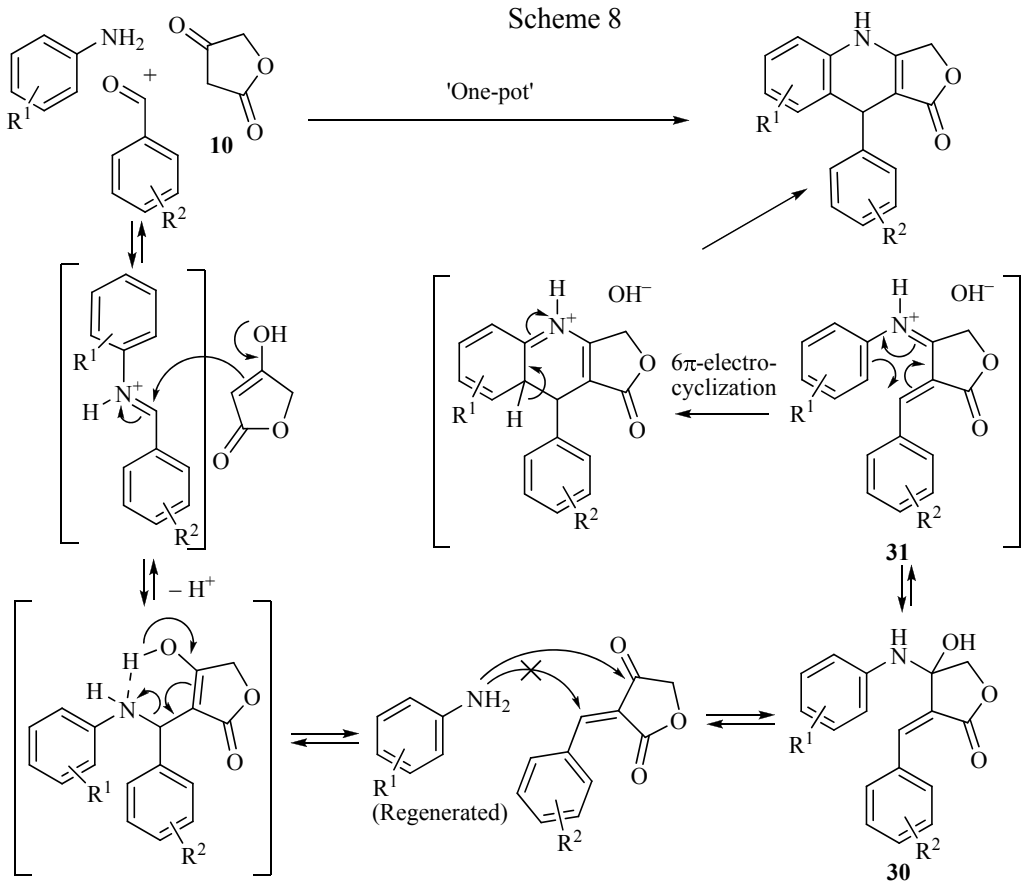
[12, 13]. This involved the reaction of aniline **24** and tetronic acid (**10**) to form anilolactone **25**, followed by reaction of this product with the benzaldehyde **12** (Reaction 2, Scheme 6). Rather than using trifluoroacetic acid and *p*-chloranil in this reaction as by Takeya and co-workers [12, 13], the reaction conditions were kept the same as for a one-pot MCR. Roche and co-workers observed a full conversion to anilolactone **25** after just 5 min. However, the addition of a suitable benzaldehyde **12** in the second step produced no expected product and only returned the unreacted anilolactone **25** [27]. Lastly, these researchers reacted the aniline **24** with the benzaldehyde **12** (Reaction 3, Scheme 6). The full conversion to imine **27** occurred within 5 min, and with the subsequent addition of tetronic acid (**10**), the 4-azapodophyllotoxin analog **26** was produced in 31% yield. Based on the third set of reaction conditions, the aniline **24** was thus deemed to serve a dual purpose, as elaborated below.

Scheme 7



Control reaction done by Roche and co-workers to support the dual-role hypothesis of aniline [27]

Scheme 8



Complete mechanism proposed by Roche and co-workers [27]

To further investigate the roles played by aniline in the three-component synthesis of 4-aza-podophyllotoxins, an electron-deficient aniline was used that did not fulfill the requirements set by Giorgi-Renault and co-workers [14], along with a "normal" aniline **24**. The electron-poor 4-chloroaniline (**28**) was then utilized to first form the intermediate imine **29**, followed by the sequential addition of tetronic acid **10** and aniline **24** (Scheme 7) [27]. It is important to note that after completion, only compound **26** was isolated in 31% yield and that none of the chlorine-containing product of the MCR was observed.

