Historic Perspectives on Annonaceous Acetogenins from the Chemical Bench to Preclinical Trials

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- Annonaceae
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- pesticidal activity
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Abstract

Studies on the Annonaceous acetogenins began after the first cytotoxic acetogenin, uvaricin, was isolated in 1982. This attractive finding made many medicinal and natural product chemists direct their efforts on the isolation and identification of these classes of compounds. As more Annonaceous acetogenins were isolated, more information about them was uncovered. From their structural identification to the total synthesis of natural product analogues and from cell-based screening and molecular-based targeting to animal testing, the mechanisms of action of the Annonaceous acetogenins became clearer. The purpose of this review is to give an account of recent studies on this class of compounds and their analogues, which will aid us not only in clarifying how the Annonaceous acetogenins act but also in establishing principles for the further development of this class of compounds.

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Abbreviations

AGE:	Annonaceous acetogenin
APICID:	atmospheric pressure in-source collision-induced dissociation
CC:	column chromatography
CCC:	countercurrent chromatography
CD:	circular dichroism
Complex I:	NADH : ubiquinone oxidoreductase
Dansyl-NH:	5-dimethylaminonaphthalen-1-yl-sulfonamide
DIP:	direct-inlet probe
EI-MS:	electron-impact mass spectrometry
ESI-MS:	electron-spray ionisation mass spectrometry
FITC:	fluorescein isothiocyanate
H125:	lung adenocarcinoma cells
H23:	non-small cell human lung cancer cells
H8:	HPV16 subgenes-immortalised human endocervical cells
HCT-8:	intestinal adenocarcinoma cells
HSCCC:	high-speech countercurrent chromatography
HT-29:	human colon cancer cells
IC_{50}:	the half maximal inhibitory concentration
ITC:	isothermal titration calorimetry
K562:	human immortalised myelogenous leukaemia line
KB 3–1:	human epidermoid carcinoma cells
LC/MS:	liquid chromatography/mass spectrometry
LT_{50}:	median lethal time
M17/Adr:	adriamycin-resistant murine mammary cells
MCF-7/Adr:	adriamycin-resistant human mammary adenocarcinoma cells
MCF-7/wt:	nonresistant human mammary adenocarcinoma cell wild type
MDA-MB-468:	human mammary adenocarcinoma cells
MDR:	multiple-drug resistant
NADH:	nicotinamide adenine dinucleotide, reduced form
NBD-NH:	7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl-amino
NCI:	National Cancer Institute
NMA:	naphthylmethoxyacetic acid
P388:	murine lymphoblastoid cells
P-gp:	P-glycoprotein mediated pumps
PI:	propidium iodide
PO:	oral administration
PO3:	pancreatic adenocarcinoma
PS:	PS system (P-388 lymphocytic leukaemia in mice)
SAR:	structure-activity relationship

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Sf9 cell: pupal ovarian tissue of the Fall armyworm *Spodoptera frugiperda*
T/C: test/control
T24: bladder cancer cells
THF: tetrahydrofuran
THP: tetrahydropyran
TLC: thin-layer chromatography
TMS: trimethysilyl
YEM: yellow fever mosquito larvae assay

Introduction

Plants have a long history of use in the treatment of human diseases. Botanical extracts have long been regarded as a source of new and useful pharmaceuticals. According to Cragg's investigation, approximately 62% of commercially available drugs have natural product origins [1]. These natural products also play important roles as direct treatments or as templates (lead compounds) that are modified for the treatment of human diseases. The commonly known example from folkloric medicinal plants is the anticancer agent paclitaxol, a diterpene from *Taxus brevifolia* (Taxaceae) discovered by Wall and Wani in 1971 [2] that is now used for the treatment of advanced ovarian cancer. Other examples of anticancer drugs derived from natural products, such as camptothecin from *Camptotheca acuminata* (Nyssaceae) [3], podophyllotoxin from *Podophyllum peltatum* [4], vincristine and vinblastine from *Vinca rosea* [5], and adriamycin from *Streptomyces peucetius* [6], have encouraged pharmaceutical chemists to search for new drugs from medicinal plant sources.

Annonaceous acetogenins (AGEs) are a unique class of C_{35} or C_{37} secondary metabolites of Annonaceous plants derived from the polyketide pathway. Extensive studies on AGEs have indicated that these naturally occurring compounds possess a broad spectrum of bioactivity, including anticancer, antiparasitic, insecticidal and immunosuppressive effects. In 27 years, more than 500 AGEs were isolated from various parts of the plants of this family [7–10]. These bioactive AGEs became more important, particularly in pharmaceutical research [11,12]. A great interest in investigating the mechanisms of action of a series of AGEs emerged from the leaps in knowledge about the processes involved in tumour cell death. Members of this class of natural compounds are regarded as "potential" candidates for future generations of anticancer drugs. AGEs have become one of the most interesting classes of natural products at present.

Annonaceous plants are important economic crops in Asia. There is an especially abundant biomass of Annonaceous plants in Taiwan. In our studies, AGEs from Formosan Annonaceous plants showed significant cytotoxicity against ovarian cancer cells, 1A9, with more potential than paclitaxol [13]. The purpose of this review is to give a short historical introduction as well as an account of the recent studies on AGEs and their analogues. This brief outline begins with a description of the sources, isolation, chemistry and biological activities of this class of natural compounds. Achievements in the studies of AGEs are noted by their significant cytotoxicity. Recent studies on the mechanisms of action of the pesticidal and antitumour AGEs are reviewed within the individual sections. In addition, modified analogues of AGEs and AGE mimics were made to verify hypotheses regarding the modes of action of the AGEs. Historically following the studies of the AGEs from the abundant natural biomass, it will be helpful for us to not only clarify how AGEs act but also establish the principles that will guide the further development of these natural products.

Previous Studies on the AGEs before the 2000s

The isolation of uvaricin (**1**) from the roots of *Uvaria accuminata* Oliv. by Jolad et al. in 1982 and its excellent bioactivity in the PS test system initiated the studies on AGEs as a hot topic in drug discovery [14]. Following the experimental and major biochemical conceptual advances, the studies on AGEs during the early 1980s to about 2000 could be summarised in three stages, including the initial stage (before 1990), the middle stage (from 1991–1995), and modern stage (1996–2000). In the initial stage (before 1990), several groups, including McLaughlin's (Purdue, USA), Cave's (CNRS, France), Fujimoto's (Tokyo, Japan), Pettit's (Arizona, USA), and Sneden's (Virginia, USA), began efforts toward the isolation and structural identification of these types of bioactive compounds.

In the isolation procedure, AGEs were first concentrated via solvent partitioning driven by their amphiphathic properties. Methanol is usually the solvent of choice for the extraction of these AGEs from plant materials. By partitioning the crude extract with chloroform and water, the chloroform layer will become enriched with the AGEs. Chromatographic techniques, such as gradient elution column chromatography (CC), flash chromatography and preparative TLC, were generally utilised for the isolation and purification of these compounds. During this period of time the major population of AGEs was discovered. For example, the first mono-tetrahydrofuran (THF) AGE, annonacin (**2**), was isolated from *Annona densicoma* Mart [15]; the adjacent bis-THF AGE, squamocin (**3**), was isolated from *A. squamosa* L. [16]. The first nonadjacent bis-THF AGE, bullatalicin (**4**) [17], was isolated from *A. bullata* A. Rich. and was also isolated from *A. cherimolia* Mill as cherimoline (**5**) [18] (renamed by Cortes et al. as cherimolins-1 and 2 [19]). The first AGE with the saturated γ-lactone moiety, laherradurine (**6**), was isolated from *A. cherimolia* seeds, of which the structure was revised by the same group later [19, 20]. The primary structures of the AGEs were determined by ^{1}H- and ^{13}C-NMR and mass spectroscopy, in which the former method (NMR) could confirm the presence of functional group substituents (the γ-lactone ring moiety and the oxygen-bearing moieties) and the latter (MS) could determine the placement of substituents along the carbon skeleton. Rupprecht et al. published the first review on AGEs in 1990 [7], reporting the strategies for their structural elucidation and revising some structures of the published compounds. At that time, three main types of AGEs, mono-THF, adjacent bis-THF and nonadjacent bis-THF, were classified based on the presence of the THF ring moieties.