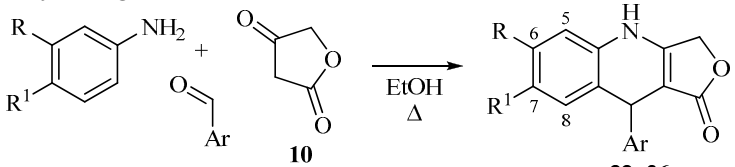
Based on these experimental results, Roche and co-workers thus proposed a modified mechanism [27], in which the aniline first serves as a catalyst to form the Knoevenagel adduct, *via* the formation of an imine from the benzaldehyde and aniline, followed by reaction with the tetronic acid (**10**) (Scheme 8). The initial aniline is then regenerated and reacts with the Knoevenagel adduct to form the product.

In further support of the proposed mechanism, the achiral intermediate **31** (obtained from the hemiaminal **30**) explains why the products are always observed as racemates. The intermediate **31** also explains why no enantiomeric enrichment was observed in the MCR products when a chiral ligand (L-proline) was used [23]. In summary, the work by Roche and co-workers gives a good indication of the importance of the aniline reactant in the discussed reaction, but it is clear that many nuances of this MCR are still poorly understood [27]. Further work is thus required before the mechanistic understanding of this important reaction can be employed to expand its scope and allow modification of the reaction conditions to prepare currently inaccessible 4-azapodophyllotoxins, perhaps even enantiomerically enriched ones.

SYNTHESES OF AZAPODOPHYLLOTOXIN LIBRARIES

Due to the simplicity of MCRs, a number of 4-azapodophyllotoxin analog libraries have been synthesized. The earlier projects tried to mimic the podophyllotoxin backbone, thus keeping the A, B, D, and E rings as close to the natural lignan as possible, for example, in the synthesis of 4-azadidehydropodophyllotoxin

TABLE 1. Synthesis of 4-Azapodophyllotoxin Analogs **32-36** Using MCR by Giorgi-Renault and Co-workers [14]



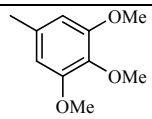
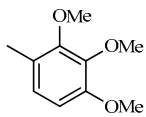
Product		Ar	Yield, %
32			59
33			63
34			94
35			78
36			83

TABLE 2. The Effect of Solvent on the Synthesis of Compound **37**

Entry	Solvent	Time, min	Yield, %
1	Ethylene glycol	12	86
2	Water	12	85
3	Acetic acid	15	71
4	Dimethyl formamide	16	63
5	Ethanol	18	60

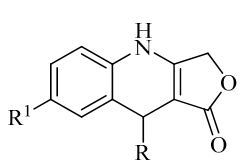
7a (Scheme 1) [12, 13, 14]. Giorgi-Renault and co-workers also reported the synthesis of libraries of compounds that had variations in the A and E rings, as well as substitutions on the nitrogen in the C ring (Table 1) [14]. These authors modified the A ring (product **32**) by annealing a benzene ring to positions 7 and 8, using 2-naphthylamine as the aniline reactant, as well as by leaving out the ring altogether (products **33**, **34**). The E ring was also modified by introducing heterocyclic (product **35**) or isomeric trimethoxyphenyl substituents (product **36**).

Following these results, it is of interest that Tu and co-workers published a procedure on MCRs using microwave (MW) heating instead of conventional heating [19]. These researchers first optimized the reaction by trying various solvents at 80°C and MW power of 300 W, as shown in Table 2.

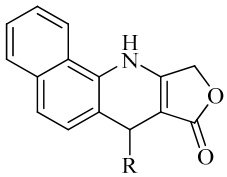
Since the use of ethylene glycol and water afforded the highest yields in the shortest times, the decision was made to use water instead of ethylene glycol for further optimization, as water is environmentally preferable. Tu and co-workers carried out the reaction in water going up to 120°C in 10°C increments [19]. As the yields levelled out at higher temperatures, 100°C was chosen as the optimal value. The authors also searched for the optimal power output. It was established that the lower power settings resulted in relatively poor yields. With the use of the optimized conditions, a library of 36 compounds was then synthesized. It is noteworthy that for this specific library, the anilines used did not have an electron-donating group in the *meta* position. Examples included α-naphthylamine, β-naphthylamine, *p*-toluidine, and aniline to vary the AB ring system. For the E ring, some variations were tried, including *para*-halo-substituted benzaldehydes (products **37**, **38, 39a**, **40a**, **41**, **42a**, **43a**), as well as a short-chain linear aldehyde (product **39c**). All of these reactions gave excellent yields in the range of 93-98%, and some of the structures are shown in Figure 4. Benzaldehyde, 3- and 4-nitrobenzaldehydes, and 4-methoxybenzaldehyde were also successfully utilized in the MW-heated MCR reactions. A few specific examples of these synthesized compounds are reported in Table 3 (the antiproliferative activity of these compounds was unfortunately not described in this work). With the use of this method, a variety of AB ring systems was then explored by Tu and co-workers, which included modifications to the D ring to generate indane-based podophyllotoxin analogs (**41**-**43**) [19]. These compounds were synthesized in excellent yields (the yields of some examples are shown in Table 3).

In 2007, Magedov, Kornienko, and co-workers investigated the use of various aminopyrazoles in MCRs with tetronic acid and 3,4,5-trimethoxybenzaldehyde to obtain the heterocyclic podophyllotoxin analogs shown in Figure 4 [28]. 5-Methylpyrazol-3-amine used for the synthesis of compound **44f** gave the highest yield, as well as the best antiproliferative activity among those tested, having an activity similar to that of podophyllotoxin (**1**) (results discussed later in this review). Based on this result, the application of different aldehydes (26 in total) was investigated, followed by testing of the antiproliferative activity of the MCR products [29]. The promising activity of some of these 4-azapodophyllotoxin analogs prompted a further investigation by Magedov, van Otterlo, Kornienko, and co-workers in 2011, which involved the use of other aldehydes, as well as an unsubstituted aminopyrazole in the MCR [30]. In this study, a total of 47 compounds was synthesized (see representative examples **45**-**48** in Figure 5), including scaffolds using indoles and other heterocycles in the AB ring system (not shown). Four of these compounds with the pyrazole moiety in the AB ring system showed an antiproliferative activity similar to that of podophyllotoxin (**1**) (see later for a more detailed description of the antiproliferative activity). The yields of these dihydropyridopyrazole compounds were in the range 54-85%. In the same study, α-naphthylamines were also used in the MCR (e.g., products **47a**-**e**, **48a**-**e**).

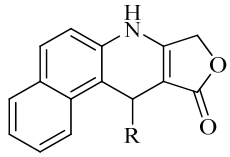
TABLE 3. Examples of Reaction Times and Yields of the Compounds Synthesized by Tu and Co-workers under MW Heating [19]



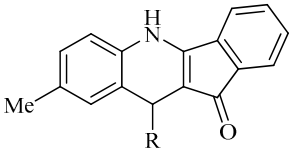
37 (R^1 = Me), **38** (R^1 = H)



39a–c



40a,b



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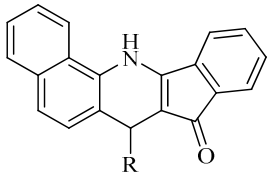
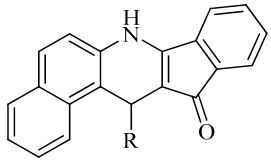
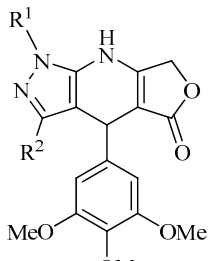


42a,b



43a,b

Compound	R	Time, min	Yield, %
37	4-BrC_6H_4	5	96
38	4-FC_6H_4	6	95
39a	4-FC_6H_4	5	95
39b	Thiophen-2-yl	6	97
39c	*n*-C_4H_9	3	94
40a	4-FC_6H_4	5	93
40b	3,4-$Cl_2C_6H_3$	3	95
41	4-FC_6H_4	7	96
42a	4-FC_6H_4	5	97
42b	Thiophen-2-yl	6	98
43a	4-FC_6H_4	7	96
43b	Ph	3	97



44a–f

	R^1	R^2	Yield, %
44a	Ph	4-BrC_6H_4	45
44b	*p*-Tol	Me	53
44c	H	4-BrC_6H_4	54
44d	*i*-Pr	Me	57
44e	Me	Me	69
44f	H	Me	80

Fig. 4. Various pyrazole-containing azapodophyllotoxin analogs synthesized by Magedov and co-workers [28] with the corresponding yields.

Although the yields of some of these MCR products shown in Figure 5 were low, the reactions were all done under conventional heating conditions and could possibly be improved by using microwave heating. It is the high anticancer activity of these compounds that makes them interesting. They exhibit antiproliferative activity similar to and greater than podophyllotoxin, yet have all been generated in a one-pot, one-step reaction (the biological data of these compounds are given later in Tables 5 and 6). The lower yields of these compounds synthesized under conventional heating can probably be overcome, as Tu and co-workers did a study to compare the differences in yield when using microwave heating instead of conventional heating [24].