The stereochemistry of the substituents of AGEs was mainly determined by organic syntheses of the partial structures and X-ray crystallography of the AGEs. Hoye et al. (Minnesota, USA) first proposed a valuable method for the quantitative correlation of the ^{1}H-NMR chemical shift data for a series of model diastereomeric bis-tetrahydrofurans [21,22]. Pettit et al. first elucidated the relative stereochemistry of the eight chiral centres of rolliniastatin 1 (**7**), by X-ray structure determination of its 15-*O*-*p*-bromophenylurethanic derivative, as 4*S**,15*S**,16*S**,19*R**,20*R**,23*S**,24-*R**,36*R** [23]. However, the absolute configuration of rolliniastatin

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1 (**7**) should be the mirror image of the proposed structure because of its CD data [n-π^* $\Delta\varepsilon$ (235) < 0 and π-π^* $\Delta\varepsilon$ (220 nm) > 0] [24,25], which indicates a 36*S* configuration. In 1990, Born et al. proposed a systematic comparison of the ^{1}H- and ^{13}C-NMR chemical shifts of the diagnostic protons for two diastereomers, which became a popular way to confirm the *erythro* and *threo* effects on the protons of the tetrahydrofuran rings [26]. Meanwhile, Hoye et al. proposed a symmetry-assisted synthesis of the bis-THF moiety [27]. Biosynthetically, the AGEs are regarded to originate from polyhydroxy C-32 or C-34 fatty acids, to which a 2-propanol unit is added to form the methylated α,β-unsaturated γ-lactone. Hoye's proposed idea really matched the biosynthetic hypothesis for the construction of these compounds (**Fig. 1**).

Following the rapid development of chromatographic techniques in the early 1990s, studies on the AGEs in the middle stage (from 1991 to 1995) focused on the isolation and purification of various types of AGEs and the identification of the stereochemistry of AGEs by chemical methods. More laboratories worldwide became active in research related to the isolation, structural elucidation, and even total synthesis and mechanisms of anticancer action of these compounds. Our group also began studying the Formosan AGEs at this time.

Since the 1970s, our group has mainly studied the secondary metabolites from plants of the Lauraceae. Because of the close phylogenic relationships of both the Lauraceae and Annonaceae families, we started to study the secondary metabolites of the Formosan Annonaceous plants (**Table 1**). In Taiwan, there are 21 species (8 genera) of Annonaceous plants, of which three, *Fissistigama glaucescens* (Hance) Merr., *F. oldhami* (Hemsl.) Merr. and *Goniothalamus amuyon* (Blanco) Merr., are native to the Taiwan island [28,29]. Our early studies on the Formosan Annonaceous plants focused on alkaloids, which showed diverse biological functions, including cytotoxicity [30], antiplatelet aggregation activity [31], cardiovascular activity [32,33] and antimicrobial activity [34]. Afterwards, diterpenoids, styrylpyrones [12,35,36] and some linear AGEs [37], mono-THF AGEs [38,39], and adjacent bis-THF AGEs [39] were isolated from various parts of the *Annona* plants in our laboratory. Two groups in China also started related research on AGEs in China during that period [40,41], one of which – Dr. Yang's group in Yun-Nan – cooperated with us to elucidate the structures of the AGEs from *A. muricata* [42].

To improve the efficiency of the chromatography, repeated open column chromatography and high-performance liquid chromatography (HPLC) were introduced for the isolation of AGEs [43–46] such that AGEs with minor structural differences could be isolated more easily and quickly. Based on the isolations of different AGEs, their general structural features can be divided into two classes: 1) the moiety of the γ-lactone rings: the α,β-unsaturated γ-lactone ring (normal form) or the ketolactone (isoform) (see **Fig. 2**); and 2) the oxygen-bearing moieties (see **Fig. 3**) [9,47]. In addition, the structure of (−)-muricatacin (**8**), the first shortened AGE with only a terminal γ-lactone moiety and an aliphatic chain, was reported in 1991 [48].

Due to the difficulty in crystallising aliphatic AGEs, the determination of the absolute stereochemistry of AGEs became the main challenge of the research work. Hoye et al. first used spectroscopic methodologies to determine the absolute configurations of stereogenic carbinol centres in the five adjacent bis-THF AGEs and four mono-THF AGEs with the refined Mosher method in 1992 [43,44]. They also validated the configuration at the C-4 carbinol centre in these bioactive AGEs as the *R* configuration [49].

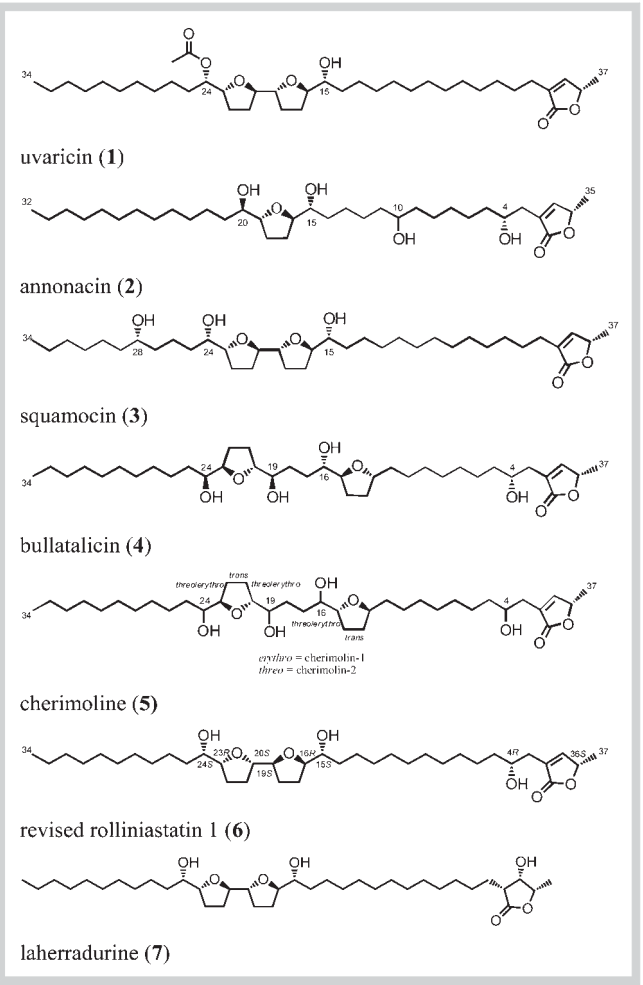

Fig. 1 Structures **1**–**7**.

Fujimoto et al. designed a series of mono-THF compounds with various conformations and reported their ^{13}C-NMR resonances, which are useful in determining the conformations of the THF ring moieties [50]. Yu et al. made a single crystal of (+)-gigantecin (**9**) from *A. coriacea* Mart for an X-ray study to determine its absolute conformation [51]. These research findings accelerated the process for the structural identification of the AGEs.

On the other hand, Duret et al. suspected that AGEs with terminal ketolactones (isoforms) were artefacts of the translactonisation of 4-hydroxy-AGEs. They further confirmed this hypothesis by performing the extraction and characterisation of the initial AGEs from fresh crude materials under the effects of alkaloids, basic media, and alcohols. These reagents affected the kinetics of the translactonisation [52], a result that was later supported by Duret et al work showing that 4-hydroxylated AGEs led to iso-AGEs under basic conditions [53].

The studies on AGEs in the modern stage (from 1996 to 2000) were concerned with the efficient identification of AGEs by hyphenated techniques and other spectroscopic methods. Although normal and reverse-phase HPLC are powerful tools for the isolation of natural products, they still have some limitations, such as the amount of sample that can be purified per unit time, the cost of the solvent, the size of the columns. Searching for new technologies to facilitate the chromatographic work is very important. Hopp et al. used countercurrent chromatography (CCC) to isolate

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Table 1 Formosan Annonaceous plants collected for investigation of their secondary metabolites

	Seed or unripe fruit	Fruit	Leaves	Stem	Bark	Root
Annona						
A. cherimola Mill.		*	*	*		
A. glabra L.		*		*		
A. artemoya (*A. cherimola X A. squamosa*)	*					
A. montana Macf.	*		*	*	*	
A. muricata L.	*		*			
A. reticulata L.	*		*			
A. squamosa L.	*		*	*		
A. purpurea L.			*	*		
Artabotrys						
A. hexaptalus (L. f.) Bhandqri						
A. uncinatus (Lam.) Merr.		*	*	*		*
Cananga						
Cananga odorata (Lam.) Hook. F. & Thomas	*		*			
Fissistigma						
F. glaucescens (Hance) Merr.#			*	*		
F. oldhami (Hemsl.) Merr.#	*		*	*		
Goniothalamus						
G. amuyon (Blanco) Merr.#			*	*		
Polyalthia						
P. longifolia Benth. et Hook. F.						
P. longifolia Benth. et Hook. F. **'pendula'**			*			
P. suberosa Hook. f.						
P. liukiuensis Hatusima#						
Rollinia						
R. mucosa Baill	*		*	*		
Uvaria						
U. rufa Bl.				*		