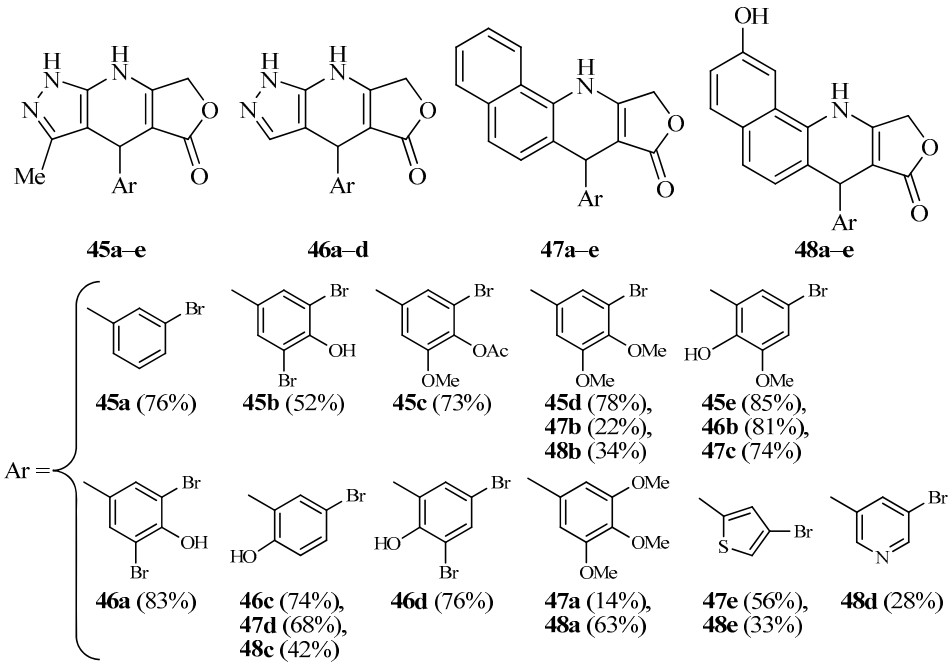
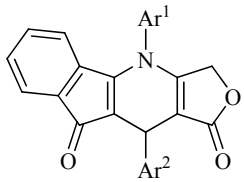


Fig. 5. Azapodophyllotoxin analogs synthesized by Magedov and co-workers found to have good antiproliferative activity [30].

They reported increases in yield by 12-19%, as well as a reduction in reaction time by up to 97% when synthesizing *N*-phenyl-4-azapodophyllotoxin analogs **49a-f** (Table 4). These results highlight the efficiency of using microwave conditions for MCRs, both with respect to high yields of the desired products obtained, and to the use of environmentally benign solvents, such as water.

Recently, the group of Ghahremanzadeh and co-workers also reported the use of ionic liquids in the synthesis of *N*-phenyl-4-azapodophyllotoxin analogs such as compound **50** (Scheme 9), which were prepared in excellent yields [31]. They demonstrated that the proposed MCRs performed in various 1-butyl-3-methylimidazolium (bmim) salt ionic liquids proceeded with great efficiency and with enhanced environmental benefits.

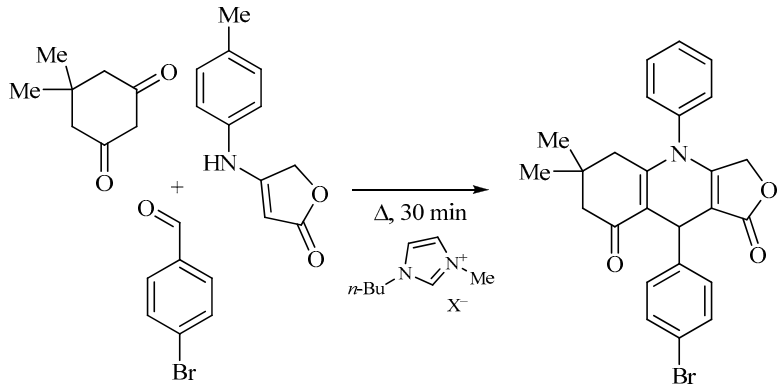
TABLE 4. Reaction Times and Yields of Synthesis of Compounds **49a-f** under MW and Conventional Heating Conditions [24]



49a–f

Com-pound	Ar^1	Ar^2	Time, min		Yield, %	
			MW	Conventional	MW	Conventional
49a	Ph	4-FC_6H_4	7	180	89	71
49b	Ph	Ph	10	270	74	61
49c	Ph	2,4-$Cl_2C_6H_3$	7	180	90	76
49d	4-MeC_6H_4	4-ClC_6H_4	10	210	88	71
49e	4-MeC_6H_4	4-BrC_6H_4	10	240	85	69
49f	4-ClC_6H_4	2,4-$Cl_2C_6H_3$	8	240	89	73