* Chemical components of the material have been studied in our laboratory. # Annonaceous plants that are native in Taiwan island

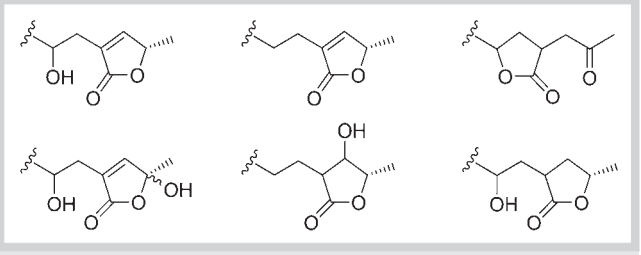

Fig. 2 γ-Lactone subunits in AGEs.

four AGEs, (2,4-*cis*- and *trans*)-9-hydroxyasimicinone (**10**), (2,4-*cis*- and *trans*)-squamoxinone B (**11**), (2,4-*cis*- and *trans*)-squamoxinone C (**12**), and isoannoreticuin (**13**), from the bark of *A. squamosa* [54]. Duret et al. also applied high-speed countercurrent chromatography (HSCCC) to the separation of AGEs from *A. atemoya* to give two major AGEs, squamocin (**3**) and bullatacin (rolliniastatin-2, **14**), and six other known AGEs [55]. Moreover, to separate mixtures of AGEs that cannot be easily purified by regular HPLC methods, some Japanese scholars and our laboratory introduced the recycle-HPLC system for isolating AGEs.

To develop a convenient spectral methodology to determine the stereochemistry of AGEs, Gawronski an Wu provided a far more reliable way of determining the absolute configuration of the γ-lactone ring moiety through the analysis of the CD spectra of butenolides [25]. Duret et al. modified the Mosher method and determined the stereochemistry of asimicin (**15**) by the long-range anisotropic effect of 2-NMA (naphthylmethoxyacetic acid), [56] (**Fig. 4**).

The other key tool for determining the structures of AGEs is mass spectrometry (MS). Generally, electron-impact mass spectrometry (EI-MS) is the preferred technique for determining the placement of the tetrahydrofuran rings and functional groups (hydroxy, ketone, acetoxy and double bond) along the hydrocarbon chain. The derivatised AGEs, such as TMS and acetyl derivatives, are helpful in the elucidation of these structures. In addition, the direct-inlet probe technique (DIP) and lower evaporator energy (e.g., 30 eV) have been suggested for use with EI-MS scanning because AGEs easily decompose thermally. The structure of squamocin (**3**) from *A. squamosa* was characterised by a combination of chemical derivatisation and precursor-ion scanning mass spectrometry. The lactone portion of squamocin (**3**) was modified with *N,N*-dimethylethylenediamine in the vapour phase to afford a strong positive charge at one end of the skeleton [57]. In 1997, Gu et al. (XenoBiotic Laboratories), cooperating with the McLaughlin group, analysed AGEs from *R. mucosa* that were amenable to liquid chromatography/mass spectrometry (LC/MS) with ionisation source-atmospheric pressure in-source collision-induced dissociation (APICID) to detect the presence of 40 known AGEs, in addition to four new AGEs of diverse structures, in a bioactive crude methanol-soluble fraction from this plant extract [58]. They also observed a unique fragmentation rule for AGEs with a hydroxy group at C-4, which had a characteristic loss of a terminal γ-lactone (112 amu) during ESI-MS scanning [58]. This

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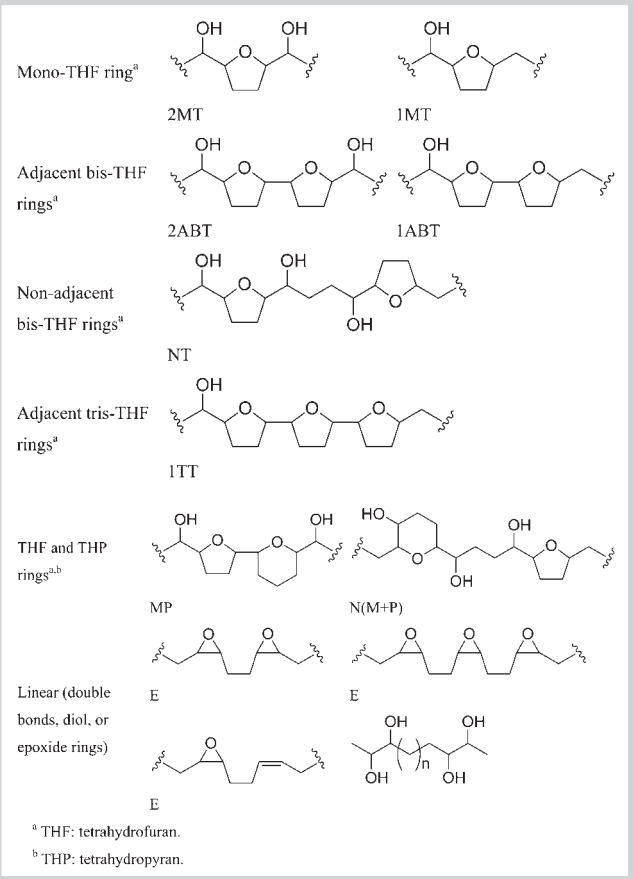

Fig. 3 Tetrahydrofuran (THF), tetrahydropyran (THP), and other oxygen-bearing subunits in Annonaceous AGEs.

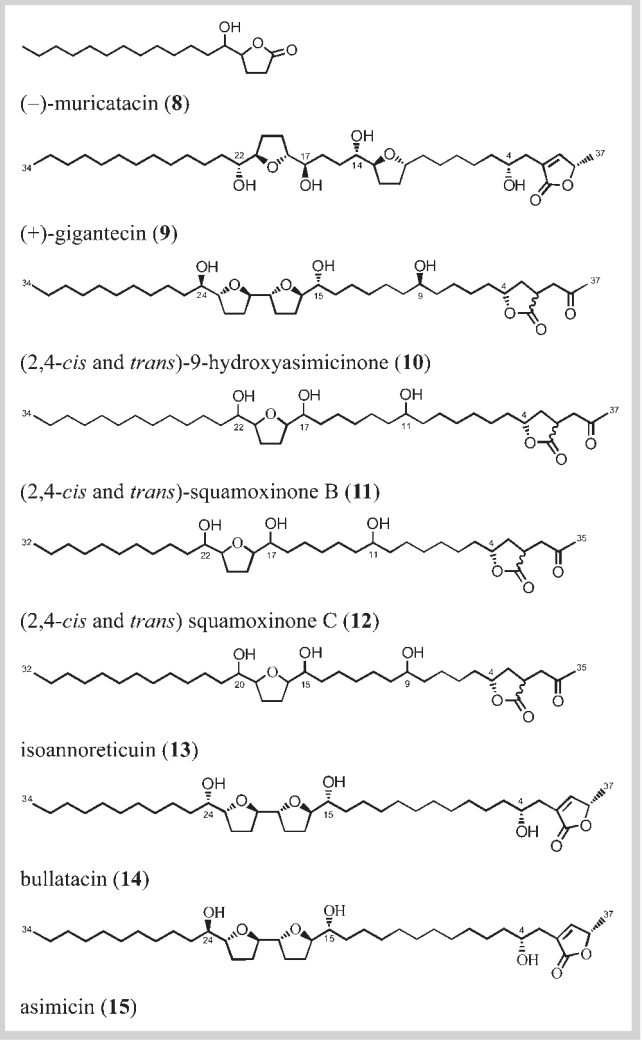

Fig. 4 Structures **8**–**15**.

rapid and relatively uncomplicated selective ionisation procedure also provided a convenient and useful method for identifying AGEs with or without a hydroxy group at C-4.

The Extensive Studies on AGEs after 2000

After 2000, we continued investigating the AGEs from Formosan plants of *Annona* species [59–62]. Among them, two epimeric AGEs, muricins A (**16**) and B (**17**) [59], were isolated and their absolute configurations were determined by the modified Mosher method. Muricin B (**17**) is the first Annonaceous acetogenin to possess a hydroxy group with the *S*-configuration at C-4, where the typical configuration of the hydroxy group was *R*. In 2003, we reported a novel skeleton of abridged AGE, rollicosin (**18**), from the unripe fruits of *Rollinia mucosa* Baill, which was the first identified compound that contained lactone moieties on both sides of an aliphatic chain [62]. Soon after this isolation, Chinese scholars reported the second abridged AGE, squamostolide (**19**), from *A. squamosa* [63] (**Fig. 5**).

During this time, the function and mechanism of action of AGEs were investigated. The link between the mitochondrial respiratory chain and cell apoptosis was clarified [64]. The latter, namely programmed cell death, is a normal physiological process that selectively and desirably destroys cells and tissues without an inflammatory response, as opposed to a necrotic cell death. Instead of focusing on the inhibition of mitochondria complex I, we found that bullatacin (**14**) could induce cell death via apoptosis based on an analysis of the morphological changes of bullatacin (**14**)-treated Hep 2.2.15, as determined by double staining with fluorescein isothiocyanate (FITC)-labelled annexin V and propidium iodide (PI) [65]. This finding also opened a new window for exploring the mechanism of action of AGEs. Herein, we summarise the studies on the cytotoxicity and pesticidal activity of AGEs in the last decade.

Biological activities and the mechanisms of action of the AGEs

Although AGEs were reported with high potential and diverse biological activities, including antibacterial [66], insecticidal, cytotoxic and immunosuppressive effects, pharmaceutical scientists were interested in how AGEs worked in cells (what the mechanism of the anticancer action of AGEs is) and whether the compounds could work *in vivo*. Combined with the traditional uses of the Annonaceous plants in North America and South-East Asia, scientists noticed the pesticidal activities of AGEs and proposed a possible mechanism of pesticidal action involved to ATP levels in pests. Thereafter, they found that both the cytotoxic and pesticidal activities should be related to ATP generation and NADH oxi-

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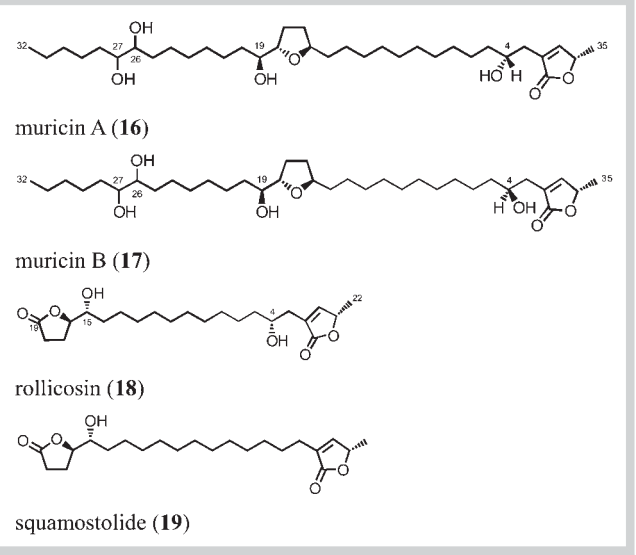

Fig. 5 Structures **16**–**19**.

dation in mitochondria, which directed many ongoing studies about the interaction of AGEs with the mitochondrial complex I.

Anticancer activity: Jolad et al. first reported the significant *in vivo* cytotoxic activity of uvaricin (**1**) by the PS screening system [14]. Ahammadsahib et al. used normal mice bearing L1210 murine leukaemia and athymic mice bearing A2780 conventional ovarian cancer xenografts to study the cytotoxic action of bullatacin (**14**) and analogues in 1993; meanwhile these compounds also have potential as insecticides in insect-derived Sf9 cells. The toxicity of AGEs in both cases probably arises from the strong inhibitory ability of mitochondrial electron transport with specific action at complex I [67]. Degli Esposti et al. (France) first used mammalian mitochondria to study the action of AGEs toward the NADH-ubiquinone reductase (complex I) and reported that bullatacin (**14**) inhibited the proton pumping function of complex I with similar efficiency under steady-state and non-steady-state conditions, as compared to the action of rotenone and piericidin [68]. Because of the ability to inhibit the mitochondrial complex I, the main gate of the energy production in the cells, AGEs have been regarded as candidates for future generations of antitumour drugs with different mechanisms.

Besides blocking the NADH : ubiquinone oxidoreductase (complex I) in the electron transport system, AGEs are also powerful inhibitors of the NADH oxidases peculiar to the plasma membranes of cancer cells. Both mechanisms of action result in the inhibition of ATP production and may account for the observation that AGEs are more effective at killing multiple-drug resistant (MDR) tumours than their nonresistant counterparts since the MDR pumps on the cell membranes require ATP to function. In addition, Oberlies et al. observed that AGEs could selectively inhibit the cell growth of cancerous cells by *in vitro* cell inhibition assays against three murine (P388, PO3, and M17/Adr) and two human (H8 and H125) cancer cell lines [69]. Interestingly, the work of Oberlies et al. proposed that this class of natural products showed a certain biological activity against some drug-resistant cancers. Currently, multidrug-resistant cancers are hard to cure because the cancer cells have developed a mechanism to overcome the anticancer agents. Based on the biochemical differences between MDR and parental cancer cells, such as the ATP-dependent P-glycoprotein-mediated pumps (P-gp) and the higher demand for ATP in the MDR cancer cells, Oberlies et al. used bullatacin (**14**) to test two cell lines, MDR human mammary adenocarcinoma (MCF-7/Adr) cells and the parental, nonresistant wild-type (MCF-7/wt) cells [70]. Therefore, ATP depletion could be another mode of action of AGEs that offers a special advantage in the chemotherapeutic treatment of MDR tumours.

Shimada et al. also proposed a model for explaining the action of AGEs [71]. They suspected that the lactone ring alone could directly interact with the binding to complex I, and the THF rings with flanking OH groups function just as hydrophilic anchors at the membrane surface that allow lateral diffusion (or random distribution) of the lactone ring in the membrane interior. To verify the model, Kuwabara et al. synthesised a series of analogues with two terminal γ-lactone rings [72]. However, the bioassay results did not show that these analogues worked twice as well as AGEs did.

To clarify the mechanism of action of AGEs, we cooperated with biochemists and pharmacologists in Taiwan due to its abundant amount of naturally occurring Formosan AGEs. Yuan et al. found that annonacin (**2**) could arrest T24 bladder cancer cells at the G1 phase and cause cytotoxicity in a Bax- and caspase-3-related pathway [73]. In addition, squamocin (**3**) was also observed to arrest the same cancer cells at the G1 phase and cause a selective cytotoxicity in S-phase-enriched T24 cells via the same pathway of cleaving the functional protein of PARP and inducing cell apoptosis [74]. Squamocin (**3**) was also found to inhibit the proliferation of K562 cells via G2/M arrest in association with the induction of p21, p27 and the reduction of Cdk1 and Cdc25C kinase activities [75]. These works connected the AGEs with cell apoptosis, which exploited the multiple functions of AGEs as new anticancer candidates.

Pesticidal activity: Following the North American folk use of Annonaceous plants as pesticides, Rupprecht et al. first noted this application. They determined the pesticidal potencies of the extracts from the paw paw tree (*Asimina triloba*) by the brine shrimp test (*Artemia salina* larvae), which paralleled the significant activities seen against the striped cucumber beetle (*Acalymma vittatum* F.), Mexican bean beetle (*Epilachna varivestis* Mulsant), mosquito larvae (*Aedes aegypti* L.), blowfly larvae (*Calliphora vicina* Meigen), melon aphid (*Aphis gossyphii* Glover), two-spotted-spider mite (*Tetranychus urticae* Koch) and free-living nematode (*Caenorhabditis elegans*). During the preliminary screening, asimicin (**15**) was isolated and its pesticidal action evaluated [76]. Moreover, by the same bioactivity-guided isolation/fractionation method, bullatacin (**14**) was isolated and its pesticidal effects observed at concentrations as low as 1 ppm, whereas bullatacinone (**20**) lacked pesticidal activities [24]. Meanwhile, Ratnayake et al. conducted a controlled study on the pesticidal potencies of extracts from various plant parts of the paw paw tree (*Asimina triloba*) using the brine shrimp test [77] (▶ **Fig. 6**).