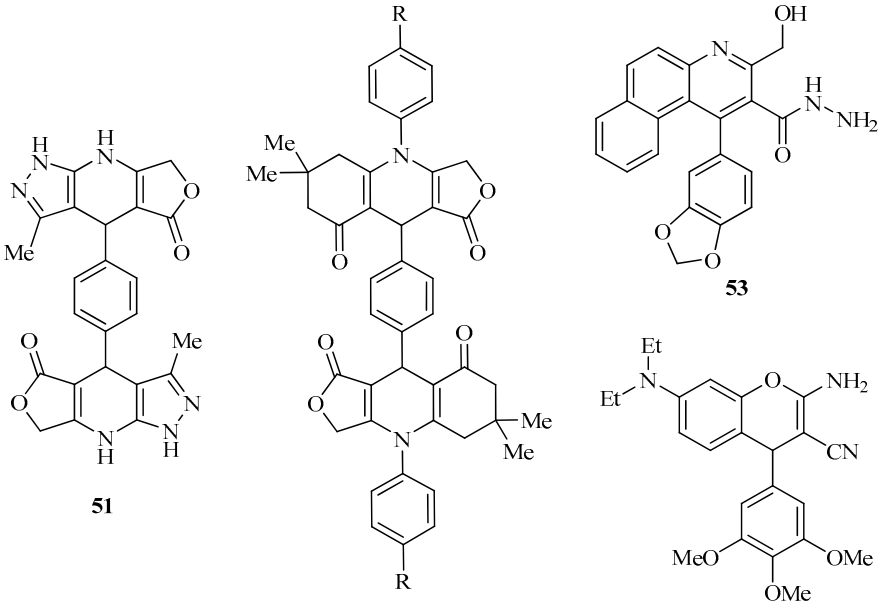
Scheme 9



X = Tf (90%), OH (75%), Br (80%), PF_6 (88%), BF_4 (83%) **50**

Synthesis of compound **50** in different ionic solvents with respective yields [31]

These yields were better (75-90%) than those achieved in organic solvents (60-75%) and were obtained in a quarter of the time. The best organic solvent, ethanol, gave a yield of 75%, while the rest produced lower yields. Following this, the use of bmim salts was investigated, and it was found that a 20 mol% loading of [bmim]Tf resulted in a 94% yield, compared to the 90% yield without the catalyst for the same reaction. Thus, the use of a catalyst did improve the yield slightly, but given the high yield of the reaction without catalyst, the use of a catalyst might only be necessary in cases where the yield is much lower. Unfortunately, compounds in this library were not tested for their ability to inhibit cell proliferation.



52 a R = Cl (85%), **b** R = H (89%), **c** R = Me (92%)

Fig. 6. Dimeric 4-azapodophyllotoxin analogs **51** [29] and **52** [32].
Two examples with modified D rings (compounds **53** and **54**).

As regards more distantly related structures, Magedov, Kornienko, and co-workers also reported the synthesis of a dimeric analog with a pyrazole moiety in the AB ring, compound **51** (Fig. 6), under conventional heating conditions and in excellent yield (92%) [29]. Tu and co-workers have synthesized dimeric *N*-phenyl-4-azapodophyllotoxin analogs **52a-c** (Fig. 6) using MW conditions [32]. The use of MW heating gave a more than twofold increase in yield of these compounds, in comparison with the conventional conditions.

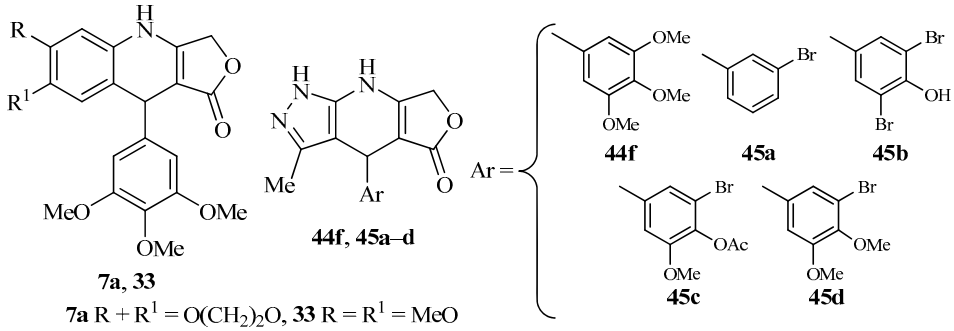
These dimeric analogs only serve to show the scope of MCRs in generating 4-azapodophyllotoxin analogs, since the antiproliferative activity of compound **51** proved to be fairly weak. It should also be noted that other variations on the 4-azapodophyllotoxin scaffold have been achieved. These examples include products where the D lactone ring has been cleaved to form a hydrazide, e.g., compound **53** (Fig. 6) [33]. These compounds were utilized for their fluorescent properties, and their ability to induce cell death in cancer cells has not been investigated. Another example of a similar elimination of the D ring was published by Shestopalov and co-workers in which compounds having a 4-oxapodophyllotoxin scaffold lacking the D ring were produced by MCR (e.g., compound **54**, Fig. 8) [34]. Of interest is that these structural variations still produced compounds with good cytotoxic activity. Even though compound **53** has not be tested as an antiproliferative agent, together with compound **54** these compounds offer new avenues for exploring different structural analogs of azapodophyllotoxins as antiproliferative agents.