He et al. further evaluated the pesticidal properties of 44 AGEs using the yellow fever mosquito larvae (YFM) assay [78]. The results clearly demonstrated that most AGEs had pesticidal properties. In addition, they indicated that the adjacent bis-THF AGEs with three hydroxy groups, for example, bullatacin (**14**) and trilobin (**21**), were the most potent. They further made AGEs, mono-THF, adjacent bis-THF, and nonadjacent bis-THF types as insecticidal baits to test the potent toxicity of these compounds against insecticide-susceptible and -resistant German cockroaches, compared to the activities of some conventional synthetic insecti-

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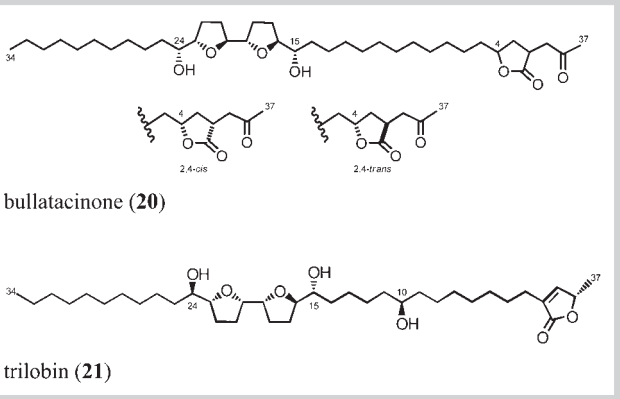

Fig. 6 Structures **20**–**21**.

cides [79]. Ohsawa et al. evaluated the insecticidal activities of AGEs from the seeds of the pond apple, *A. glabra* L. with a micro-sprayer on the cabbage leaf or the filter paper [80]. Meanwhile, Guadano et al. also found that annonacin (**2**) showed antifeedant effects on *L. decemlineata* and squamocin (**3**) was toxic to *L. decemlineata* and *M. persicae*. They also proved that both AGEs were not mutagenic but were toxic in the absence of a metabolic activation system [81].

Londershausen et al. also noticed that extracts of ground seeds from *A. squamosa* revealed interesting insecticidal properties. AGEs were determined to be the active components through an activity-monitored fractionation. The investigation of ATP-levels (at the LT_{50} value) in *Plutella xylostella* under treatment with squamocin (**3**) and antimycin A revealed values of 1.45 and 1.35 µmol/g fresh weight, respectively. Further studies revealed that squamocin (**3**) showed an inhibitory effect on NADH-cytochrome *c*-reductase and complex I of insect mitochondria with IC_{50} values of 4–8 µmol/g protein and 0.8 µM, respectively. Similar results for squamocin (**3**) were observed for the inhibition of complex I from bovine heart muscle (IC_{50}: < 0.1 µM) or *Neurospora crassa* cells (IC_{50}: 0.3 µM), but no effects on other coupling sites of mitochondrial complexes were observed [43]. These assembled experimental results were the work of Lewis et al. in 1993 [82]. Friedrich et al. and Hollingworth et al. simultaneously reported the insecticidal action of AGEs to be a result of the inhibition of mitochondria complex I [83,84]. Friedrich et al. found that the inhibition of mitochondrial and bacterial NADH : ubiquinone oxidoreductase (complex I) by AGEs was not purely competitive [83]. They demonstrated that AGEs should affect the electron-transfer step from the high-potential iron-sulfur cluster to ubiquinone by directly acting at the ubiquinone-catalytic site of complex I.

The Studies on Modifications and Analogues of the AGEs

The structure-activity relationship (SAR) studies of AGEs are always interesting for medicinal and natural product chemists. Miyoshi et al. noticed that the alkyl spacer between the γ-lactone and hydroxylated THF ring moieties elicited potent inhibitory activities on the NADH oxidase [85]. They summarised the SAR rules of AGEs as follows: 1) the adjacent bis-THF ring moiety is not an essential structural factor for inhibition, and the mono-THF ring compounds can maintain potent activities; 2) this stereochemical factor was also not essential for potent activity irrespective of the number (one or two) of THF rings; 3) the THF rings of the AGEs had strong interactions with the interface of lipid bilayers irrespective of the stereochemistry in the THF region; and 4) the spacer moiety is very important for potent activity [86]. Takada et al. also tested the NADH oxidase activity of two naturally occurring AGEs, bullatacin (**14**) and diepomuricanin (**22**), and several synthesised analogues in a comparison with that of piericidin A [87]. They concluded that both ring moieties, the γ-lactone ring and the tetrahydrofuran ring, acted in a cooperative manner on the enzyme and that the optimal length of the alkyl spacer was 13 carbon atoms. These results supported the above hypothesis that Miyoshi et al. offered.

To consider solely the role of the THF ring moieties, Murai et al. synthesised Δlac-AGE (**23**) (AGE without the *α,β*-unsaturated γ-lactone ring), which was also shown to be a novel type of inhibitor that acts at the terminal electron transfer step of mitochondrial NADH-ubiquinone oxidoreductase (complex I). They also synthesised a photolabile Δlac-AGE (**24**) connected to a biotin probe to trace the labelled peptide without the use of a radioisotope. This photolabile Δlac-AGE (**24**) elicited potent inhibition of bovine heart mitochondrial complex I at nanomolar levels [88]. Ichimaru et al. further synthesised a series of Δlac-AGEs, in which the stereochemistry around the hydroxylated tetrahydrofuran (THF) ring moiety was systematically modified, and examined their inhibitory effects on complex I. The results revealed that the bis-THF ring analogues are much more potent than the mono-THF ring analogues and that the stereochemistry around the bis-THF ring moiety played a significant role in the inhibitory effects on complex I [89]. Compound **25** showed a similar IC_{50} value as was observed for bullatacin (**14**) in the reduction of NADH oxidase activity (0.60–0.65 mmol NADH/min/mg of protein) in submitochondrial particles. Intriguingly, Ichimaru et al. demonstrated that the inhibitory site of complex I on which Δlac-AGEs acted might be different from that at which natural AGEs did.

On the other hand, these featured structures, such as the γ-lactone ring moiety, one to three THF/THP rings with multiple chiral centres, and an alkyl side chain make AGEs difficult and challenging synthetic targets. Because substantial amounts of pure samples are required for further biological and clinical studies, a number of total syntheses of AGEs have been reported in the literature since the 1990s. Recent advances in the total syntheses of AGEs include mono-THF AGEs: murisolin (**26**) [90,91], longicin (**27**) [92,93], and *cis*-solamin (**28**) [94,95], adjacent bis-THF AGEs: bullatacin (**14**) [96], rolliniastatin 1 (**7**) [23,97], rollimembrin (**29**) [97,98], 10-hydroxyasimicin (**30**) [99,100], membranacin (**31**) [97,101], asimicin (**15**) [76,102], longimicin D (**32**) [103–104], and mucoxin (**33**) [105,106], non-adjacent bis-THF AGEs: *cis*-sylvaticin (**34**) [107,108] and gigantecin (**9**) [109,110], and others, jimenezin (**35**) [111,112], mucocin (**36**) [113,114], pyranicin (**37**) [115,116], pyragonicin (**38**) [115,117,118], rollicosin (**18**) [62,119] and squamostolide (**19**) [63,120] (**Fig. 7**).

In addition to the total syntheses of various AGEs, some special analogues were designed to improve the bioactivities through, for example, modifications of the γ-lactone ring, the THF ring, and hydroxy moieties on the aliphatic chain.