ANTICANCER ACTIVITIES OF 4-AZAPODOPHYLLOTOXIN ANALOGS AND MODE OF ACTION STUDIES

As described in the previous sections of this review, libraries containing a significant number of 4-azapodophyllotoxin analogs, with multiple points of variation, have been synthesized following the advent of the MCR approach to these compounds. However, it should be noted that the early libraries focused more on the synthesis of these compounds and on improving the yields of the reactions. Therefore, many of these compounds were not tested for their antiproliferative action (or, alternatively, these results were not reported in the literature). Nonetheless, it was clear from the initial studies that many of these compounds had very similar potency to the cyclolignan podophyllotoxin (**1**), but with the substantial advantage that they could be generated in good to excellent yields by environmentally friendlier procedures [29]. This was in contrast to the large scale production of podophyllotoxin (**1**), which is still extracted from the *P. peltatum* and *P. hexandrum* species, producing only 0.25 and 4% compound **1** by dry weight, respectively [35]. Since 4-azapodophyllotoxin derivatives with good activity could be generated in one step, Magedov, Kornienko, and co-workers used the libraries of compounds synthesized in their group to generate systematic structure-activity relationship (SAR) data [10]. This group [28-30] had synthesized libraries of dihydropyridopyrazole analogs of 4-azapodophyllotoxin, as well as analogs based on α-naphthylamine. Table 5 shows some of the results involving the antiproliferative and apoptosis-inducing effect of some of the dihydropyridopyrazole analogs on the HeLa, MCF-7/AZ, and Jurkat cell lines [29]. The most active examples, compounds **33**, **44f**, and **45a-d**, are shown, and their activities are compared to those of podophyllotoxin (**1**) and 4-aza-2,3-didehydropodophyllotoxin **7a** originally synthesized by Giorgi-Renault and co-workers [14] (Table 5).

As shown in Table 5, these compounds all have apoptosis-inducing properties comparable to that of the naturally occurring cyclolignan **1**, inducing apoptosis in more than half of the Jurkat cells in the assays performed. As to the remaining cell viability, only compound **45d** (Fig. 10) had activity similar to that of podophyllotoxin (**1**), both showing almost the same values against HeLa and MCF-7/AZ cell lines (details in Table 5). Compounds **45a-d** proved to be less active than the aminopyrazole analogs without the methyl group (compounds **46a-d**), which had GI_{50} values very similar to that of podophyllotoxin (**1**) (Table 6). The naphthylamine derivatives **47** and **48**, however, showed very high potency, inhibiting HeLa and MCF-7 cells at concentrations as low as 2 nM.

TABLE 5. Antiproliferative and Apoptosis-inducing Properties of Podophyllotoxin (1) and some 4-Azapodophyllotoxin Analogs Synthesized by Magedov, Kornienko, and Co-workers [29]



7a R + R^1 = $O(CH_2)_2O$, **33** R = R^1 = MeO

Compound	Cell viability*, %			Apoptosis*[2], %
	HeLa	MCF-7/AZ	Jurkat	Jurkat
1	19 ± 5	55 ± 3	18 ± 5	54 ± 2
7a	54 ± 6	52 ± 3	54 ± 1	55 ± 4
33	53 ± 5	58 ± 4	35 ± 6	49 ± 4
44f	50 ± 2	58 ± 5	47 ± 3	55 ± 4
45a	51 ± 3	63 ± 4	68 ± 3	58 ± 2
45b	58 ± 7	60 ± 6	58 ± 3	53 ± 0
45c	47 ± 2	46 ± 2	36 ± 6	58 ± 1
45d	16 ± 3	47 ± 1	29 ± 3	56 ± 1

*Percent of remaining cell viability after 48 h treatment with compounds at a final concentration of 5 μM relative to DMSO control ±SD from two independent experiments, each performed in eight replicates. Determined by MTT assay.

*[2]Percent of apoptotic cells after 48 h treatment with compounds at a final concentration of 5 μM relative to DMSO control ±SD from two independent experiments, each performed in eight replicates. Determined by flow cytometric Annexin-V/propidium iodide assay.

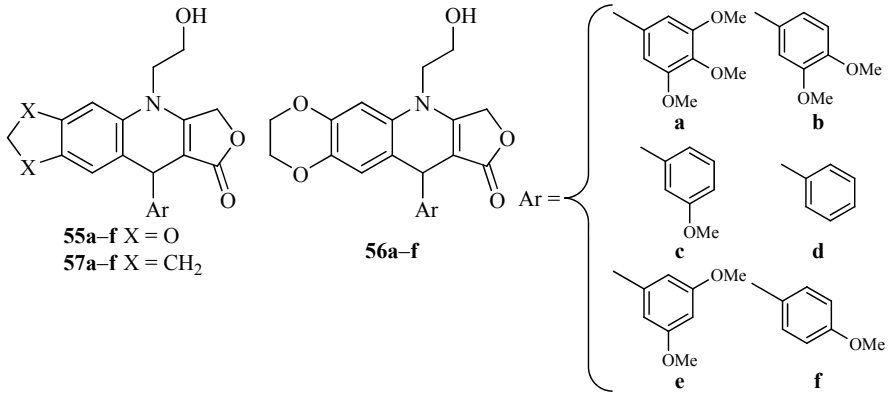
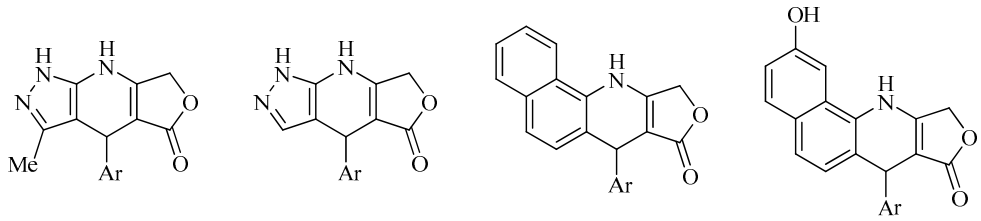


Fig. 7. Library of *N*-hydroxyethyl-4-azapodophyllotoxins synthesized by Kumar and Alegria [36, 37].

TABLE 6. GI_{50} Values of Pyrazole- and α-Naphthylamine-based 4-Azapodophyllotoxin Analogs by Magedov, Kornienko, and Co-workers [28, 29]*

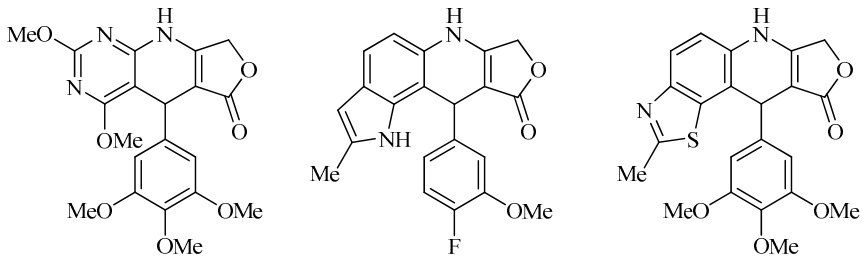


45a–e **46a–d** **47a–e** **48a–e**

Compound	GI_{50}*[2], μM		Compound	GI_{50}*[2], μM	
	HeLa	MCF-7		HeLa	MCF-7
1	0.020 ± 0.002	0.010 ± 0.003	**47a**	0.003 ± 0	0.003 ± 0
45a	3 ± 0.1	4.0 ± 0.3	**47b**	0.003 ± 0	0.003 ± 0
45b	8 ± 2	—	**47c**	0.003 ± 0	0.003 ± 0
45c	4 ± 1	—	**47d**	0.003 ± 0	0.003 ± 0
45d	0.75 ± 0.1	1.0 ± 0.1	**47e**	0.003 ± 0	0.003 ± 0
45e	0.035 ± 0.004	0.10 ± 0.04	**48a**	0.002 ± 0	0.003 ± 1
46a	0.025 ± 0.008	0.025 ± 0.003	**48b**	0.002 ± 0	0.003 ± 0
46b	0.025 ± 0.003	0.030 ± 0.005	**48c**	0.003 ± 1	0.003 ± 1
46c	0.050 ± 0.005	0.025 ± 0.004	**48d**	0.003 ± 0	0.003 ± 0
46d	0.020 ± 0.005	0.010 ± 0.003	**48e**	0.003 ± 0	0.003 ± 0

*General structures given – for substitutions of the E ring, see Fig. 5.
*[2]Concentration required to reduce the viability of cells by 50% after 48 h treatment with compounds, relative to DMSO control, ±SD from two independent experiments, each performed in eight replicates, determined by MTT assay.

Regarding other compounds tested for their antitumor activities, it should be noted here that Kumar and Alegria also synthesized a series of *N*-substituted 4-azapodophyllotoxin analogs by MCR [36]. The same group then expanded this library to 18 *N*-hydroxyethyl-4-azapodophyllotoxins in a follow-up study (compounds **55-57**, Fig. 7) and screened this library against the National Cancer Institute's 60 human tumor cell lines [36, 37]. These compounds had good activity (most compounds showing GI_{50} values in the sub-micromolar range), with compounds **56a-f** proving to be more active *in vitro* than the related scaffolds **55a-f** and **57a-f**.



58 **59** **60**

Fig. 8. Three most active compounds from the library synthesized by Kamal and co-workers [38].

Kamal and co-workers also synthesized a small library of 4-azapodophyllotoxin analogs and tested these against five human cancer cell lines (MCF-7, KB, Colo 205, A-549, and A-2780). Out of the 23 compounds contained in this library, three compounds **58**-**60** (Fig. 8) had activity comparable to podophyllotoxin (**1**) and better than etoposide **6a** against certain cell lines (data shown in Table 7) [38]. Tu and co-workers also reported the synthesis of a small library of 12 compounds with the benzothiazole moiety in the AB ring system. These compounds were similar to compound **60**, albeit with variations in the E ring by the use of different aldehydes [39]. These compounds were then screened against three carcinoma cell lines (M14, MCF-7, and SW1116) and exhibited cytotoxic activities ranging from moderate to strong.

An interesting variation to the 4-azapodophyllotoxin scaffold was also investigated by Labruère and co-workers, where various linkers, including methylene and phenylene groups, were incorporated in between the C ring and E ring, e.g., compounds **61** and **62** (Fig. 9) [40]. The goal was to design novel antivascular agents that maintained a potent activity towards tubulin, but with lower toxicity towards normal, healthy cells. These compounds proved to be fairly inactive in the destabilization of tubulin, thus showing that the linker has a deactivating effect [40]. Compound **61** did, however, show promising results as an antivascular agent.