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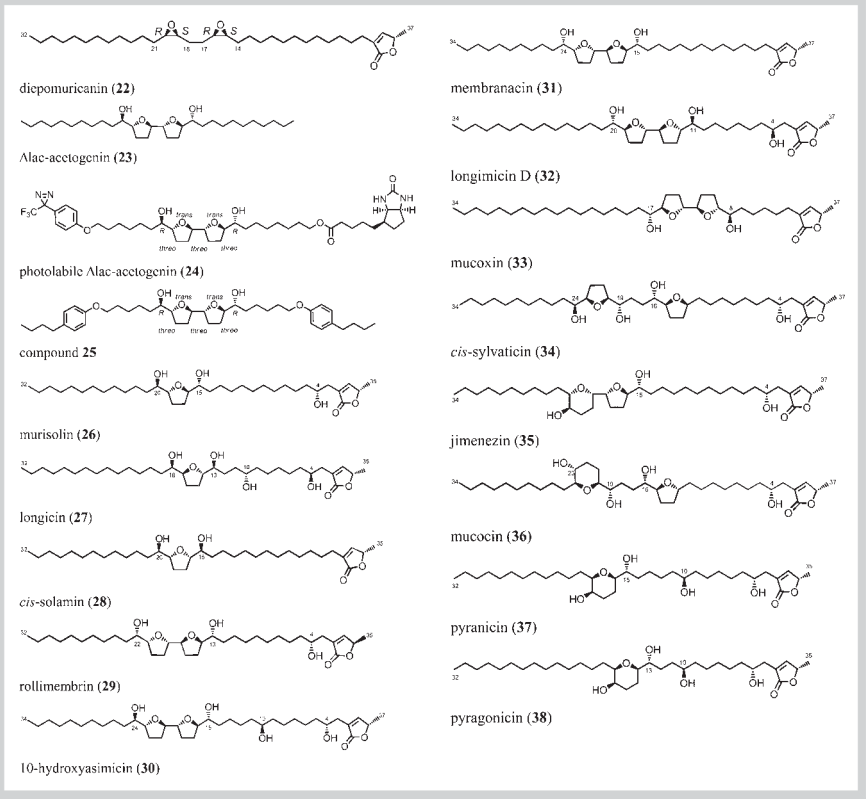

Fig. 7 Structures **22–38**.

Modifications of the γ-lactone ring moiety

Hoppen et al. designed and prepared quinone-mucocin (**39**) and quinone-squamocin D (**40**) to elucidate the mechanisms of action of the AGEs. The IC_{50} values of **39** and **40** in the inhibition of the mitochondrial NADH-ubiquinone oxidoreductase complex were 3.6 and 1.7 nM, respectively [121]. These results supported their hypothesis that AGEs are competitive inhibitors at the ubiquinone binding site of complex I based on the structural similarity between the butenolide and the quinine. Arndt et al. synthesised a systematic variation of featured structures, the butenolide and the ether components, to evaluate the critical factors for the interaction of the AGEs with complex I. Their results and data from the smaller substructures indicated that the substructures of the AGEs, the polyether component and the lipophilic side chain, would be necessary for strong binding of the AGEs to complex I [122].

In addition, aromatic heterocycles are commonly found as base structures of potent complex I inhibitors. Duval et al. tried to replace the α,β unsaturated γ-lactone moiety of squamocin (**3**) with benzimidazole via an unusual condensation-oxidative decarboxylation reaction with 1,2-diamines in the presence of acetic acid and oxygen. Although they did not clarify the inhibitory ability of the modified squamocin toward complex I, one of the benzimidazole analogues (**41**) showed cytotoxicity (KB 3–1) with an IC_{50} value of 2.2×10^{-3} µM and induced a 61% accumulation of the G1 phase of the KB 3–1 cell cycle at concentrations of 1–5 nM, with apoptosis above 10 nM [123]. In 2006, Duval et al. semisynthesised a series of heterocyclic analogues of squamocin (**3**). Their results suggested that the binding of this hybrid inhibitor (**41**) was responsible for a negative allosteric effect at the level of the first ubiquinone-binding site of mitochondrial complex I [124].

Duval et al. also prepared a small library of the γ-keto ester derivatives of squamocin (**3**) and screened their biological activities, including their cytotoxicity against KB 3–1 cells, inhibition of mitochondrial complex I and of complex III. However, these modified analogues with an open γ-lactone ring did not show better activity than that of the parent compound, squamocin (**3**) [125].

Except for the adjacent bis-THF and nonadjacent bis-THF AGEs, Kojima et al. made a series of α,β-unsaturated-γ-lactone-free, nitrogen-containing heterocyclic analogues of solamin (**42**), a mono-THF acetogenin. The cytotoxicities of the compounds were investigated against 39 tumour cell lines. One of them, a 1-methylpyrazol-5-yl derivative (**43**), showed a selective increase in cytotoxicity against NCI-H23 with a potency 80 times higher than that of solamin [126] (**Fig. 8**).

Modification of the THF ring moiety

The AGEs are a large class of naturally occurring polyketides that exhibit potent anticancer activities. In 2000, based on both the difficulty associated with total syntheses of AGEs and the straightforward means by which their structures can be simplified, Chinese and French scholars both proposed to replace the ethylene bridge in the THF rings with normal and iso-terminal lactone moieties, respectively [127, 128].

Yao and coworkers further studied simplified AA005 (**44**) and its analogues, which showed potent antitumour activities and significant selectivity between normal cells and cancer cells (≥ 7, [129]). Zeng et al. designed and synthesised (4*R*)-hydroxylated analogue **45** based on the structure of bullatacin (**14**). The preliminary screenings showed that the IC_{50} values of **45** were 1.6×10^{-3} and 8×10^{-2} µg/mL against HT-29 and HCT-8 cells, respectively. A remarkable enhancement of cytotoxic effect for the 4(*R*)-hydroxy analogue (**45**) was observed [130]. The results support a very interesting piece of information, namely that both the butenolide and ethylene glycol subunits play essential roles in the cytotoxicities of the compounds against tumour cell lines. Their precise

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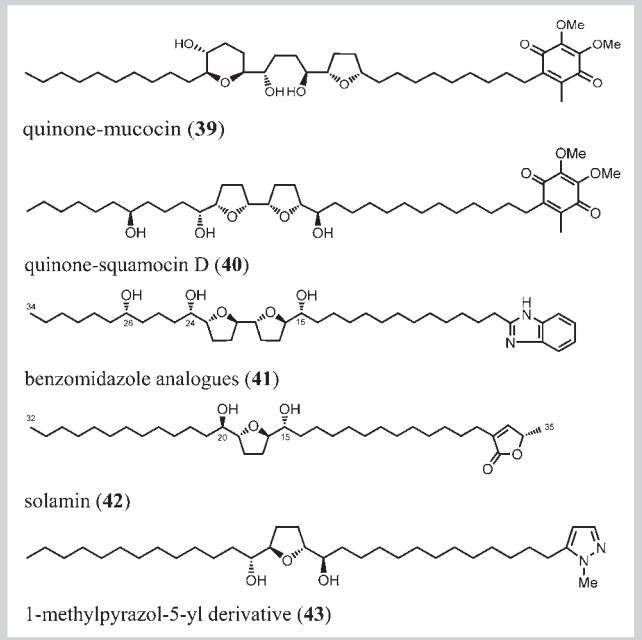

Fig. 8 Structures **39**–**43**.

role is not yet clear. Additionally, the presence of the hydroxy group at C-10 and the absolute configuration of the methyl group on the butenolide moiety (**46**) are less important for their activity [131].

Rodier et al. tried to introduce a benzoyl group to fix the moiety between the ether linkage; these analogues display interesting cell cycle effects but are less potent than bullatacinone (**20**), a compound with the same terminal lactone [132]. Fujita et al. also tried to replace the bis-adjacent THF ring by a 1,2-cyclopentanediol bis-ether skeleton to obtain simplified mimics **47**–**50**. Based on the evaluation of the inhibitory effects on mitochondrial NADH : ubiquinone oxidoreductase (complex I), the 1,2-cyclopentanediol bis-ether motif also showed very potent inhibitory activity at the nanomolar level [133].

For the study of AGE mimics, Liu et al. further designed, synthesised and evaluated a new series of mimics containing a terminal lactam [134]. They found that the *N*-methylated lactam-containing compounds **51, 52**, and **53** exhibited comparable potencies to that of AA005 (**44**) and similar selectivity among cancer cells. *N*-Methyl compound **51** shows comparable activities to that of AA005 (**44**) and retains similar cell selectivity. It was also revealed that the stereogenic centre on the lactam is not essential for antitumour activity. Recently, Liu et al. synthesised a series of analogues by replacing the acyclic bis-ether functionality of AA005 (**44**) with certain conformationally constrained fragments. Interestingly, most newly synthesised mimetics were found to exhibit potent activities against breast cancer cells and showed satisfactory selectivities between cancerous and non-cancerous cells. Among them, an *N,N'*-dimethyl bis-amide compound (**54**) exhibits more potency against MDA-MB-468 cells than does its parent molecule AA005 (**44**). Studies by Lin et al. indicate that bisamide analogues of AA005 make this unique class of anticancer agents much simpler and allow more flexibility for their future development [135] (**Fig. 9**).