As mentioned earlier, podophyllotoxin (**1**) is known to inhibit cell mitosis through microtubule destabilization [3]. In 2011, Magedov and co-workers investigated the mode of action of the 4-azapodophyllotoxin analogs synthesized by them to see if it was similar to that of the natural product, the latter thus being mimicked by the MCR products [30]. Using flow cytometric cell cycle analysis, it was found the pyrazole-based 4-azapodophyllotoxin analogs, like compounds **45a-e** and **46a-d** (Fig. 5), retained the microtubule-destabilizing mechanism of the natural cyclolignan **1**. The retention of the microtubule-destabilizing activity was also confirmed *in vitro* using a fluorimetry-based tubulin polymerization assay.

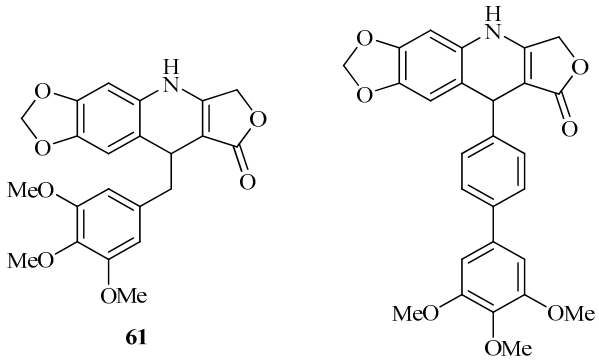


Fig. 9. 4-Azapodophyllotoxin analogs with linkers between C and E rings [40].

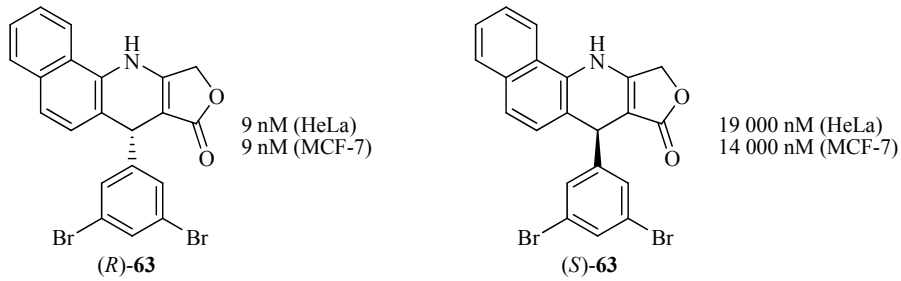


Fig. 10. Comparison of GI_{50} values of the enantiomers of compound **63** against two cell lines [30].

In the same study, the effect of the stereocenter in the C ring was also investigated. Using chiral HPLC to separate the enantiomers of compound **63** (Fig. 10), these researchers found the *R*-isomer to be four orders of magnitude more active than the *S*-isomer [30]. The bioactivity results obtained, and shown in Fig. 10, indicates

that the isomer that mimics the stereochemistry of podophyllotoxin (**1**) clearly is the more active of the two isomers. This evidence thus adds support for the hypothesis that these particular synthetic structures mimic the natural product prototype very closely, perhaps even in terms of biological interactions with the target site.

TABLE 7. Cytotoxic Activity of Compounds **6a**, **58**-**60** (GI_{50}, μM) against the Five Human Cancer Cell Lines

Com-pound	Breast MCF7	Oral KB	Colon Colo 205	Lung A-549	Ovarian A-2780
6a	2.1	0.3	0.13	3.08	1.3
58	2.4	0.17	0.16	2.1	—
59	2.6	—	—	2.1	2.4
60	—	2.1	2.5	2.4	2.3

The application of MCR has become a powerful synthetic tool in modern drug discovery, due to its atom economy and the fact that molecular diversity is readily achievable through one-step syntheses [41]. This has been demonstrated by the advances in podophyllotoxin-inspired medicinal chemistry research, with many active 4-aza-podophyllotoxin analogs being generated by way of MCRs in good to excellent yields. A fair number of research groups have thus generated libraries of new 4-azapodophyllotoxin analogs of considerable structural variety, thereby demonstrating the versatility of these reactions. The fact that these active compounds are now accessible through one-step syntheses means that more effort can now be invested in improving the selectivity and potency of these compounds, instead of focusing on linear total syntheses to generate simple podophyllotoxin analogs. Downstream medicinal chemistry issues such as ADMET properties can now also be addressed, as many of the currently active compounds are very insoluble (for instance, this could involve the synthesis of the phosphate salts of the MCR compounds, in effect mimicking the prodrug, Etopophos). Since the MCR also allows the synthesis of *N*-substituted analogs, the activity of these compounds can now be compared to etoposide and teniposide. The biggest challenge of all that still needs to be addressed is enantioselectivity, as Magedov and co-workers have shown that the *R*-isomer is significantly more active than the *S*-isomer [30]. Thus, an enantioselective MCR could possibly produce active compounds with single digit nanomolar or even sub-nanomolar activities (for a recent review on advances made in asymmetric MCRs, see [42]).

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