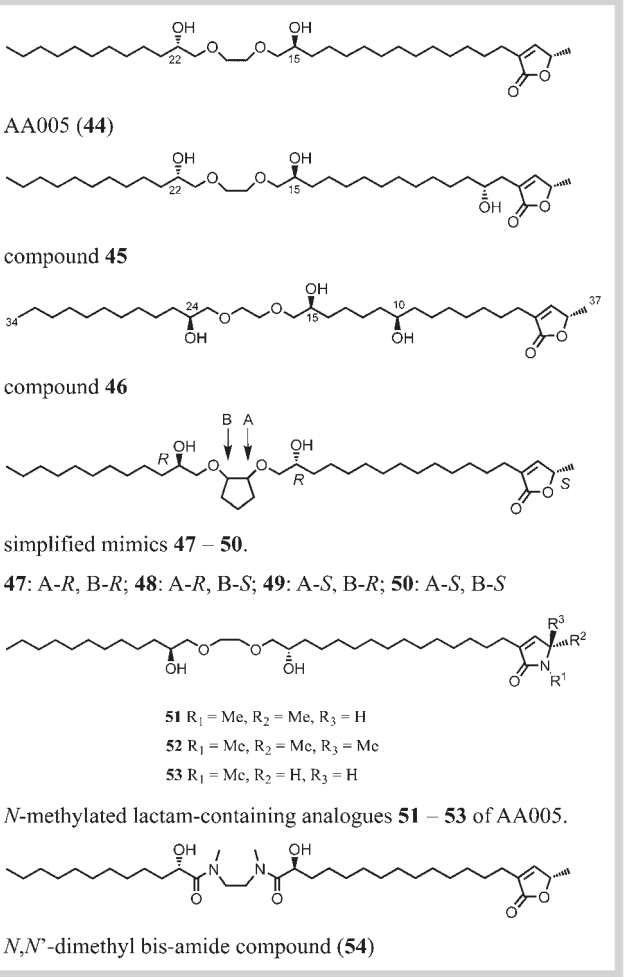

Fig. 9 Structures **44**–**54**.

Replacement of the hydroxy moiety on the aliphatic chain

Some scholars have studied the replacement of the hydroxy moieties on the aliphatic chain of AGEs. Ye et al. obtained halogen-substituted AGEs, 4(*S*)-chloro-4-deoxygigantetrocin A and 4(*S*)-18-dichloro-4,18-dideoxy-asimilobin, by treating gigantetrocin A with triphenylphosphine and CCl_4. The chlorinated compounds showed decreased bioactivities in the brine shrimp lethality test and against human tumour cell lines [136]. Kojima et al. made C4-fluorinated analogue (**55**) of solamin and evaluated its antitumour activities against 39 tumour cell lines. They found that C4-fluorinated solamin (**55**) showed more potent growth inhibitory activity against cancer cell lines than did solamin [137].

On the other hand, Gallardo et al. made 10-oximeguanacone (**56**), the first bioactive nitrogenated acetogenin, which showed potent inhibition towards complex I by the titration of the NADH oxidase and NADH : ubiquinone oxidoreductase activities [138]. Duret et al. semisynthesised amino derivatives from two natural AGEs, rolliniastatin-1 and squamocin. Although it is noteworthy that these amino-AGEs still retain some activity, more studies are required to confirm the potencies of these derivatives as new specific and efficient anticancer agents [139].

A variety of chemical strategies has been applied to investigating biological processes. Recently, fluorescent modifications became powerful tools for visualising the distribution of bioactive natural

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products in cells and investigating their targeting. In 2005, Derbre et al. synthesised hybrids consisting of an AGE tail connected to a fluorescent tag. Using fluorescent microscopy, both **57** and **58** were initially observed in Jurkat cell mitochondria, but they diffused into the cytosol of apoptotic cells, supporting the conclusion that squamocin (**3**) passes through the plasma membrane and targets the mitochondria. Indeed, both semisynthesised fluorescent derivatives were shown to be potent apoptosis inducers that were directed to this organelle. Besides, they proposed that the lactone moiety seems not to interfere with the mitochondrial targeting but apparently influenced the bioactivity of AGEs [140]. Alexander et al. attached ethyl 7-dimethylaminocoumarin 4-acetate to the diols of (−)-mucocin (**59**) through amide coupling chemistry. Although coumarin-labelled mucocin can also induce fluorescently coded morphogenic responses, no expected response was found [141]. This result might be due to the occupation of the mitochondrial recognition site by the fluorescent coumarin group. To overcome the above disadvantage, Maezaki et al. and Kojima et al. labelled the terminal aliphatic chain of solamin (**60, 61**) with fluorescent groups, 7-nitrobenzo [*c*][1,2,5]oxadiazol-4-yl-amino (NBD-NH-) and 5-dimethylaminonaphthalen-1-yl-sulfonamide (dansyl-NH-), in 2007 and 2009, respectively [142,143]. It was anticipated that these compounds would be used to explore the targeting of AGEs.

Among AGE mimics, Liu et al. tried to modify the C-10 hydroxy group of the AGE mimic to introduce a label based on the results of the anticancer-activity screenings of parallel synthetic analogues. Fluorescent-imaging studies revealed that AA005-flu's (**62** and **63**) distribution in normal human cells was significantly different from that in cancer cells. AA005-flu accumulated in the mitochondria of the cancer cells. This direct and visible evidence suggests that membrane recognition of AA005 (**44**) is involved in its selective bioactivity [144] (**Fig. 10**).

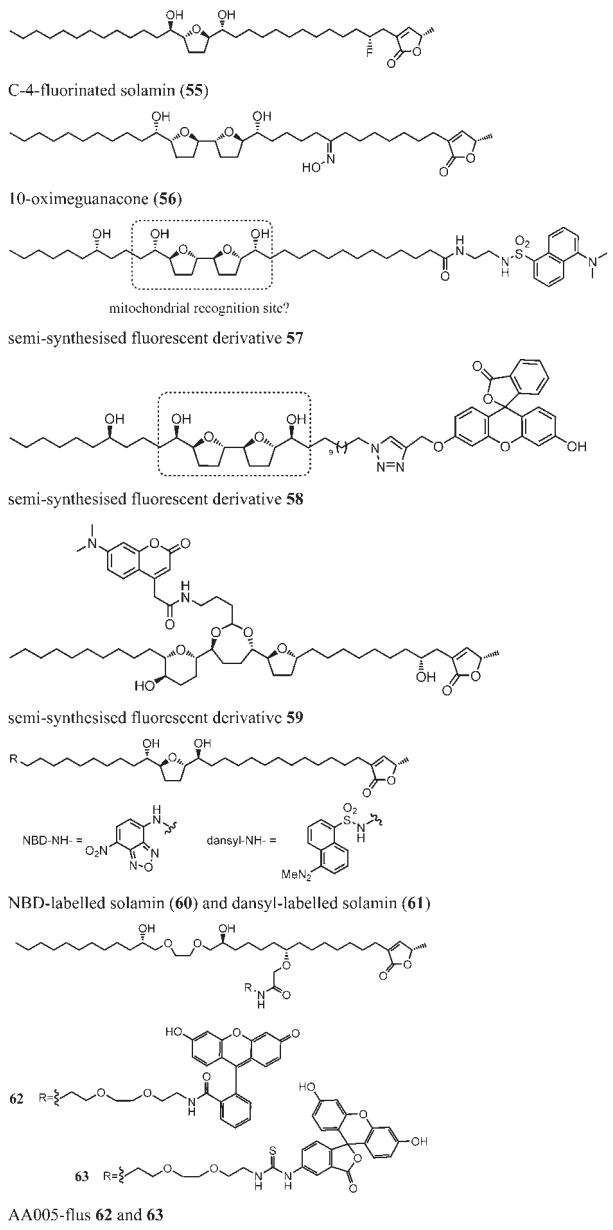

Fig. 10 Structures **55**–**63**.

AGEs as cation ionophores

Although many research results mentioned the mechanisms of AGEs, for instance, the inhibitory action of mitochondria complex I (NADH : ubiquinone oxidoreductase) [145], induction of programmed cell death by the expression of the pro-apoptotic proteins Bax, Bad, caspase-3 [74] and the structure-activity relationships of either natural, semisynthesised or synthesised compounds, the diverse bioactivities of the various types of AGEs still seem difficult to explain. Some researchers notice the chemophysical features of various AGEs and more direct evidence was provided as a new structure-activity relationship for AGEs.

Sasaki et al. first reported the ionophore activity of the AGEs. It was revealed by NMR studies that the structurally-related analogues of AGEs form supramolecular complexes with metal cations [146]. These studies indicated that hydroxylated bis-THF derivatives, structural components of the potent antitumour AGEs, formed supramolecular complexes with metal cations. In particular, some formed 2 : 1 ligand : metal complexes with calcium cations with high selectivity [146]. Although Araya et al. evaluated the ion-transport and ion-binding activities of AGEs using apparatus W-08 in 1995 and did not find any special activity [147], in1998 Sasaki et al. indicated that two AGEs, bullatacin (**14**) and asimicin (**15**), and their structurally related analogues binding bivalent cations such as Ca^{2+} and Mg^{2+} [148,149]. Peyrat et al. evaluated the ^{13}C-NMR longitudinal relaxation times (T_1) of both annonacin (**2**) and squamocin (**3**) in the absence and presence of Ca^{2+} ions to assess the structural changes that accompany complexations. They thought that the interesting cytotoxic activities of the THF-γ-lactone derivatives could be explained by their ionophoric ability. Their results also show differences in the stoichiometry of the complexes for mono-THF AGE and bis-THF AGE with Ca^{2+} ions [150].

In biological studies with living cells, we assumed that AGEs play a role in the bioavailability of the cations in the cell membranes due to their amphiphilic nature. While culturing smooth muscle cells of the human coronary artery with squamocin (**3**) in our study, we observed that squamocin (**3**) (an adjacent bis-THF acetogenin) could induce a transient but strong increase in the large-conductance Ca^{2+}-activated K^+ channels [151]. In a whole-cell configuration, squamocin (**3**, 0.3–100 µM) induced a Ca^{2+}-activated K^+ current [$I_{K(Ca)}$] in a concentration-dependent manner with an EC_{50} value of 4 µM. When cells were exposed to a Ca^{2+}-free solution, squamocin (**3**, 3 µM) induced a transient increase in $I_{K(Ca)}$. In the continued presence of squamocin (**3**), an additional increase in ex-

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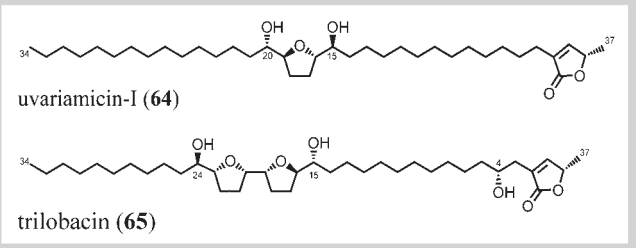

Fig. 11 Structures **64** and **65**.

tracellular Ca^{2+} (1 mM) caused a significant increase in $I_{K(Ca)}$. In the cell-attached configuration of the single-channel recordings, squamocin (**3**) applied to the bath increased the activity of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels without altering the single-channel conductance. These findings provide evidence that squamocin (**3**) can activate $I_{K(Ca)}$ in coronary arterial smooth muscle cells. The initial transient activation of $I_{K(Ca)}$ may reflect squamocin-induced Ca^{2+} release from intracellular Ca^{2+} stores, whereas the sustained activation of $I_{K(Ca)}$ may arise from the squamocin-induced Ca^{2+} influx across the cell membrane. The stimulatory effects of squamocin (**3**) on these channels should affect the functional activity of vascular smooth muscle cells [151].

We speculate that AGEs could use their hydrophilic centres (THF rings with flanking hydroxy groups) to bind cations like Ca^{2+} and surround the ion core by its peripheral hydrophobic regions (long chains). This arrangement allows the molecules to dissolve effectively in the membrane and diffuse transversely into cells as ionophores. We clarified the interaction between mono-THF AGEs and Ca^{2+} by isothermal titration calorimetry (ITC), which is an extremely powerful and highly sensitive technique for measuring the heats of interaction of reacting species in dilute solution. Interestingly, we found that the mono-THF AGEs annonacin (**2**) and uvariamicin-I (**64**) interacted with Ca^{2+} by an exothermic process, indicating the formation of AGE-calcium complexes [152] (**Fig. 11**).

From the Chemical Bench to Preclinical Trials

In 1976, Ratnayake et al. found that extracts of the leaves and twigs of the native paw paw tree, *Asimina triloba*, were bioactive in the antitumour screens of the U.S. National Cancer Institute (NCI). Following the sound phytochemical studies on AGEs [77], Gu et al. used the three most active AGEs, bullatacin (**14**), asimicin (**15**) and trilobacin (**65**), to establish quality control over AGE extracts of the paw paw tree by LC/MS/MS [153]. In this study they identified that small twigs from the months of May and June were the optimum plant sources for the commercial harvest of biomass for extraction. They further tried to develop some useful commercial products containing the AGEs, including head lice shampoo (in 2001), ointment, lotion, spray for plant pests, and paw paw capsules (in 2003) for human administration. The entire process from the safety and toxicology of the AGEs to the success of commercial products was described in McLaughlin's 2008 review [154]. More recently, Cuendet et al. reported the potential of the standardised extract from the twigs of *A. triloba* to mediate a cancer chemopreventative effect in the *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis model. As McLaughlin et al. did, they used three potent bioactive AGEs, bullatacin (**14**), asimicin (**15**), and trilobacin (**65**), in their standardised extract. Mammary tumour latency was increased from 55 to 66 days in Sprague-Dawley rats given a diet containing paw paw extract (1250 and 2500 mg/kg diet; based on maximum tolerated dose studies) [155].

In Taiwan, plants of the genus *Annona* are important economic crops for their edible fruits. The abundant material obtained from the seeds and the excellent cytotoxicities of the AGEs from this material attracted us to further develop pharmaceutical products. In addition to the aforementioned achievements, oral gavage (PO) animal studies have been performed by MDS Pharma Services and us. The extract from *A. muricata*, WYC-AA07, was applied to the xenograft tumour model of human MCF-7 breast tumour cells on SCID mice (assay 580000). WYC-AA07 at 10 mg/kg was administered daily by PO for a total of 10 doses. The tumour size, body weight and signs of overt animal toxicities after dosing were monitored and recorded for 25 days. WYC-AA07 at 10 mg/kg PO caused a significant decrease in the tumour weights from day 13 to day 25. However, it also caused a significant decrease in body weight on days 9, 13, 17, and 21. The other experiment was done on the xenograft tumour model of human HT-29 colon tumours on SCID mice (assay 580100). WYC-AA07 at 20 mg/kg PO caused death in one half of the animals and a significant decrease in body weight on day 8. Recently, we tested the toxicity of squamocin (**3**) on nude mice at 20 mg/kg PO. Although most of the animals showed an abnormal neuron function that caused the mice to move uncontrollably in a lateral direction, the mice recovered after we stopped the administration of squamocin (**3**). On the other hand, a ethnopharmacological investigation reported the similar side effect of AGEs that a neurodegenerative tauopathy endemic to the Caribbean island of Guadeloupe was suspected to be linked to the consumption of Annonaceous plants. Escobar-Khondiker et al. further found that annonacin (**2**) induced the retrograde transport of mitochondria to decrease ATP levels, which induces changes in the intracellular distribution of tau in a way that shares characteristics with some neurodegenerative diseases [156]. The possible adverse effect of AGEs on neuron cells should be a concern in advanced pharmaceutical applications for alternative therapy.

Perspectives

The AGEs are one of the most interesting classes of natural products appearing in the past two decades. They exhibit a wide variety of biological activities, and, impressively, some of them have comparable cytotoxic to taxol against various cancer cells. Both featured structures, the hydroxylated THF and γ-lactone ring moieties, are thought to be the pharmacophores that block the electron transport system of mitochondrial complex I. Much effort has been dedicated to elucidating the underlying cytotoxic mechanisms of the AGEs and to synthesising AGE analogues by altering the spacing between two moieties, removing either one of two critical featured structures (*Δ*Lac AGEs or muricatacin), or mimicking the THF rings by ether linkages. Although none of the modified AGEs obtained thus far have demonstrated activities comparable to those of the naturally occurring AGEs, studies on the synthesis and mechanisms of action of these compounds established solid fundamental knowledge and drug discovery experience. For example, the studies on analogues of AGEs created a series of compounds that contain completely different skeletons, of which some also show excellent bioactivities against various can-

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cer cells. In addition, *in vivo* tests in mice showed the antitumour effects and even some possible adverse effects of AGEs. However, there was no released data about the *in vivo* pharmacokinetic study of AGEs. Thus, more *in vivo* studies are necessary. Combined with the above information, it will be substantially helpful to further develop these types of compounds as new drugs.

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